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PAHs would alter cyanobacterial blooms by affecting the microcystin production and physiological characteristics of *Microcystis aeruginosa*

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ABSTRACT

The wide presence of polycyclic aromatic hydrocarbons (PAHs) in lakes necessitates a better understanding of cyanobacteria metabolites under the contamination of PAHs. The *M. aeruginosa* strain PCC7806 was selected to investigate the effects of naphthalene and pyrene on the physiological and biochemical reactions of cyanobacteria, including antioxidant defense system (superoxide dismutase, catalase), intracellular microcystin (MC) content, phycobiliprotein (phycocyanin, allophycocyanin) contents, and specific growth rate. Naphthalene and pyrene altered the growth of the *M. aeruginosa* strain, reduced the contents of phycocyanin and allophycocyanin, and stimulated the activities of antioxidant enzymes without lipid peroxidation. Remarkably, the intracellular MC content was significantly increased by 68.1% upon exposure of *M. aeruginosa* to 0.45 mg L^{-1} naphthalene, and increased by 51.5% and 77.9% upon exposure of *M. aeruginosa* to 0.45 mg L^{-1} pyrene and 1.35 mg L^{-1} pyrene, respectively ($P < 0.05$). Moreover, significant correlations were observed between these physiological reactions, referring that a series of physiological and biochemical reactions in *M. aeruginosa* worked together against the PAH contamination. Considering that MCs are the most studied cyanobacterial toxins, our results clarified that the promoting MC production by PAH contamination cannot be neglected when making related risk assessments of eutrophic waters.

1. Introduction

Toxic cyanobacterial bloom, which not only causes taste and odor problems but also produces toxins, has become a widespread and critical problem in aquatic environments all over the world (Ding et al., 1999). Among the dominant cyanobacteria, *Microcystis* is the most widespread cyanobacterial genus in eutrophic lakes and is known to produce cyanobacterial hepatotoxins termed microcystins (MCs) (Carmichael, 1992). MC contamination in eutrophic lakes can be accumulated in fish, bivalves and other invertebrates and then cause harmful effects to human health by transference through the food web. Previous research has suggested that MCs can cause liver damage by inhibiting essential enzymatic functions in hepatic tissue (Yoshizawa et al., 1990).

MC concentrations in water bodies depend not only on the abundance of toxin-producing *Microcystis* strains but also on the MC production by toxigenic strains (Yang et al., 2015). The population of cyanobacteria (Pomati et al., 2004; Parsons et al., 2015) and MC production (Wang et al., 2007; Al-Ammara et al., 2015) can be affected by some environmental stresses, including pollutants. Al-Ammara et al. (2015) noted that some environmental factors could alter MC concentrations by affecting the abundance of toxin-producing strains in a cyanobacterial population and/or their toxin production (Al-Ammara et al., 2015). As a ubiquitous toxic freshwater cyanobacterium isolated from a lake in China, the *M. aeruginosa* strain PCC7806 has usually been used to investigate the ecophysiology of *M. aeruginosa* (Downing et al., 2005; Frangeul et al., 2008).

Significant levels of PAHs, which are common constituents of combustion residues, have been detected in lakes in China (Lu et al., 2010). Even at low dosages, the exposure of *M. aeruginosa* to PAHs would have effects on its growth (Stoichev et al., 2011). In the surface layers of Taihu lake, corresponding to sedimentation from 1980 to 1990, the surface sediments of ΣPAH_s concentrations were 1180 and 530 μg kg^{-1} in Meiliang Bay and Xukou Bay, respectively (Liu et al., 2009). Meanwhile, PAHs in sediments have a profound deleterious effect on benthic communities (Bennett et al., 2000). Although growth inhibition (in term of specific growth rates) of *M. aeruginosa* by several contaminations has been widely recognized as direct evidence of contamination (Cerezo et al., 2015), reduction of cell growth was only observed at specific levels of PAHs. PAHs could promote the growth of *M. aeruginosa* at specific levels (Zhu et al., 2012), suggesting that colonies of *M. aeruginosa* have a high tolerance to PAH contamination. Thus, increasing PAH contamination in an aquatic system might alter the occurrence of cyanobacterial blooms and MC contamination in lakes, which could not be neglected as making risk assessments of eutrophic waters.

Due to the poor understanding of the effects of PAHs on *M. aeruginosa*, related risk assessments for PAHs and cyanobacterial blooms are significantly complicated. To better elucidate the bloom sustainment of colonial *M. aeruginosa* in eutrophic reservoirs and lakes, the physiological and biochemical responses of *M. aeruginosa* to PAH contamination should be clarified. The present study investigated the effects of PAHs on a series of physiological and biochemical processes of the *M. aeruginosa* strain PCC7806 to determine the main factors of PAHs affecting cyanobacterial blooms.

2. Material and methods

2.1. Experimental design

The *M. aeruginosa* strain PCC7806 used in this study was provided by the Institute of Hydrobiology, Chinese Academy of Sciences.

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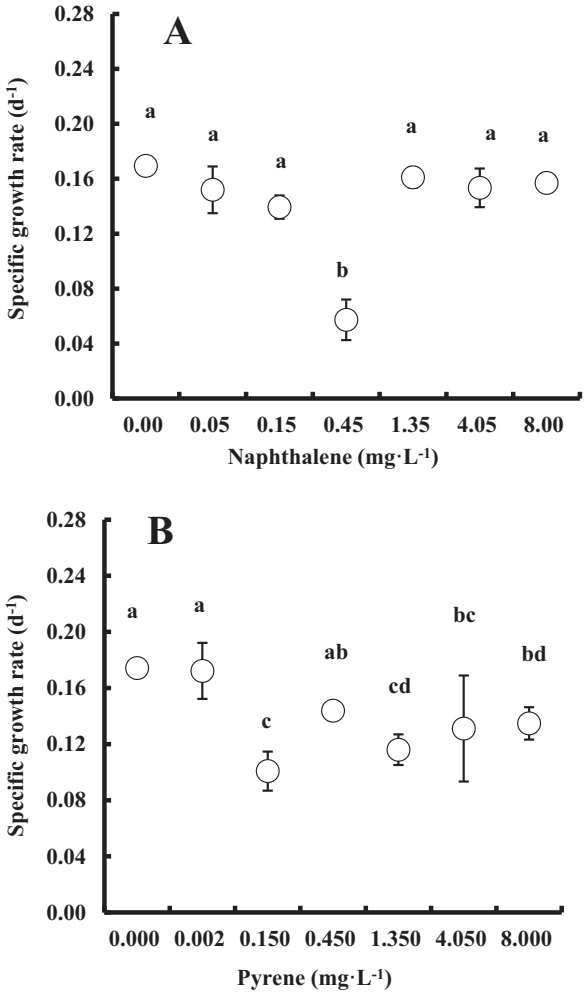

Fig. 1. The growth of the *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

Naphthalene and pyrene were selected as representative PAHs. The exposure media were prepared by adding different levels of naphthalene (0 mg L^{-1}, 0.05 mg L^{-1}, 0.15 mg L^{-1}, 0.45 mg L^{-1}, 1.35 mg L^{-1}, 4.05 mg L^{-1}, respectively) or pyrene (0 mg L^{-1}, 0.002 mg L^{-1}, 0.15 mg L^{-1}, 0.45 mg L^{-1}, 1.35 mg L^{-1}, 4.05 mg L^{-1}, respectively) in 500-mL flasks containing 300 mL of blue-green medium (BG11). The lowest exposure levels of PAHs were designed based on the average PAH levels in lakes in China (Liu et al., 2009), and the NOEC values of PAHs were measured in terms of their population growth inhibition of *Scenedesmus subspicatus*, a green alga (Djomo et al., 2004). The stock solution of PAHs was prepared in methanol and used in all the exposure experiments. The methanol level in the blue-green medium (BG11) did not exceed 0.01%, at which level methanol has no effects on the growth of *M. aeruginosa*. The same methanol levels were added to the control. Each treatment was performed in triplicate. The exponentially growing cells were used as inocula for the start of the experiments (the initial cell density was approximately 10^4 cells·mL^{-1}). For testing the acute toxicity of the PAHs, after 120 h of incubation, specific volumes of algal suspensions were retrieved for further measurement (Chen et al., 2016a, 2016b).

All cultures were incubated at 25 ± 1 °C under cool white fluorescent light at an intensity of 1600 lx with a light: dark period of 12 h:12 h. The BG11 medium contained 0.075 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.036 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.006 g L^{-1} citric acid, 0.006 g L^{-1} ferric citrate, 0.001 g L^{-1} EDTA sodium salt, 0.02 g L^{-1} Na_2CO_3, and 1 mL of trace elements mix (containing in g·L^{-1}: H_3BO_3, 2.86; $MnCl_2{\cdot}2H_2O$, 1.81; $ZnSO_4{\cdot}7H_2O$, 0.222; $Na_2MoO_4{\cdot}2H_2O$, 0.39; $CuSO_4{\cdot}5H_2O$, 0.079; and $Co(NO_3)_2{\cdot}6H_2O$, 0.0494) (Stanier et al., 1971).

2.2. Cell density analyses

The biomass of *M. aeruginosa* was determined by measuring their optical density (OD) at 450 nm with a spectrophotometer (Downing et al., 2005; Wang et al., 2017), as the Eq. (1):

$$N = 70.575 \quad OD_{450} + 6.323, \tag{1}$$

where N represents the cell numbers (cells mL^{-1}), and OD_{450} are the optical density (OD) at 450 nm with a spectrophotometer.

For testing the growth rate of *M. aeruginosa*, specific volumes of algal suspensions were retrieved for measuring the density of cells at 0, 24, 48, 72, 96, 120, 144 h after cultivation. The specific growth rate of the *M. aeruginosa* strain is identified as the correlation slope of cell densities and the cultivation time, and was computed by the regression analysis as Eq. (2) (Al-Ammara et al., 2015) for the period between samplings therefore representing the mean μ for that period.

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}, \tag{2}$$

where μ represents the algal specific growth rate, d^{-1}, and N_0 and N_n are the cell numbers at time t_0 (beginning of the test) and time t_n, respectively (cells mL^{-1}).

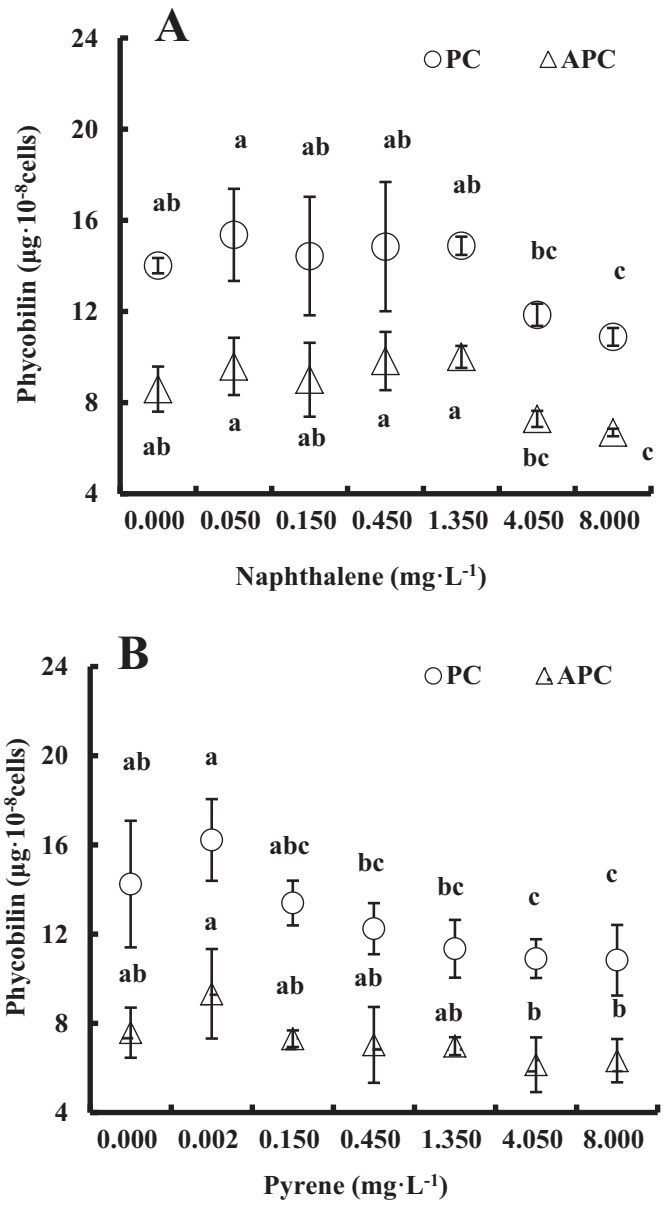

Fig. 2. The levels of PC and APC in the *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

2.3. Intracellular microcystin extraction and analyses

Cells were harvested by centrifugation at 6000 rpm for 15 min, frozen in liquid N_2, and thawed at 4 °C for 3 cycles in methanol-water solution (75:25, v-v). The cells were then sonicated for 30 min and centrifuged for 10 min at 6000 rpm. The cell pellets were extracted thrice. All the supernatants were pooled and diluted with distilled water. The distilled supernatants were filtered through Mixed Cellulose Ester-Microporous Filtering Membranes (0.45 μm) and then concentrated using solid phase extraction cartridges (C18, 0.5 g), eluted with 100% (0.1% TFA) methanol. These were then blown dry using nitrogen at 40 °C, following which the residue was re-suspended in 2 mL of methanol-water solution (75:25, v-v) prior to HPLC analysis (Yu et al., 2015).

MCs were analyzed using high-performance liquid chromatography (HPLC, LC-20AT; Shimadzu, Kyoto, Japan) equipped with a 4.6 × 250-mm Zorbax SB2C18 column with methanol-water (75:25, v-v) as the mobile phase at a flow rate of 0.6 mL min^{-1}. The chromatography was performed at 40 °C using a detection wavelength of 238 nm (Barco et al., 2005; Vichi et al., 2012; Yang et al., 2014).

The amount of MC-LR was quantified by the external standard quantification procedure, referring to a calibration curve (correlation coefficient $R^2 = 0.9997$), obtained with 7 known amounts (from 0.1 to 5 mg L^{-1} in duplicate) of the MC-LR analytical standards (Sigma, München, Germany).

2.4. Oxidative damage and antioxidant responses

The cells were homogenized in a 0.05 mol mL^{-1} phosphate buffer (pH 7.4) by an ultrasonic cell pulverizer at 200 W for 10 min under ice-bath cooling. Then, the homogenate was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant, cell-free enzyme extract, was used for enzymatic and non-enzymatic antioxidant measurements. Superoxide dismutase (SOD) and catalase (CAT), which accompanied the oxidative stress on cyanobacteria elicited by PAHs (Vega-López et al., 2013), were selected to assess the antioxidant defenses in this study. SOD activity was measured using a total superoxide dismutase assay kit with WST-1 (Dojindo, China) according to the manufacturer's instructions. CAT

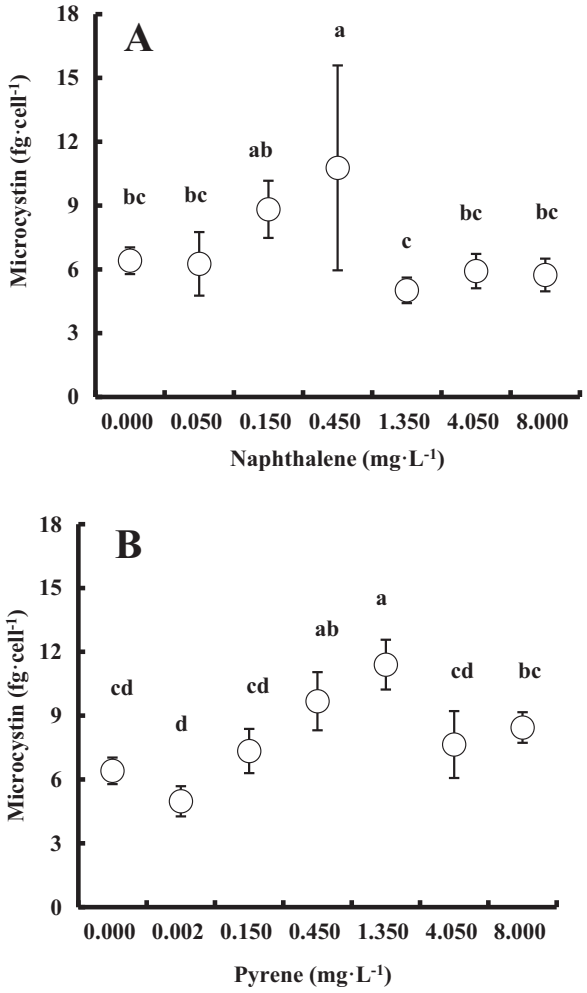

Fig. 3. Levels of intracellular MC-LR in the *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

activity was measured by H_2O_2 decomposition at 240 nm according to (Hong et al., 2009). One unit of CAT was defined as the decrease in absorbance at 240 nm up to 0.1 per min. The activities of these enzymes were expressed on a basis of protein content to eliminate the measurement deviation caused by the varied extraction efficiency of protein. The total protein content of the supernatant was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as the protein standard (Bradford, 1976).

The level of lipid peroxidation was expressed as the content of malondialdehyde (MDA) (Hong et al., 2009). The algal cells from each treatment were homogenized in 5 mL of 10% (w: v) trichloroacetic acid (TCA), centrifuged at 4000 rpm for 10 min. Then, 2 mL of thiobarbituric acid (TBA) reagent (0.6% in 10% TCA) was added to each 2-mL aliquot of the supernatant. The mixtures were heated at 100 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 4000 rpm for 10 min, the absorbance of the supernatant was read at 450 nm, 532 nm and 600 nm. The MDA content was calculated using Eq. (3).

$$C_{MDA} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} \quad (3)$$

where C_{MDA} represents the MDA content (μmol L^{-1}) and A_{450}, A_{532}, and A_{600} represent the absorbance values detected at 450 nm, 532 nm and 652 nm, respectively.

2.5. Phycocyanin and allophycocyanin

For phycobiliprotein analysis, the exponentially grown cells were repeatedly frozen 3 times in liquid N_2 and thawed at 4 °C in the presence of a 0.05 mol mL^{-1} phosphate buffer (pH 6.7). The samples were centrifuged at 10,000 rpm for 10 min, and after the samples were kept in the dark for 24 h, the absorbance of the supernatant was determined. The concentrations of phycocyanin (PC) and allophycocyanin (APC) were calculated according to Bennett and Bogorad (1973).

$$C_{(PC)} = [1000 \cdot (A_{615} - 0.474 \times A_{652})/5.34]/Nn \quad (4)$$

$$C_{(APC)} = [1000 \cdot (A_{652} - 0.208 \times A_{615})/5.09]/Nn \quad (5)$$

where $C_{(PC)}$ and $C_{(APC)}$ represents the concentration of PC and APC (μg·10^{-8}cells), respectively, N_n represents cell density values (cells·mL^{-1}), and A_{615} and A_{652} represent the absorbance values detected at 615 nm and 652 nm, respectively.

2.6. Statistical analyses

The statistical analysis of data was performed using SPSS 16.0 for Windows (SPSS Inc. Chicago, USA). The statistical significance of the data was tested with one-way analysis of variance (ANOVA), with the significance level set at 0.05. Pearson correlation coefficients were calculated between the growth parameters and the concentrations of PAHs.

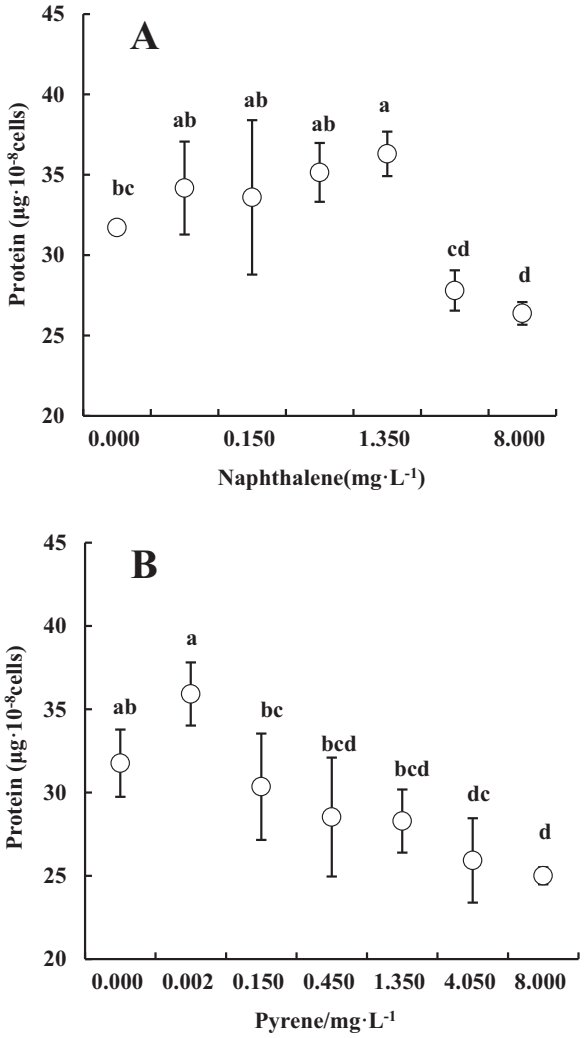

Fig. 4. Levels of protein in *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

3. Results

3.1. Growth of M. aeruginosa

The growth of the *M. aeruginosa* strain was significantly inhibited for the strain grown in the media containing 0.15 mg L^{-1} naphthalene or more than 0.45 mg L^{-1} pyrene (Fig. 1). The maximum inhibition was observed for those *M. aeruginosa* grown in the media containing 0.45 mg L^{-1} naphthalene or 0.15 mg L^{-1} pyrene. Compared to the control, the specific growth rate of the *M. aeruginosa* exposed to 0.45 mg L^{-1} naphthalene was significantly decreased by 55%, and the specific growth rate of *M. aeruginosa* exposed to 0.15 mg L^{-1} pyrene was also significantly decreased by 42.2% ($P < 0.05$).

3.2. Phycocyanin (PC) and allophycocyanin (APC)

The quantity and composition of phycobiliproteins were significantly modified by naphthalene and pyrene, when their concentrations were higher than 4.05 mg L^{-1} in the media (Fig. 2). There were significant negative correlations between the concentrations of naphthalene/pyrene and the levels of PC/APC in *M. aeruginosa* ($P < 0.05$). APC content was significantly negatively correlated with the levels of naphthalene ($\rho = -0.695^{**}$) and pyrene ($\rho = -0.445^{**}$), and PC content was significantly negatively correlated with the levels of naphthalene ($\rho = -0.700^{**}$) and pyrene ($\rho = -0.555^{**}$). Compared to the control, high level of naphthalene (8 mg L^{-1}) significantly reduced the content of PC by 22.3%, and the content of APC by 22.3%. High levels of pyrene (4.05 mg L^{-1}, 8 mg L^{-1}) significantly decreased the content of APC by 16.6–19.0%. Moreover, the ratio of APC/PC was also increased by both naphthalene and pyrene.

3.3. Intracellular Microcystin

The MC content ranged from 4.974 fg $cell^{-1}$ to 11.397 fg $cell^{-1}$ in the *M. aeruginosa* cells grown in the medium containing pyrene. It should be noted that the MC content was increased with the increasing pyrene level ($\rho = 0.777^{**}$), as the pyrene concentration was less than 1.35 mg L^{-1}. Meanwhile, the intracellular MC content was significantly increased by 68.1% upon exposure of *M. aeruginosa* to 0.45 mg L^{-1} naphthalene, and increased by 51.5% and 77.9% upon exposure of *M. aeruginosa* to 0.45 mg L^{-1} pyrene and 1.35 mg L^{-1} pyrene, respectively ($P < 0.05$). Under high-level contamination of PAHs, the content of MC increased more in the *M. aeruginosa* cells exposed to pyrene than that in the *M. aeruginosa* cells exposed to naphthalene (Fig. 3).

3.4. Oxidative damage and antioxidant responses

The protein content in the *M. aeruginosa* strain was significantly increased by the low level of naphthalene and significantly decreased by the high levels of naphthalene and pyrene (Fig. 4) ($P < 0.05$). The

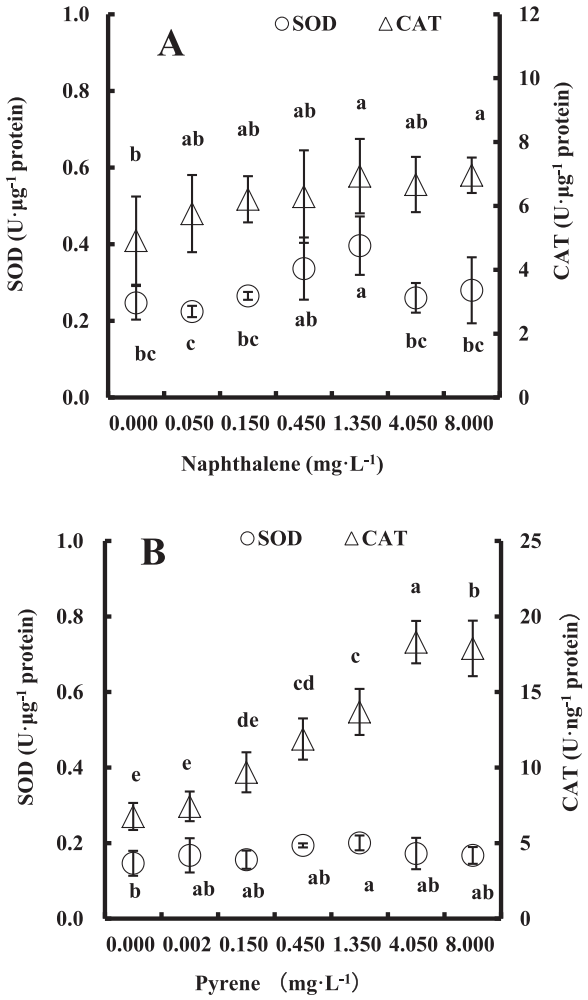

Fig. 5. The activities of SOD and CAT in *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

lowest protein content was observed in the *M. aeruginosa* strain exposed to 8.0 mg L^{-1} pyrene. The total protein contents in *M. aeruginosa* were significantly reduced by exposure to naphthalene (≥4.05 mg L^{-1}) or pyrene (≥4.05 mg L^{-1}) and were negatively correlated to the levels of naphthalene ($\rho = -0.731^{**}$) and pyrene ($\rho = -0.659^{**}$). On the other hand, the concentration of protein was significantly increased by 14.5% after exposure to 1.35 mg L^{-1} naphthalene ($P < 0.05$). Phycobiliproteins were also reduced with the decreasing total protein in *M. aeruginosa*, and their co-relations were significant.

As antioxidant defense enzymes, the activities of SOD and CAT were significantly enhanced in the *M. aeruginosa* cells (Fig. 5). In particular, a significant positive correlation was found between the activity of CAT and the pyrene level ($\rho = 0.749^{**}$). The activity of SOD significantly increased by 60.4% ($P \leq 0.05$) upon exposure to 1.35 mg L^{-1} naphthalene and by 36.9% ($P \leq 0.05$) upon exposure to 1.35 mg L^{-1} pyrene. The maximal activity of CAT in *M. aeruginosa* was 17.89 U·μg^{-1} protein (increased by 164.5%) upon exposure to 4.05 mg L^{-1} pyrene ($P \leq 0.05$).

The malondialdehyde (MDA) content remained stable in the *M. aeruginosa* strain exposed to naphthalene and pyrene (Fig. 6). The stable MDA content, which is an important index of lipid peroxidation to reflect cellular oxidative damage, suggested that naphthalene and pyrene did not initiate lipid peroxidation in *M. aeruginosa*. The results also referred that the stimulated antioxidant defense in the *M. aeruginosa* strain was efficient in eliminating ROS, and preventing the algal cells from oxidative damage (as shown in Fig. 5).

4. Discussion

M. aeruginosa cells could eliminate or reduce the toxicity of superoxide radicals produced by PAH contamination as Giannuzzi et al. (2016) reported. Naphthalene and pyrene increased the levels of ROS due to the increased activities of SOD and CAT, according to the research reported by Turja (Turja et al., 2014). And MDA is an important index of lipid peroxidation to reflect cellular oxidative damage (Zhang et al., 2011; Chen et al., 2016a). The stable MDA content indicated that there was no cellular oxidative damage in the *M. aeruginosa* cells exposed to PAHs. Meanwhile, these results also suggested that the increased activities of SOD and CAT in *M. aeruginosa* cells are effective in eliminating ROS and protecting the *M. aeruginosa* strain from the oxidative damage caused by naphthalene and pyrene.

In this study, the alteration differed slightly between SOD and CAT. Compared to the activity of CAT, no significant effects of high level PAHs on the activity of SOD might result from increasing hydrogen peroxide, which would inhibit the SOD activity of *M. aeruginosa* cells (Wang et al., 2016). SOD, which is responsible for converting superoxide radicals into either ordinary molecular oxygen or hydrogen peroxide, is normally regarded as the first defense against ROS. CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen. Moreover, in the *M. aeruginosa* strain exposed to pyrene, there were significant correlations between the phycobiliproteins and the activity of SOD ($\rho = 0.587^{**}$ for PC, $\rho = 0.694^{**}$ for APC) / CAT ($\rho = -0.685^{**}$ for PC, $\rho = -0.525^{**}$ for APC) and significantly negative correlations

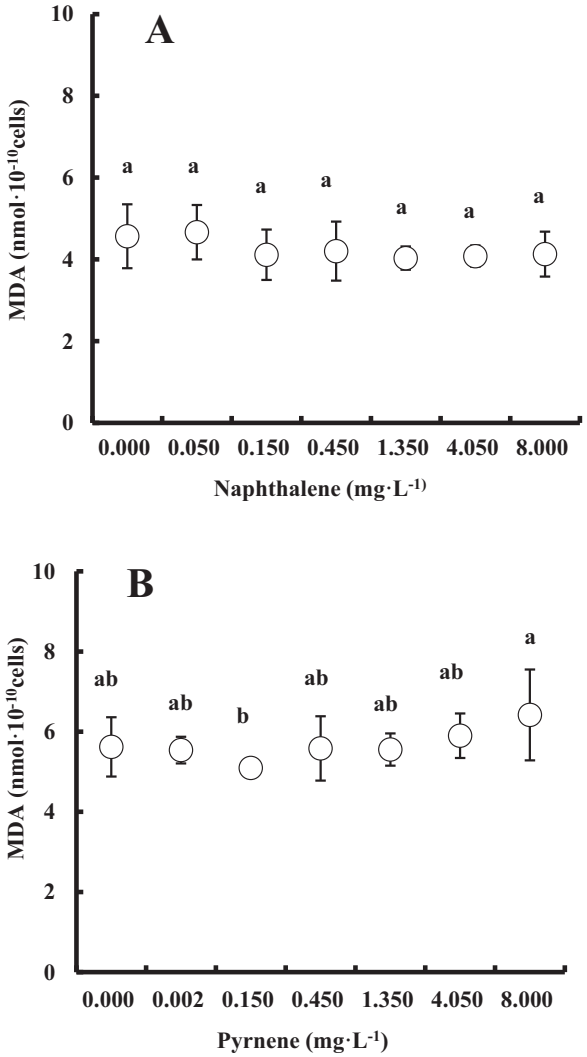

Fig. 6. The levels of MDA in the *M. aeruginosa* strain PCC7806 exposed to naphthalene (A) and pyrene (B). Data are means ± SD of the three replicates. Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

between intracellular MCs and APC ($\rho = -0.400^{**}$) / TP ($\rho = -0.567^{**}$), which referred that phycobiliproteins and MCs were also involved in the antioxidant defense of *M. aeruginosa* to pyrene contamination. The reduction of phycobiliproteins is a general acclimation response occurring under PAH contamination as various stress conditions reported by Lafabrie (Lafabrie et al., 2013). Phycobiliproteins are reported to be good antioxidants under oxidative stress due to their nucleophilic ability to neutralize reactive oxidants (Cano-Europa et al., 2010), and they are both sources and targets of singlet oxygen ($^1O_2$), with consequent loss of the macromolecular biological functions (Giannuzzi et al., 2016). In this study, MCs in the *M. aeruginosa* cells played a role as a protectant against oxidative stress, stabilizing the photosynthetic apparatus, and protein-modulating metabolites as previous reports (Zilliges et al., 2011; Yang et al., 2015). Remarkably, the intracellular MC content was found to be increased with increasing pyrene contamination ($p = 0.777^{**}$), which inferred that the increasing production of MC and the reduction of PC and APC in *M. aeruginosa* cells were attributable to the protection against the stress caused by pyrene. Unfortunately, MCs are the most commonly occurring class of cyanobacterial toxins (Carmichael, 1992) and can cause serious health problems (Yoshizawa et al., 1990; Nishiwaki-Matsushima et al., 1992).

Moreover, some other significant correlations showed that many physiological reactions worked together against the PAH contamination. The concentration-dependent decrease in the total protein in the *M. aeruginosa* strain exposed to naphthalene ($\rho = -0.731^{**}$) / pyrene ($\rho = -0.659^{**}$) showed that, similar to other pollutants (Carfagna et al., 2013; Chia et al., 2015), naphthalene and pyrene inhibited the protein synthesis in cyanobacteria. Since sub-lethal concentrations of PAHs could disturb cyanobacteria cell division by reducing DNA synthesis and decreasing the percentage of cells entering mitosis and then reducing population growth and protein production (Cerezo and Agustí, 2015), the decreased growth observed in this study might also result from the inhibition of protein synthesis in the *M. aeruginosa* strain. Additionally, with the addition of the negative effects of naphthalene and pyrene on APC/PC, which are the principal light-harvesting antenna pigments of cyanobacteria (Sampath-Wiley et al., 2008), naphthalene and pyrene would reduce carbon dioxide assimilation and thus inhibit the growth of *M. aeruginosa*.

It can be concluded that exposure of *M. aeruginosa* to pyrene would elevate the levels of ROS in algae cells without lipid peroxidation but that the increasing ROS would result in a decrease in phycobiliproteins and an increase in intracellular MC contents.

5. Conclusions

Overall, both naphthalene and pyrene significantly affected the growth of the *M. aeruginosa* strain PCC7806 and promoted MC production, which resulted from the decreasing levels of phycobiliproteins

and increasing activities of antioxidant enzymes. Since MCs are the most commonly occurring class of cyanobacterial toxins and can cause serious health problems, the effects of PAH contamination on cyanobacterial bloom cannot be neglected when assessing eutrophic lakes.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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