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Effects of nonylphenol on the growth and microcystin production of *Microcystis* strains

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Abstract

Both organic pollution and eutrophication are prominent environmental issues concerning water pollution in the world. It is important to reveal the effects of organic pollutants on algal growth and toxin production for assessing ecological risk of organic pollution. Since nonylphenol (NP) is a kind of persistent organic pollutant with endocrine disruptive effect which exists ubiquitously in environments, NP was selected as test compound in our study to study the relationship between NP stress and *Microcystis* growth and microcystin production. Our study showed that responses of toxic and nontoxic *Microcystis aeruginosa* to NP stress were obviously different. The growth inhibition test with NP on *M. aeruginosa* yielded effect concentrations E_bC_{50} values within this range of 0.67–2.96 mg/L. The nontoxic *M.aeruginosa* strains were more resistant to NP than toxic strains at concentration above 1 mg/L. Cell growth was enhanced by 0.02–0.2 mg/L NP for both toxic and nontoxic strains, suggesting a hormesis effect of NP on *M. aeruginosa*. Both toxic and nontoxic strains tended to be smaller with increasing NP. But with the increased duration of the experiment, both the cell size and the growth rate began to resume, suggesting a quick adaptation of *M. aeruginosa* to adverse stress. NP of 0.05–0.5 mg/L significantly promoted microcystin production of toxic strain PCC7820, suggesting that NP might affect microcystin production of some toxic *M. aeruginosa* in the field. Our study showed that microcystin excretion was species specific that up to 75% of microcystins in PCC7820 were released into solution, whereas >99% of microcystins in 562 remained in algal cells after 12 days' incubation. NP also significantly influenced microcystin release into cultural media. The fact that NP enhanced growth and toxin production of *M. aeruginosa* at low concentrations of 0.02–0.5 mg/L that might be possibly found in natural freshwaters implies that low concentration of NP may favor survival of *M. aeruginosa* in the field and may play a subtle role in affecting cyanobacterial blooms and microcystin production in natural waters.

Keywords: *Microcystis aeruginosa*; Microcystin; Nonylphenol (NP); Algal growth

1. Introduction

With the escalation of industrial processes and the expansion of urban population, a vast amount of organic pollutants in industrial waste, residue pesticide and sewage have been released into the environment. Many of the organic pollutants are persistent organic pollutants (POPs) and endocrine disruptors with potential of persistence, half-volatile and bioaccumulation in the environment. These pollutants show potential toxicity, carcinogenicity and/or mutagenicity to human, causing great concerns to the society.

Eutrophication is one of the most serious problems concerning water pollution in the world. The bloom of cyanobacterium *Microcystis aeruginosa* is a ubiquitous phenomenon in eutrophic lakes and reservoirs and polluted water in many countries of the world. Many strains of *Microcystis* are known to produce cyanobacterial hepatotoxins called microcystin. The toxin, a soluble peptide, is lethal to many kinds of aquatic organisms and damages zooplankton, fish, and the liver of higher animals (Penaloza et al., 1990; Sivonen, 1996; Watanabe et al., 1989). Large amounts of organic pollutants enter into

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waters, though in low concentrations in the waters, having caused great influence on the growth of microalgae. As a primary producer in the aquatic ecosystem, algae provide food and oxygen by photosynthesis for invertebrate, fish and bird. Its phyletic diversity and primary production can directly affect structure and function of the ecosystem. On the other hand, alga is a good surveillant for risk assessment with its sensitivity to toxicant, easy acquisition, small size and fast reproduction. It is important to study the effects of organic pollutants on algal growth for controlling algal bloom, monitoring and eliminating organic pollution, and assessing ecological risk of organic pollution. There are many studies about effects of nutrients, such as nitrogen and phosphorus and heavy metals on the growth of algae. In recent years, attentions have focused on the effects of organic pollutants on algae (Semple and Cain, 1995; Wei et al., 1998; Nakai et al., 2001; Sabater and Carrasco, 1996). However, limited information is available on toxic effects of *M. aeruginosa* caused by persistent organic pollutants and endocrine disrupters.

Not all of *M. aeruginosa* can produce microcystin. There is no obvious difference in phenotypic characteristics between toxic and nontoxic *M. aeruginosa.* Some toxic strains isolated from field experiments can spontaneously lose their toxicities under laboratory conditions (Schatz et al., 2005). It is recognized that toxicity occurrence of a *M. aeruginosa* strain is determined by its microcystin production genes (mcy genes) (Dittmann et al., 1997; Kaebernick and Neilan, 2001). On the other hand, environmental factors can adjust and control the expression of microcystin genes. Several field studies have shown that certain environmental factors are associated with the amount of toxins found in cyanobacterial blooms (Wicks and Thiel, 1990; Zheng et al., 2004). A wide range of laboratory studies also have examined the effects of various environmental factors on microcystin production, including trace metal supply (Lukac and Aegerter, 1993; Utkilen and Gjølme, 1995), nitrogen and phosphorus (Sivonen, 1990), light and temperature (van der Westhuizen and Eloff, 1985), pH (de Maagd et al., 1999), culture medium (Song et al., 1998), water column stability (Huisman et al., 1999) and grazing pressure by zooplankton (Paerl, 1996; Hyenstrand et al, 1998). However, there are still many controversial issues from both field and laboratory studies on the mechanisms of microcystin production.

In recent years, *Microcystis* bloom occurs frequently in bodies of freshwater all over the world (Codd et al., 1999). This phenomenon is necessarily related with the increasing living and industrial pollution, which contains a vast amount of organic pollutants. Organic pollutants in industrial waste, pesticide residue and sewage enter into waters and exert side effects on ecological and human being health. It is essential and helpful to study the effects of organic pollutants on algal growth and microcystins production for exploring the mechanism of cyanobacterium bloom and microcystins production.

Nonylphenol (NP) is a degradation product of the alkylphenol polyethoxylates, an important class of non-ionic 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., 1987; Ahel et al., 1994; Giger et al., 1984). NPs are as high as 325 μg/L in surface water and reach 72 mg/kg in sediments (Ahel et al., 1994; Bennie, 1999; Gross-Sorokin et al., 2003; Kvestak and Ahel, 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 (Baldwin et al., 1997; Jobling et al., 1996; Schmude et al., 1999; Höss et al., 2002; Hense et al., 2003). However, studies about effects of NP on growth of *M. aeruginosa* and microcystin production are still lacking.

In this paper, we studied the toxic effect of NP on the growth of both toxic and nontoxic *M. aeruginosa* strains and effect of NP on microcystin production of toxic *M. aeruginosa* strains. Batch culture experiments were conducted with toxic and nontoxic *Microcystis* strains exposed to various concentrations of NP (0–2 mg/L). Changes of chlorophyll *a*, carotinoid and protein affected by NP stress were also investigated. The main objective of this study was to explore responses of *Microcystis* to NP stress and the possible effects of NP on *Microcystis* bloom and microcystin production.

2. Materials and methods

2.1. Experimental organisms and growth conditions design

Four axenic *Microcystis 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 major toxin. The toxic 562 also produces microcystin-YR in minor quantity. The nontoxic subculture of PCC7820N emerged from the toxic one which spontaneously succeeded the toxic one under laboratory conditions and was not capable of producing toxins.

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/mL. The laboratory conditions were controlled at 25 ± 1 °C using cool white fluorescent lights (48 μE/m^2/s) 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 (4-NP >98%, Sigma-Aldrich, Seelze, Germany) 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. Effects of the carrier were tested by running controls with and without DMSO. In the DMSO control and in all the NP treatments, the DMSO concentration was kept constant at 0.02% (v/v). Eight different NP concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 mg/L) were chosen for algal exposure experiment. The experiment was repeated three times and each treatment had at least two replicates.

2.2. Growth measurements

Cells were exposed on Day 0, growth was then firstly monitored on Day 4 and every 2 days subsequently (Days 6, 8, 10, 12) and the test was terminated on Day 12. The growth test includes the optical density at 650 nm (uv-1601, Shimadzu Corporation, Kyoto, Japan), cell size, cell number, chlorophyll *a* and carotinoid. Approximately 10 mL of each culture was removed for analysis of these parameters. Growth curves based on changes in the optical density at 650 nm were constructed. The percent inhibition of cell growth at each NP concentration was calculated by comparison of areas under growth curves for each NP concentration (OECD, 1984). The percents inhibition were transformed into probit values and then represented as log doses of NP in order to calculate the E_bC_{50} values. In this study, E_bC_{50} is the concentration of NP which results in a 50% reduction in algal growth on the 12th day relative to the control values.

Mean cell diameter was measured using a micrometer under an Olympus microscope (× 100) and cells were counted microscopically using a hemacytometer (improved Neubauer). Wet weight was calculated from cell size and cell number by the following equation: $G = N \times V/10^9$, where G represents wet weight; N the cell number, V the cell volume and is calculated by the equation: $V = 4/3\ \pi\ (d/2)^3$, with d the cell diameter.

Chlorophyll *a* and carotinoid were measured spectrophotometrically according to Jeffrey and Humphrey (1975). After extraction with 90% acetone at 4 °C in dark for 24 h, optical density was measured at 665, 663 and 645 nm and chlorophyll *a* and carotinoid were calculated according to the following equations: $C_a = 12.7A_{663}–2.69A_{645}$; $C_k = 4.70A_{440}–5.45A_{645}–2.16A_{663}$. C_a represents chlorophyll *a* and C_k represents carotinoid.

At the end of the experiment of 12 days' incubation, 50 mL of the growth medium was used to analyze protein concentration; another 50 mL was collected for microcystin detecting. The two supernatants were combined (100 mL) and filtered for dissolved toxin analysis. Total protein content was determined colorimetrically (Bradford, 1976) using Bradford reagent purchased from Sigma-Aldrich. Bovine serum albumin was selected as a reference and the average protein standard curve was

$$y = (0.019 \pm 0.003)x + (0.056 \pm 0.009)$$
$$(R^2 = 0.987 \pm 0.005),$$

y is the absorbance of Coomassie Brilliant Blue G-250 dye determined at 595 nm, and x is protein content μg/mL.

2.3. Toxin analysis

The harvest cells were immediately centrifuged at 12000*g* at 4 °C for 20 min. For toxin analysis, the samples were prepared and measured as previously described (Park and Lwami, 1998; Martin et al., 1999) with some improvements. For the cell material, the microcystins were extracted three times with 8 mL of 75% methanol (v/v) for 3 h at 4 °C. The extract was centrifuged at 10000*g* for 30 min, and the supernatants were pooled and were applied to a C_{18} cartridge (Dalian Institute of Chemical and Physical, China). The cartridge containing the microcystin was rinsed with 10 mL water and the microcystins were finally eluted from the C_{18} cartridge with 10 mL of methanol. The eluate was evaporated under reduced pressure and then the residue was dissolved in 100 μL methanol. Toxins dissolved in the growth medium were concentrated and analyzed in a similar manner from 100 mL of filtered supernatants.

Microcystin in cell material and those dissolved in growth medium were analyzed by a reverse-phase high performance liquid chromatography (HPLC) (Shimadzu, LC-10AD) equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nacalai, Japan) and a SPD-10A UV–VIS spectrophotometer set at 238 nm. The sample was separated by using linear gradient elution of 50–70% aqueous methanol containing 0.05% trifluoroacetyl (TFA) in 25 min at a flow rate of 1 mL/min. The microcystins were identified by their UV spectra and retention times, and by using commercial microcystin-LR RR and YR (Wako Pure Chemical Industries, Japan) as standards. In addition, the toxin peaks were isolated and identified according to their mass spectra by using a Finnigan LC-MS system.

2.4. Statistic analysis

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 OK, USA).

3. Results

3.1. Growth patterns of *M. aeruginosa* strains

The growth patterns of the two nontoxic strains were very similar, but differed from those of the toxic strains (Fig. 1, data of 0.02, 0.1, 0.2 mg/L treatments and solvent control were not shown). For the nontoxic strains compared with the control, the presence of 0.02–0.2 mg/L NP in the culture media stimulated cell growth, whereas 1–2 mg/L NP began to inhibit cell growth. For the toxic strains, growth patterns were similar to nontoxic strains at low NP concentrations (0.02–0.2 mg/L) exposure, but were different at high NP concentration exposure. A total of 2 mg/L NP completely inhibited cell growth of the two toxic strains. A 1 mg/L NP obviously inhibited cell growth of toxic strain 562, but this concentration of NP had no obvious effect on growth of toxic strain PCC7820. Solvent DMSO in the designated concentration in the study had no obvious effect on algal growth.

The 12th day' E_bC_{50} values for the four species of *M. aeruginosa* affected by NP are listed in Table 1. Toxic strain 562 was more sensitive to NP than toxic strain PCC7820

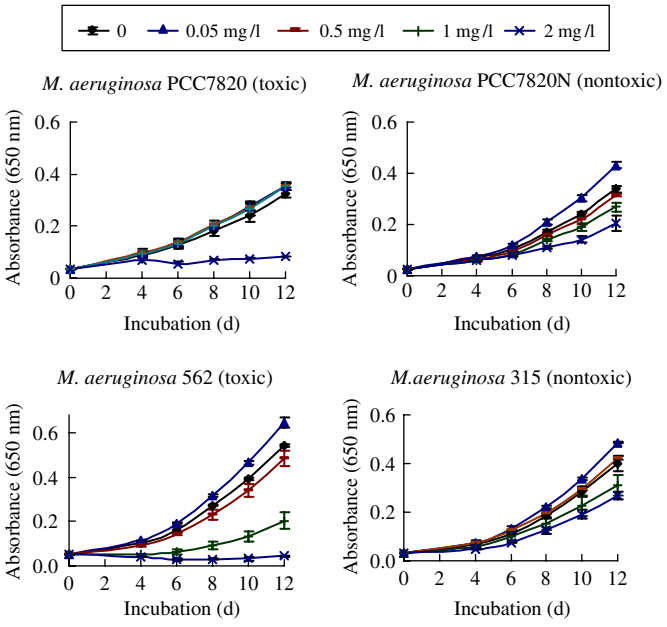

Fig. 1. Growth curves of the toxic *M. aeruginosa* PCC7820 and 562 and the nontoxic *M. aeruginosa* PCC7820N and 315 exposed to different concentrations of nonylphenol. Vertical bars show standard deviation.

Table 1
Probit regression equations and E_bC_{50} values of the four *M. aeruginosa* strains after the 12 days' growth with different concentration of NP

	Probit regression equations	E_bC_{50} (mg/L)
Toxic PCC7820	$Y = 5.55x + 2.18$	1.66 (1.42–1.78)
Toxic 562	$Y = 5.25x + 7.07$	0.67 (0.60–0.78)
Nontoxic PCC7820N	$Y = 2.42x + 2.69$	2.60 (2.40–2.92)
Nontoxic 315	$Y = 2.77x + 2.00$	2.96 (2.94–3.46)

Note: Y = Probit of % inhibition; x = ln(mg of NP/L).

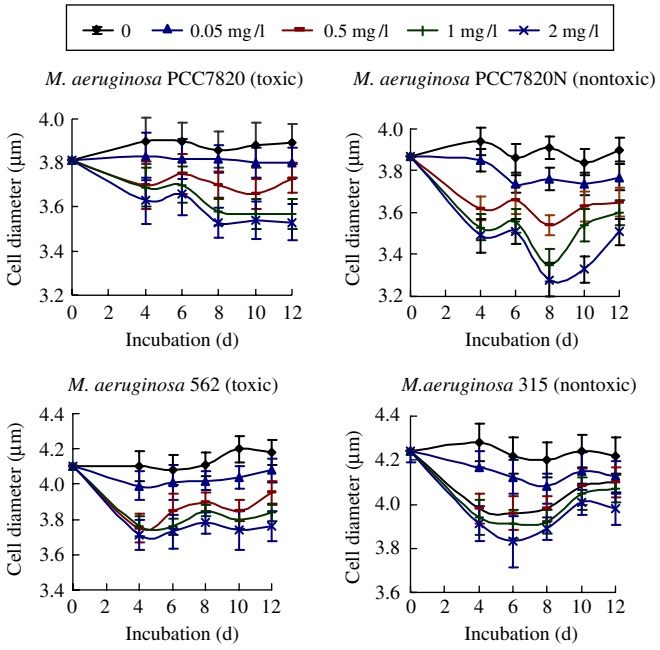

Fig. 2. Cell sizes of the toxic *M. aeruginosa* PCC7820 and 562 and the nontoxic *M. aeruginosa* PCC7820N and 315 exposed to different concentrations of nonylphenol. Vertical bars show standard deviation.

and the toxic strains were less resistant to high NP concentration than the nontoxic strains.

The cell diameter of the four *M. areuginosa* strains tended to grow smaller with increasing NP concentrations (Fig. 2, data of 0.02, 0.1, 0.2 mg/L treatments and solvent control were not shown). Solvent DMSO had no obvious effect on cell diameter in the study. The cell diameters of the nontoxic strains exposed to 1–2 mg/L NP began to resume after a decreasing period of 6 or 8 days, but such phenomenon was not so obvious for the toxic strains.

3.2. Effect of NP on chlorophyll, carotinoid and protein syntheses of *M. aeruginosa* strains

The four *M. aeruginosa* strains showed different changes for chlorophyll *a*, carotinoid and protein contents (normalized by fresh wet weight of algal cells) after 12 days' exposure to different NP concentrations (Fig. 3–5). The toxic strain PCC7820 produced relatively higher chlorophyll *a* and carotinoid at low NP concentrations

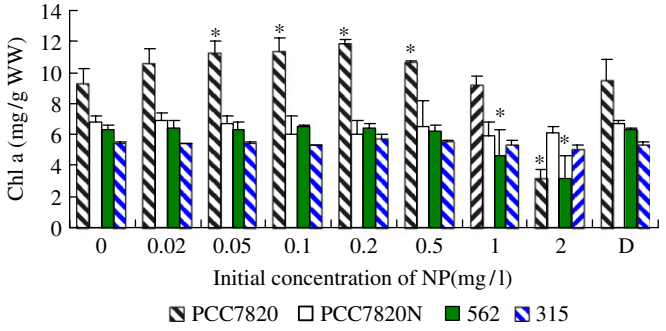

Fig. 3. Chlorophyll *a* contents of toxic and nontoxic *M. aeruginosa* strains after 12 days' exposure to different NP concentrations. Vertical bars show standard deviation. DMSO represents solvent control. WW indicates wet weight of algae and *indicates a significant difference from control ($P<0.05$).

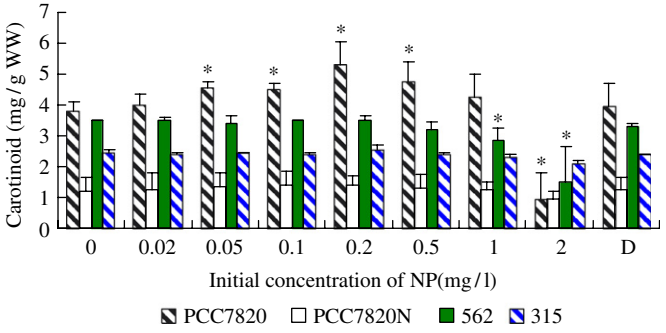

Fig. 4. Carotinoid contents of toxic and nontoxic *M. aeruginosa* strains after 12 days' exposure to different NP concentrations. Vertical bars show standard deviation. DMSO represents solvent control. WW indicates wet weight of algae and *indicates a significant difference from control ($P<0.05$).

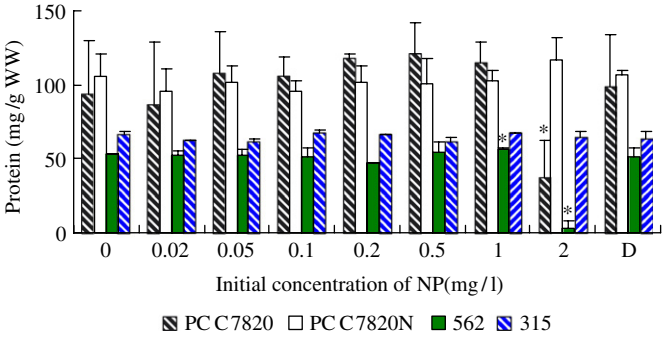

Fig. 5. Protein contents of toxic and nontoxic *M. aeruginosa* strains after 12 days' exposure to different NP concentrations. Vertical bars show standard deviation. DMSO represents solvent control. WW indicates wet weight of algae and *indicates a significant difference from control ($P<0.05$).

(0.05–0.2 mg/L) exposure. At high NP concentration (2 mg/L), syntheses of chlorophyll *a*, carotinoid and protein for the toxic strains PCC7820 and 562 were obviously inhibited, but that for the nontoxic strains PCC7820N and 315 were not obviously changed.

3.3. Intra- and extra microcystin content of *M. aeruginosa* strains

Both toxic strains produced predominantly microcystin-LR, and strain 562 also produced in extremely low content microcystin-YR (were 1000 times less than microcystin-LR content), which was confirmed qualitatively by LC/MS. Toxic strain PCC7820 produced relatively higher toxin treated with low NP concentrations (0.05–0.5 mg/L) compared with control (Fig. 6). The highest intracellular microcystin-LR content of the toxic PCC7820 at 0.2 mg/L NP exposure was average 75% higher than that of the control. But the highest microcystin-LR content in strain 562 was only 10% higher than control. Microcystin-YR was detected by HPLC only occasionally in strain 562 cells at 0.2–1 mg/L NP exposure, ranging between 0.59 and 5.59 μg/g wet weight of algae. Only microcystin-LR was detected in the growth media of both strains. Both of the extra- and intracellular microcystin LR contents were expressed with milligram per liter of algal culture medium and then the ratios of extracellular microcystin to total toxin were calculated (Table 2). For strain PCC7820, as high as 33–90% of total microcystins were present in the growth media with the maximum at 2 mg/L NP treatment. On the contrary, for the strain 562, intracellular toxins comprised over 99% of the total microcystins.

Table 2
Ratio of extracellular microcystin-LR to total toxin contents for *Microcystis aeruginosa* strains grown in CT medium with different concentrations of NP

NP concentrations (mg/L)	Ratio of extra-microcystin to total toxin	
	Toxic strain PCC 7820	Toxic strain 562
Control	0.756±0.004	0.0039±0.0003
0.02	0.765±0.015	0.0040±0.0001
0.05	0.687±0.001	0.0010±0.0002
0.1	0.662±0.023	0.0006±0.0001
0.2	0.330±0.156	—
0.5	0.407±0.066	—
1	0.486±0.141	—
2	0.904±0.025	—

Note: Both of the extra- and intracellular microcystin-LR contents were expressed as milligram per liter of algal culture medium and then the ratios of extra microcystin to total toxin were calculated.

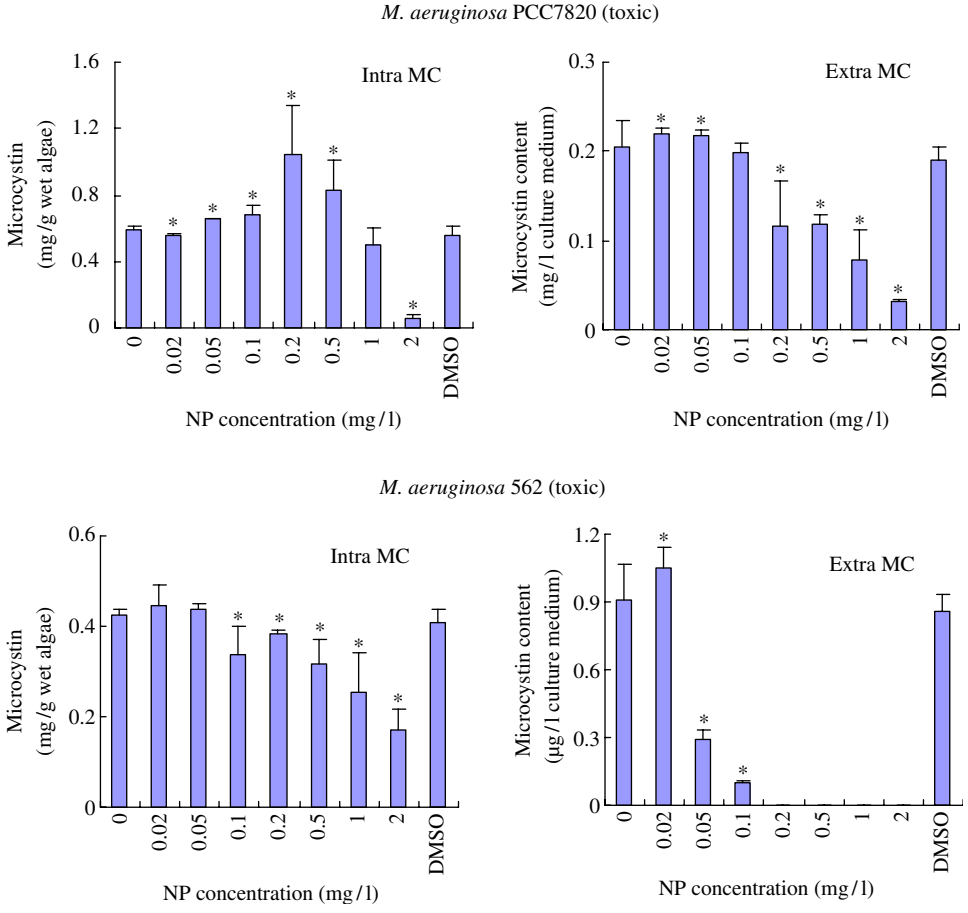

Fig. 6. Intra- and extracellular microcystin-LR contents of toxic *M. aeruginosa* strains PCC7820 and 562 after 12 days' incubation with different concentrations of NP. Vertical bars show standard deviation. DMSO represents solvent control. *indicates a significant difference from control ($P<0.05$).

4. Discussion

In algal toxicity experiment, biomass or growth rate is in general used as indicator of growth inhibition and safety evaluation for algae and the concentration that results in a 50% reduction in algal growth (E_bC_{50}) is usually used to assess the toxicity of the test compound. The growth curves of the algae in the present study indicated that all algal cells were on the logarithmic phase of growth. When NP concentration was increased, growth of algal cells was inhibited or promoted in different degree and the growth curves presented dose–effect relationship. Our growth inhibition test with NP on *M. aeruginosa* yielded effect concentrations E_bC_{50} values within this range of 0.67–2.96 mg/L. In previous reports, effect concentrations of NP on planktonic algae are from >0.4 to 2.5 mg/L (Servos, 1999). Nakai et al. (2001) reported that *M. aeruginosa* growth was inhibited by plant-producing phenols with EC_{50} values of 0.3–5.5 mg/L. Hense et al. (2003) reported that *S. subspicatus* growth was inhibited by NPs with EC_{50} values of 0.87–0.98 mg/L. The EC_{50} values in these reports are close to our results. Our study discovered that responses of toxic and nontoxic *M. aeruginosa* to NP stress were different. The E_bC_{50} values of toxic strains PCC7820 and 562 were 1.66 and 0.67 mg/L, respectively, lower than that of nontoxic strains PCC7820N and 315 (2.60 and 2.96 mg/L, respectively). The growth curves showed that the two toxic strains had negative growth under 2 mg/L NP exposure, whereas the two nontoxic strains still grew well. Therefore, nontoxic *M.aeruginosa* strains were more resistant to NP than toxic strains at concentration above 1 mg/L.

Cell size of both toxic and nontoxic strains tended to become smaller with increasing concentration of NP. Krüger and Eloff (1981) suggest that cell size is a likely indicator of the physiological state of a cell with stressed cells being larger. Visual observation of ecdysteroid—treated *C. vulgaris* cells shows that ecdysteroids stimulate enlargement of the algal cells (Bajguz and Dinan, 2004). However, our results showed oppositely that stressed cells tended to be smaller. On the sixth day of the growth, cell size of *M. aeruginosa* strains began to resume and this resumption was less obvious in toxic strains than in nontoxic strains. This indicated that cells became smaller as an emergency physiological response to environmental stress. More obvious resumption in nontoxic strains than in toxic ones also indicated that nontoxic *M. aeruginosa* strains were more resistant to high concentration of NP (1–2 mg/L) than toxic ones. After 4 days' incubation, the algal growth in the cultures was monitored at an interval of 2 days by Olympus microscope (× 100). The destruction of Chl*a* and the photo-oxidation of photo synthetic pigments could result in yellow color occurrence in culture media (Oberholster et al., 2004). Colony formation by cyanobacteria may be stress responses to abiotic factors such as temperature, salinity, light intensity and nutrients and/or a common defence strategy against biotic factors such as the grazing in aquatic ecosystems (Jang et al., 2003). No yellow color and algal agglomeration in cultures were observed indicated that photo synthetic system was not destroyed by NP and the chemical NP could not cause colony formation of *Microcystis* in the experiment. Especially, the strains with high NP concentration did not appear deformed or dead cells observed by the microscope based on changes in cell morphology. After 6 or 8 days' incubation, the inhibition growth of the toxic strains with high concentration of NP began to resume to stimulation growth. For the nontoxic strains, fast growing tendency even occurred after 10 days' incubation. This indicated that cells were in lag phase or prolonging the lag phase in cope with the toxicity of high NP concentration. The resumption phenomenon at the end of the experiment might be due to decreased concentration of NP by biodegradation, sorption or other reasons. Therefore, we thought that *M. aeruginosa* had formed strong ability to adjust and rehabilitate itself to survive under adverse stress in the long process of evolution. Further studies needed to study the ultrastructure of *M. aeruginosa* in response to NP stress to probe into the reactive mechanism.

Variable environmental conditions may affect cyanobacterial phenotypes based on expressed proteins, such as photosynthetic pigments and isozymes, or plasmid content (Neilan et al., 1995). Prasad (1989) suggests that NP most likely interferes with photo synthesis and cell division in macrophytes. In present study, changes of chlorophyll *a*, carotinoid and protein (normalized to wet weight algae) under NP stress were investigated. Syntheses of chlorophyll *a*, carotinoid and protein of nontoxic *M. aeruginosa* strains were not influenced by NP, but that of toxic ones were obviously inhibited by 2 mg/L NP. This may be due to the toxicity of NP on toxic algal cells. On the other hand, low concentration of NP (0.05–0.2 mg/L) stimulated slightly to the synthesis of chlorophyll *a* and carotinoid of toxic strain PCC7820. Shi et al. (1995) found that microcystin was associated with the thylakoid membranes of *M. aeruginosa* PCC7820, suggesting a close physiological association between microcystins and the photosynthetic machinery of the cell. The finding in our study may suggest that NP had taken part in the physiological activity of some toxic *M. aeruginosa* cells and such activity had a certain extent relationship with toxin production.

Many organic pollutants can stimulate algal growth at low concentrations (less than EC_{50} values). For example, glyphosate of 0.02 mg/L stimulates growth of *Scenedesums quadricauda* (Wong, 2000). Dimethoate of 0.4, 0.8, 1.2, 1.6 mg/L stimulates growth of *Ghlorella vulgans* (Tian et al., 1997). The absence of an inhibitory effect and even a small positive action of moderate-strength treatment is known as hormesis—where a modest stimulation of response occurs at low doses and an inhibition of response occurs at high ones (Calabrese and Baldwin, 2003). Recognition of hormetic-like biphasic dose responses is important for elucidating the bioregulatory actions of various peptides and their biomedical implications. The

stimulation of growth of both toxic and nontoxic *M. aeruginosa* strains with low concentration of NP (0.02–0.2 mg/L) in our study might be the effect of hormesis which is needed to be testified further.

Since many organic compounds do not dissolve in water, carrier solvents are frequently used in toxicity measurement. But carrier solvents themselves or carrier solvents added with tested organic compounds can cause synergic, additive or antagonistic effects on experimental biomaterial. Studies of El Jay (1996) on toxic effects of organic solvents on the growth of *Chlorella vulgaris* and *Selenastrum capricornutum* showed that 1% (v/v) DMSO had no effect on the two algae. Hess (1980) discovered that DMSO of concentration higher than 1% obviously inhibited the growth of *Chlamdomonas eugametos*. In our study, DMSO was chosen to be the carrier solvent to prepare different concentrations of NP. Concentration of DMSO in culture medium did not excess 0.02% (v/v) and solvent control of 0.02% DMSO was set throughout the experiment. The effects of DMSO at the designated concentration on the growth of algal cells and changes of physiological index, such as chlorophyll *a*, carotinoid, protein content and the production of microcystins in toxic *M. aeruginosa* were not statistically significant from media control.

The toxicity of a cyanobacterial strain may be altered depending on varying culture conditions (Doers and Parker, 1988). Environmental stimuli (e.g., a chemical signal or feeding related activity of fish) could also cause levels of toxin expression in *Microcystis* strains to vary, and each strain showed a different extent of variation (Jang et al., 2003, 2004). Our study showed that NP had a certain extent influence on microcystin production of the two toxic monoclonal *M. aeruginosa* strains. Toxic production in PCC7820 with 0.05–0.5 mg/L NP increased obviously. The highest toxin level occurred in the treatment with 0.2 mg/L NP was 75% higher than control. However, toxic production in 562 was not affected by NP evidently. The highest toxin production in 562 with 0.02–0.05 mg/L NP was only 10% higher than control. This demonstrated that the promotion of NP on microcystin production of *M. aeruginosa* was strain dependence.

Microcystin excretion is a much debated subject since dissolved microcystin contributes up to 40% of total toxin in some experiments (Orr and Jones, 1998; Jähnichen et al., 2001) but amounts to less than 5% in other experiments (Rapala et al., 1997; Lyck and Christoffersen, 2003; Wiedner et al., 2003). These extremes were observed in our experiment: 75.6% microcystins of *Microcystis* PCC7820 without NP treatment were dissolved into culture media after 12 days' incubation, whereas only 0.39% microcystins of the control of strain 562 were released. This indicated that microcystin releasing was species specific. Our study clearly showed that NP could influence microcystin releasing. 33.0–90.4% microcystins of PCC7820 with different concentration of NP were released into cultural medium, whereas 0.06–0.40% microcystins of 562 were released. Microcystins usually remain in cyanobacteria and are only released in the water due to cell lysis (Lahti et al., 1997). Increased extracellular microcystin concentrations after exposure to high light intensities have usually been attributed to cell lysis and subsequent leakage of the peptide (Lehtimaeki et al., 1994; Rapala et al., 1997). Strain PCC7820 with 2 mg/L NP releasing 90.4% of its microcystins into solution demonstrated that toxicity of NP on *M. aeruginosa* might have destroyed the structure of algal cell and released microcystin molecular. It is assumed that microcystin excretion increases as the cell number in the culture reaches a maximum (Lyck, 2004). However, in our study, only 33.0% of microcystins in PCC7820 treated with 0.2 mg/L NP were released into the solution, which the growth of algal cell was obviously promoted. Microcystins releasing in 562 with 0.05 and 0.1 mg/L NP treatment also tended to decrease. The reason is not clear. The observation by Shi et al. (1995) of some localization of microcystin in the cell wall and sheath suggest that microcystin may act as transmembrane transporters of calcium, magnesium or other metals. Stimulation of membrane receptors is believed to be a cause for the activation of cell functions. Under stronger actions, the activity of membrane receptors is suppressed (Kuzin, 1995). Study of Okai et al. (2000) suggest that one major target site of NP-induced reactive oxygen species (ROS) attack seems to be lipids in the cellular membrane. Therefore, we speculated that NP might have taken part in the physiological activity in algal cell and have influenced the cell membrane receptors. Further experiments are needed to clarify the problem.

In summary, this is the first report on the effects of NP, a persistent organic pollutant and endocrine disrupter on the growth and microcystin production of *M. aeruginosa* strains. The effects were NP concentration dependent and strain-specific. Stimulatory effects of *M. aeruginosa* by low concentration of NP (0.02–0.2 mg/L) might be the effect of hormesis, and further studies are needed to investigate the possible relationship between such hormesis effect with cyanobacteria bloom. The finding of this study that NP enhanced growth and toxin production of *M. aeruginosa* at low concentrations of 0.02–0.2 mg/L that can be found in natural freshwaters (Ahel et al., 1994; Bennie, 1999; Johnson et al., 1998; Kvestak and Ahel, 1994) might imply that low concentration of NP favor survival of toxic *M. aeruginosa* in the field. At the mean time, microcystin releasing also influenced by NP hint us that NP and may include other POPs have taken part in the process of microcystin synthesis and releasing. Further studies are warranted to clarify if organic pollutants like POPs play a subtle role in affecting cyanobacterial blooms and microcystin production in natural waters.

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