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Characteristics of growth and microcystin production of *Microcystis aeruginosa* exposed to low concentrations of naphthalene and phenanthrene under different pH values

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ABSTRACT

Here, *Microcystis aeruginosa* (*M. aeruginosa*) was studied to analyze the effects of 0.5 mg L^{-1} naphthalene and 0.05 mg L^{-1} phenanthrene on profiles of cell growth, chlorophyll-*a* content and Microcystin-LR (MC-LR) production at different pH values. The results indicated that for both the naphthalene and phenanthrene treatments, the specific growth rates were higher in pH 10.0 than in either pH 7.0 or pH 5.0. In the presence of low concentrations of naphthalene or phenanthrene, chlorophyll-*a* in medium increased significantly more in pH 10.0 than pH 5.0. chlorophyll-*a* in cell was significantly lowered when exposed to naphthalene in both pH 10.0 and pH 7.0, and was higher when exposed to phenanthrene in pH 10.0 than pH 5.0. HPLC analysis revealed that the extracellular MC-LR concentrations in *M. aeruginosa* exposed to either naphthalene or phenanthrene were lower than in control *M. aeruginosa* at pH 5.0. The intracellular MC-LR levels in toxic *M. aeruginosa* cells exposed to naphthalene or phenanthrene were higher than in the controls at pH 10.0. Our study suggests that the MC-LR production of *M. aeruginosa* was affected by the pH value when low concentrations of either naphthalene or phenanthrene were present in the water. These results indicate that the pH value should not be ignored when evaluating the risk of chemicals that promote MC-LR production in eutrophic waters.

1. Introduction

Blooms of the cyanobacterium *Microcystis* sp. have become severe and widespread in eutrophic lakes, ponds and reservoirs, thereby causing critical environmental problems in many countries (Lin et al., 2018; Sun et al., 2016). Some *Microcystis* species are known to produce microcystins (MCs), which are cyclic hepatotoxic heptapeptides with over 85 natural structural variants that cause health problems in terrestrial and aquatic organisms (Carmichael, 1992). MCs produced by cyanobacteria occur worldwide (Chen et al., 2005) and have been recognized as potent liver toxins and tumor promoters (Lankoff et al., 2004; Carmichael, 1994). Furthermore, MCs have been blamed as one of the main contributors to the high incidence of human liver tumors in southern China (Yu, 1995; Ueno et al., 1996). *Microcystis aeruginosa* (*M. aeruginosa*) is one of the most common freshwater cyanobacteria in nature that produces MCs. (Chen et al., 2009).

It has been reported that the production of toxigenic MCs strains is regulated by environmental factors, such as temperature (Tao et al., 2012), light intensity (Kaebernick et al., 2000), nutrient concentration (especially nitrogen and phosphorus) (Che et al., 2018; Sevilla et al., 2008; Jahnichen et al., 2011) and pH value (Song et al., 1998). In recent years, many increasingly abundant organic environmental pollutants are contributing to the growth of cyanobacteria in lakes and rivers (Li et al., 2009). Both the cyanobacterium species and their production of MCs can be affected by the types of available organic environmental pollutants (Wang et al., 2007).

Significant concentrations of Polycyclic Aromatic Hydrocarbons (PAHs), an important class of environmental pollutant, have been detected in many lakes and rivers in China (Lu et al., 2010). In Taihu Lake, the ΣPAH concentrations of surface sediment are 1180 and 530 μg kg^{-1} in Meiliang Bay and Xukou Bay, respectively (Liu et al., 2009). It is reported that the dry weight of ΣPAH levels in the sediment in some parts of Taihu Lake range from 1207 to 4754 μg kg^{-1} (Qiao et al., 2006).

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It has been reported that low concentrations of PAHs act as growth-promoting factors for *M. aeruginosa*, which indicates that *M. aeruginosa* has a high tolerance to PAH pollution (Zhu et al., 2012). A recent study found that PAHs can exacerbate the harms of algal blooms by promoting the growth of *M. aeruginosa* and the production of MCs (Zhang et al., 2018). Djomo et al. reported that algae cell culture conditions affect how toxic the effects of PAHs are on *Scenedesmus subspicatus* (Djomo et al., 2004). Thus, the presence of PAHs in water is not only a direct threat to human health, but it is also an indirect hazard because PAHs promote the growth of cyanobacteria and the generation of specific toxins from cyanobacterial blooms.

Here, a laboratory incubation of *M. aeruginosa* was cultivated in a medium with a low concentration of either naphthalene (NAP) or phenanthrene (PHE), which are two types of PAH, under varying pH values to investigate how PAHs affect growth and microcystin production in blooms at different pH values.

2. Materials and methods

2.1. Reagents

The NAP and PHE (purity 98%) were obtained from the Aldrich Chemical Company. MC-LR was from Sigma (Agent technology Co. Ltd, Germany). Other reagents and chemicals applied in this study were obtained from the Sinopharm Chemical Reagent Co. (Shanghai, China).

2.2. Experimental design

In this study, *M. aeruginosa* was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Experiments were carried out at three different pH values (pH = 5.0, 7.0 and 10.0). The pH of the medium was adjusted using HCl or NaOH. The experimental media were prepared by adding 0.5 mg L^{-1} of NAP or PHE to 500-mL flasks containing 300 mL of medium. The concentrations of either NAP (0.5 mg L^{-1}) or PHE (0.05 mg L^{-1}) in the experimental media were meant to mimic the PAH levels of lakes in China (Liu et al., 2009). The no observed effect concentration (NOEC) values were analyzed to see if they inhibited population growth in the green alga (Djomo et al., 2004). The stock solutions of NAP and PHE were prepared in methanol and used for all experiments. The methanol concentration in the medium (BG11) was lower than 0.01% and had no toxic effect on *M. aeruginosa*. In the experiment, the solution with no methanol was considered control 1 (CK1), whereas the solution with methanol was considered control 2 (CK2). Each experiment was repeated three times. The exponentially-growing *M. aeruginosa* were used as inocula to start the experiments. Specific volumes of algal cell suspensions were harvested to perform additional tests regarding the toxic effects of NAP and PHE after 7 d of incubation (Chen et al., 2016a, 2016b).

The method of culturing *M. aeruginosa* was proposed by Stanier et al. (1971). *M. aeruginosa* was grown in culture medium Blue-Green Medium 11 (BG11) at 25 °C under cool white fluorescent light at an intensity of 40 μmol quanta·m^{-2} s^{-1}, with a light:dark cycle of 12:12 h. The experiments were performed for seven days, also at 25 °C under a 12:12 light:dark cycle, with light provided at an intensity of 40 μmol quanta·m^{-2} s^{-1}. Each treatment and control was repeated three times.

The BG11 medium contained gL^{-1}: $MgSO_4{\cdot}7H_2O$, 0.075; $CaCl_2{\cdot}2H_2O$, 0.036; citric acid, 0.006; ferric-citrate, 0.006; EDTA sodium salt, 0.001; and Na_2CO_3, 0.02, as well as 1 mL of a trace-element mixture composed of gL^{-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. Samples were collected after seven days of culturing, and they were divided into subsamples to determine cell counts (Cell Analyzer, Muse, Darmstadt, Germany), chlorophyll-*a*, and extracellular and intracellular MC-LR levels.

2.3. Cyanobacterium growth assay

The method employed to determine *M. aeruginosa* biomass in each treatment was created by Wang et al. (2017), where the optical density (OD) was measured under a microscope with a spectrophotometer at 450 nm (TU-1810, Puxi Co., China). Then Eq. (1) was calculated:

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

where N represents the cell count (cells mL^{-1}), and OD 450 is the optical density (OD) at 450 nm under a microscope.

To test the growth rate of *M. aeruginosa*, specific volumes of algal suspensions were retrieved to measure the change in density of cells after cultivation. The specific growth rate of the *M. aeruginosa* strain is identified as the correlation slope of cell density and cultivation time. This was calculated using the regression analysis in Eq. (2) (Al-Ammara et al., 2015) for the period between samplings, so it therefore represents the mean μ for that period.

$$\mu = \frac{\mathrm{In}N_n - \mathrm{In}N_0}{t_n - t_0} \tag{2}$$

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 (end of the test), respectively (cells mL^{-1}).

M. *aeruginosa* generation time was computed with Eq. (3), where G is the generation time of *M. aeruginosa* (d).

$$G = \frac{0.693}{\mu} \tag{3}$$

2.4. Measurement of chlorophyll-a

Chlorophyll-*a* was quantified using spectrophotometry. First, a certain volume of the test solution was concentrated via suction filtration. Next, the algal cells on the filter membrane were frozen and thawed several times, and then they were extracted with acetone. After centrifugation at 10,000 × *g*, acetone was used to determine the absorbance of the extract solution at a wavelength of 665 nm (TU-1810, Puxi Co., China). The amount of chlorophyll-*a* in this medium ($M_{\text{chlorophyll-a}}$, μg mL^{-1}) was computed with Eq. (4):

$$M_{chlorophyll-a} = 13.9 \times OD_{665} \tag{4}$$

The amount of chlorophyll-*a* in the cell ($C_{\text{chlorophyll-a}}$, μg mL^{-1}) was computed with Eq. (5):

$$C_{\text{chlorophyll-a}} = \frac{M_{\text{chlorophyll-a}}}{N_7} \tag{5}$$

where N_7 is the cell count on day seven.

2.5. MC-LR extraction and analysis

The dynamics of the extracellular and intracellular MC-LR of the toxic strain were analyzed in the monoculture experiment. Aliquots of 20 mL were filtered through glass microfiber filters (Whatman GF/C; pore size: 1.2 μm). The filtrates were then enriched with octadecylsilyl (ODS) cartridges and subsequently eluted with 10 mL of methanol solution (containing 0.1% trifluoroacetic acid). After the eluates were evaporated with N_2, the residues were re-dissolved with 100% methanol and stored at −20 °C until the extracellular MC-LR concentration could be analyzed.

To measure the intracellular MC-LR concentration, 3 mL of 75% (v/v) aqueous methanol were added to a centrifuge tube containing a filter, sonicated for 10 min to disrupt the algal cells, and then centrifuged at 5000 rpm. Each sample was extracted three times. The supernatants were evaporated with N_2, re-dissolved with 100% methanol and stored at −20 °C until analysis. For measurement of MC-LR, reverse-phase high-performance liquid chromatography separations were

performed on a Waters HPLC system (Waters 2487 , Agilent, Palo Alto, CA, USA) equipped with a Waters 2487 UV-diode array detector performed as described by Stewart et al. (2018) and Wiedner et al. (2003). The UV-diode array detection was set to scan over the wavelength range 190–430 nm with a resolution of 1 nm at a scan rate of 60 scans min^{-1}. Isolation was monitored by low-resolution ESI mass spectra, recorded on a Micromass ZQ with electrospray ionization operated in positive ion mode (Waters Corporation). UV data were acquired onto a PC-based data system via an IEEE interface and PerkinElmer software. The total MC-LR concentration was calculated as the sum of all MC-LR peaks.

2.6. Statistical analyses

The statistical analysis 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 to 0.05. Pearson correlation coefficients were calculated between the growth parameters and the pH values.

3. Results

3.1. Growth rate of M. aeruginosa

The specific growth rates and generation times are shown in Fig. 1. At low levels of NAP and PHE exposure, the specific growth rate of *M. aeruginosa* was significantly higher in pH 10.0 than in pH 7.0 or pH 5.0 (Fig. 1A and C), and the generation time decreased significantly in pH 10.0 (Fig. 1B and D). The specific growth rates for the NAP treatment in pH 10.0 were 21.1% and 35.3% higher than in pH 7.0 and pH 5.0, respectively (Fig. 1A). In the PHE treatment in pH 10.0, they were 25.0% and 42.8% higher than in pH 7.0 and pH 5.0, respectively (Fig. 1C). For *M. aeruginosa* undergoing the NAP treatment, the generation times were 14.3% and 33.3% lower in pH 10.0 than in pH 7.0 and pH 5.0, respectively (Fig. 1B). For the PHE treatment, the generation times were 17.2% and 54.5% lower in pH 10.0 than in pH 7.0 and pH 5.0, respectively (Fig. 1D). More importantly, the specific growth rates in both NAP and PHE treatments were higher than in the controls (CK1 and CK2) at both pH 10.0 and pH 7.0 ($p < 0.05$). This phenomenon indicates that exposing *M. aeruginosa* to pH 10.0 and pH 7.0 could readily promote its growth rate in the presence of low concentrations of NAP or PHE.

3.2. Chlorophyll-a content

Chlorophyll-*a* is an important component in the leaf cells of plants and the key element in photosynthesis. The chlorophyll-a content in the media and the cells were tested here to determine how different pH values affected the growth of *M. aeruginosa* in water with low concentrations of NAP or PHE (Fig. 2).

Fig. 2A and C indicate that M chlorophyll-*a* increased significantly more at pH 10.0 than pH 5.0 in the presence of low concentrations of NAP or PHE. No significant difference was observed between the treatments with and without NAP (Fig. 2A). The results of this study show that M chlorophyll-*a* was lower in the treatments with PHE than the controls at pH 10.0 (Fig. 2C). No significant difference was observed in the C chlorophyll-a in *M. aeruginosa* among NAP treatments at different pH values, though levels were significantly lower than in the controls at pH 10.0 and pH 7.0 (Fig. 2B). Meanwhile, C chlorophyll-*a* was higher in *M. aeruginosa* undergoing PHE treatments in pH 7.0 and pH 10.0 than pH 5.0 (Fig. 2D).

3.3. Intracellular and extracellular MC-LR concentrations

Low concentrations of NAP negatively affected extracellular MC-LR levels at pH 5.0 and pH 7.0, while they positively affected the intracellular MC-LR levels at pH 10.0 (Fig. 3A and B). The extracellular MC-LR concentrations in *M. aeruginosa* exposed to NAP were 66.1% and 67.3% lower than for algae in the controls at pH 5.0 and pH 7.0, respectively (Fig. 3A). The intracellular MC-LR levels in toxic *M. aeruginosa* cells exposed to low concentrations of NAP were 37.5% higher than cells in the controls at pH 10.0 (Fig. 3B). Low concentrations of PHE negatively affected extracellular MC-LR levels at pH 5.0, while they positively affected the intracellular MC-LR levels at pH 10.0

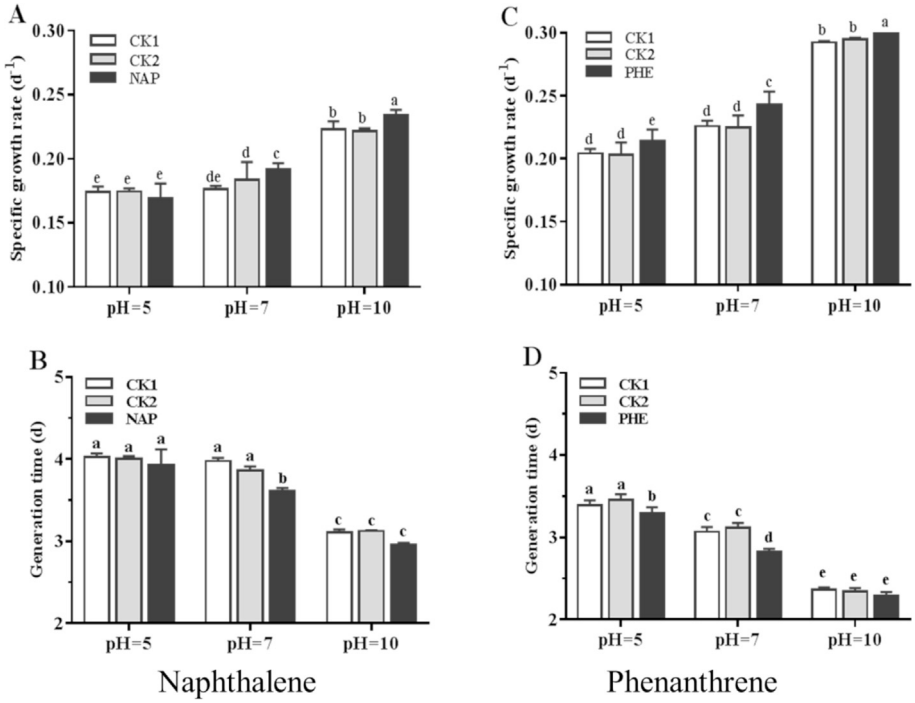

Fig. 1. Specific growth rate (A, C) and generation time (B, D) of *M. aeruginosa* for each treatment. The data are the mean ± SD (n = 3). Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

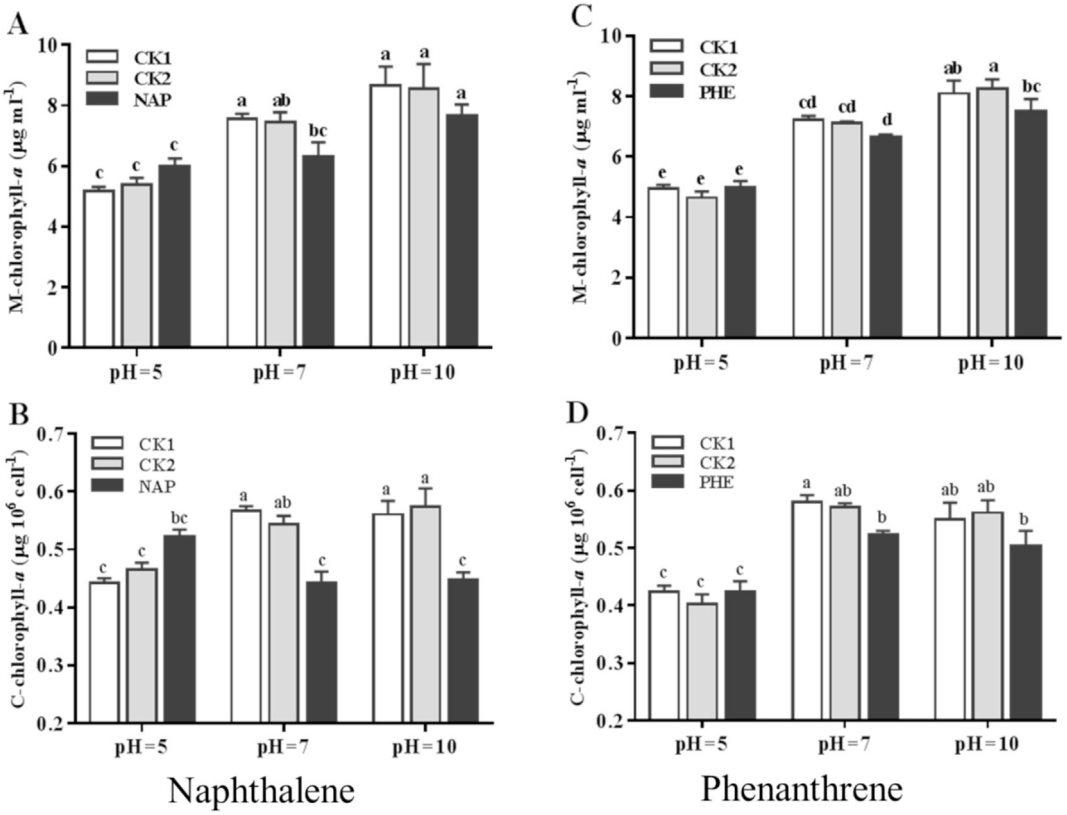

Fig. 2. M chlorophyll-a (A, C) and C chlorophyll-a (B, D) for each treatment. The data are the mean ± SD (n = 3). Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

(Fig. 3C and D). The extracellular MC-LR concentrations in *M. aeruginosa* exposed to PHE were 44.4% lower than the algae in the controls at pH 5.0 (Fig. 3C). The intracellular MC-LR levels in toxic *M. aeruginosa* cells exposed to low concentrations of PHE were 40.1% higher than cells in the controls at pH 10.0 (Fig. 3D).

4. Discussion

Both the cell growth and MCs production of toxigenic algae are regulated by many physical (such as temperature, light intensity and pH value) and chemical (such as nutrient and contaminant) factors. Thus,

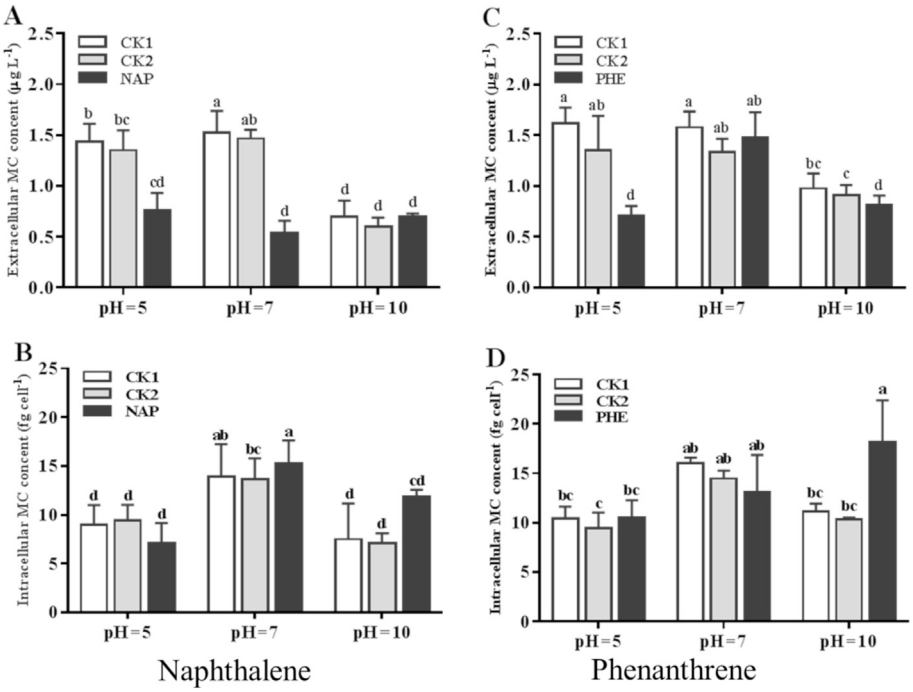

Fig. 3. Levels of intracellular (A, C) and extracellular (B, D) MC-LR in the *M. aeruginosa* exposed to 0.5 mg L−1 NAP or PHE at various pH values. The data are the mean ± SD (n = 3). Different lowercase letters indicate significant differences between the treatments ($P < 0.05$).

the occurrence of cyanobacteria and MCs in eutrophic waters is the result of a complex interplay between toxigenic algae and environmental factors. The pH values differ between various lakes and rivers, even within the same lake or river during different seasons. For example, in a study by Wu et al. the pH values varied from 6.4 to 8.5 among eight study lakes in the Yangtze River area (Wu et al., 2008). Another investigation into the occurrence and spatial distribution of microcystins in Poyang Lake was carried out by Zhang et al., in 2012, who reported that several physicochemical and biological parameters varied over a 12-day span, with pH ranging from 7.80 to 9.18 (Zhang et al., 2015). In this study, we found that the specific growth rate of *M. aeruginosa* was significantly higher in pH 10.0 than pH 7.0 or pH 5.0 for both the NAP and PHE treatments.

It was reported that PAHs and algae interact with each other and PAHs were found to affect the growth and metabolism of bacteria according to their density (Verrhiest et al., 2002). However, another study reported finding the opposite effect when PAH levels increased (Echeveste et al., 2010). The results obtained in our study were consistent with those reported by Djomo et al. (2004), where it was found that the toxic effects of PAHs seem to be affected by both the culture conditions of cyanobacteria cells and the PAH concentration. In this study, the specific growth rates of the culture exposed to either NAP or PHE were higher than the control cultures at pH 10.0 and pH 7.0. This result suggests that exposing *M. aeruginosa* to alkaline or neutral environments would readily promote its growth rate in the presence of low concentrations of PAHs in water.

The correlation between growth rate and chlorophyll-*a* content was determined here. Our study found that M chlorophyll-*a* markedly increased in the presence of low concentrations of either NAP or PHE in pH 10.0 more than in pH 5.0, and that levels were lower in the treatments with PHE than in the controls at pH 10.0. C chlorophyll-*a* contents were significantly lower with the NAP treatment than in the controls at pH 10.0 and pH 7.0. Meanwhile, C chlorophyll-*a* was higher in cultures exposed to PHE at pH 7.0 and pH 10.0 than pH 5.0.

It is generally assumed that an increase in M chlorophyll-*a* content can be attributed to an increase in cells. Thus, an increase in chlorophyll-*a* would be favorable for the growth of *M. aeruginosa*. However, C chlorophyll-*a* significantly decreased in the presence of low concentrations of either NAP or PHE in pH 10.0 and pH 7.0. In this study, no correlations were found between C chlorophyll-*a* and the specific growth rate or cell density, which indicates that exposing *M. aeruginosa* to a low-dose of PAHs could inhibit the chlorophyll-*a* content in cells in alkaline or neutral water. The stimulation of *M. aeruginosa* growth in alkaline, but not acidic, water might be attributed to the increase in photosynthesis efficiency that is caused by PAHs (Pokora and Tukaj. 2010), as well as to the stimulated production of some proteins and the defensive and repair machinery of cells (Tukaj and Tukaj, 2010). These physiological changes result in an increased growth rate for *M. aeruginosa*.

It has been reported that physical and chemical characteristics of water can alter MC-LR levels by affecting the production of MCs in a cyanobacterial population (Al-Ammara et al., 2015). In this study, HPLC analysis revealed that low concentrations of NAP negatively affected extracellular MC-LR levels at both pH 5.0 and pH 7.0, but they positively affected intracellular MC-LR levels at pH 10.0. Low concentrations of PHE negatively affected extracellular MC-LR levels at pH 5.0, but they positively affected intracellular MC-LR levels at pH 10.0. The intracellular MC-LR levels were 40.1% higher in toxic *M. aeruginosa* cells exposed to low concentrations of PHE than the controls at pH 10.0.

MCs are synthesized non-ribosomally by enzyme complexes containing non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS). Genes encoding the enzyme complex are part of a large gene cluster containing at least two operons, *mcy ABC* (peptide synthetase) and *mcy DEFGHIJ* (hybrid polyketide-peptide synthetase) (Tillett et al., 2000). The *mcy* gene has been proposed to be regulated by a complex network involving nutrient limitation, dual transcriptional regulation and stress-inducing factors (Boopathi and Ki, 2014; Neilan et al., 2013). Several batch culture experiments have demonstrated that *mcyD* transcription correlates to MCs content in cases where the MC-producing *Microcystis* cell was cultivated under different iron or nutrient conditions (Kuniyoshi et al., 2013). Our study indicates that *M. aeruginosa* produces more MC in alkaline water containing low levels of PAH pollution than in acidic water with PAH pollution.

5. Conclusions

Overall in this study, pH value markedly affected the growth and MC-LR production of *M. aeruginosa* in the presence of low concentrations of either NAP or PHE in water. The exposure of *M. aeruginosa* to low concentrations of either NAP or PHE in alkaline environments markedly promoted cell density and MC production, more than when in acidic mediums. Thus, the presence of PAHs in water directly threatens the health of algal populations, and also indirectly stimulates the growth of cyanobacteria and the generation of toxins via cyanobacterial blooms. MCs are among the most common of cyanobacterial toxins and have serious, harmful effects on human beings. Thus, the potential effects of PAH pollution in public bodies of water cannot be overlooked when assessing eutrophic rivers and lakes due to the harmful cyanobacterial blooms that are likely to be produced.

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Ethical statement

We have carefully read the information pages on ***Ethics in publishing*** and ***Ethical guidelines for journal publication*** and have abided by the statement of ethical standards for manuscripts “Characteristics of *Microcystis aeruginosa* growth and microcystin production exposed to low concentrations of naphthalene and phenanthrene under different pH values” submitted to “***Toxicon***”.

The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed.

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