Effect of *Iris pseudacorus* L. on polysaccharide composition and microcystins content of *Microcystis aeruginosa*

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Abstract. The present study aimed to explore the feedback mechanism of *M. aeruginosa* under the stress of *I. pseudacorus* L., by determining the polysaccharide composition and microcystins (MCs) synthesis and release of *M. aeruginosa* through co-cultivation of *I. pseudacorus* L. and *M. aeruginosa*. The results of our investigation and observation have shown that, under the stress of 20 or 40 g/L of *I. pseudacorus* L., the contents of intracellular and extracellular MC-LR, and the intracellular polysaccharide (IPS), the bound extracellular polysaccharide (bEPS) of *M. aeruginosa* increased at first and then began to decrease gradually afterwards. And the maximum contents were (71.03 ± 10.20) fg/cell, (0.66 ± 0.07) fg/cell, (1.84 ± 0.10) pg/cell, (1.11 ± 0.11) pg/cell and (70.33 ± 8.62) fg/cell, (0.64 ± 0.10) fg/cell, (1.74 ± 0.17) pg/cell, (0.90 ± 0.12) pg/cell. *I. pseudacorus* L. had an adsorption-enrichment effect on MCs. The content of MC-LR in the roots were (8.37± 1.58) ng/g and (4.33± 1.31) ng/g when *I. pseudacorus* L. was 20 or 40 g/L, respectively.

1. Introduction
In eutrophic water bodies, cyanobacteria "bloom" frequently occur, among which *Microcystis aeruginosa* is the most common dominant population. In the process of growth, *M. aeruginosa* synthesize and secrete two important defensive substances, microcystins (MCs) and polysaccharides (PS), which help them adapt to various unfavorable environments, thus making them dominant in cyanobacteria [1]. At present, more than 90 MCs isoforms have been identified [2]. The synthesis and release of MCs in the cells of *M. aeruginosa* has important biological significance. For example, MCs can not only participate in gene regulation and signal transmission in *M. aeruginosa* cells [3, 4], which helps *M. aeruginosa* resist the stress of H₂O₂ [5], but also inhibit other aquatic plants and algae [6, 7]. PS is a large molecular substance produced by *M. aeruginosa* cells. It is divided into extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) and it can help *M. aeruginosa* cells form protective barriers to resist ultraviolet radiation, drought, and et al. [8]. And, EPS can promote the aggregation of algae cells in groups, thus helping to form their competitive advantage to a certain extent [9]. The existence of MCs also has an impact on the synthesis of PS. Studies have shown that, the presence of MCs of appropriate concentrations can activate some polysaccharide synthesis genes and promote the synthesis of PS in algae cells [10].

At present, the restoration of aquatic plants is an important biological measure to control water eutrophication and cyanobacteria bloom [11]. *Iris pseudacorus* L., an ornamental emerged plant with a clear effect of purification, can allelopathically inhibits algae [12]. Therefore, *I. pseudacorus* L. has a broad application prospects in the prevention and control of water eutrophication. Studies have shown that, under co-cultivation, the photosynthetic system and anti-oxidase system of *M. aeruginosa* would be affected significantly by *I. pseudacorus* L. [12, 13].
In this study, the content and composition changes of the defensive substances MCs and PS of *M. aeruginosa* were studied when *M. aeruginosa* was co-cultivated with *I. pseudacorus* L.. The production and release of MCs and PS and the absorption of MCs by *I. pseudacorus* L. was analyzed to explore the feedback mechanism of *M. aeruginosa* under the stress of *I. pseudacorus* L., and provide theoretical basis for further elucidation of the allelopathic interaction between *I. pseudacorus* L. and *M. aeruginosa*.

2. Materials and methods

2.1. Plants pre-cultivation

*I. pseudacorus* L. plants with 20 cm long top shoots were purchased from a local flower market (Xiamen, Fujian, China). They were washed with distilled water firstly and then washed with sterile water three times. And then, they were pre-cultivated for 5 days with BG-11 nutritional solution[14]. *M. aeruginosa* (FACHB-905) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences, and was cultured with BG-11 nutrient solution for 1 week to expand in MGC-450BPY-2 intelligent light incubator, with the culture conditions were constant temperature and illumination (25 ± 1 ℃, 2000 lx) and light to dark ratio 12 h/12 h.

2.2. Experiment design

The well-grown *I. pseudacorus* L. plants were selected to be implanted in the glass cylinder with 2 L of BG-11 nutrient solution. The final biomass were set to 20 g/L (fresh weight) and 40 g/L (fresh weight). Then, *M. aeruginosa* was inoculated, and the final densities were 1.0×10^7 cells/mL which was set based on the abundance of microcystis in the water of Meiliang Bay and Zhushan Bay in Taihu Lake[15]. At the same time, the group without *I. pseudacorus* L. plants was treated as the control group. There were three replicates for each group. Then, the glass cylinders were put in MGC-450BPY-2 intelligent light incubator for 15 days, and samples were obtained every 3 days. The density of *M. aeruginosa* and the contents of intracellular and extracellular MCs and PS were determined. At the end of the experiment, the contents of MCs in the roots were measured.

2.3. Determination of *M. aeruginosa* Densities

Algal solution was shaken uniformly. 5 mL of algal solution was obtained and then was fixed using 10 μL formaldehyde. *M. aeruginosa* densities were determined using hemocytometer. Each sample was repeated 3 times.

2.4. Determination of PS contents

Algal solution was shaken uniformly and then 10 mL of algal solution was obtained. The algal precipitate was obtained by centrifugation at 10,000 × g for 20 min at 4 ℃. 10 mL deionized water was added to the algal precipitate and the pH value was modulated to 10 using NaOH solution. The mixture was shaken during a 5-hours water bath at 45 ℃. The supernatant was obtained for the assay of the bound extracellular polysaccharide (bEPS) by centrifugation at 10,000 × g for 20 min at 4 ℃. The concentration of bEPS was determined by sulfuric acid-anthrone method. The average bEPS content in each algae was calculated according to the algae densities. 0.5 mg / L NaOH solution was added to the second algae precipitation. After a boiling water bath for 10 min, trichloroacetic acid was added to precipitate the pigment and protein. The supernatant was obtained for the assay of the intracellular polysaccharide (IPS) by centrifugation at 10,000 × g for 20 min at 4 ℃ [16].

2.5. Determination of MCs contents

Extraction of extracellular MCs: 15 mL of algal solution was obtained, and subsequently centrifuged at 10000 g for 20 min. The MC-LR in the supernatant was loaded onto solid-phase extraction cartridges (Poly-ser) HLB, 6 mL/500 mg, CNW), and then was eluted with methanol.
Extraction of intracellular MCs: The algal residue, repeatedly being frozen and thawed, was shaken for 3 h in a thermostatic oscillator after adding 10 mL 75% methanol and subsequently centrifuged at 10000 g for 20 min. The supernatant was collected into the 500 mL glass beaker. The residue was extracted 3 times. The total supernatant was diluted to 250 mL by adding deionized water. The MC-LR in the supernatant was loaded onto solid-phase extraction cartridges (Poly-sery HLB, 6 mL/500 mg, CNW), and then was eluted with methanol.

Extraction of MCs from plant roots: The root was cleaned with deionized water 3 times. 1.0 g root was ground in a chilled mortar in 4 mL of 75% methanol, and subsequently treated by ultrasonic fragmentation. The mixture was shaken for 3 h in a thermostatic oscillator after adding 6 mL 75% methanol and subsequently centrifuged at 10000 g for 20 min. The supernatant was collected into the 500 mL glass beaker. The residue was extracted 3 times. The total supernatant was diluted to 250 mL by adding deionized water. The MC-LR in the supernatant was loaded onto solid-phase extraction cartridges (Poly-sery HLB, 6 mL/500 mg, CNW), and then was eluted with methanol.

Determination of MCs: The MC-LR was analyzed using enzyme linked immunoassay kit (detection limit 20 ng/L) (ShangHai HengYuan Biological Technology Co., Ltd, China), on a SpectraMax M2 multimode plate readers (Molecular Devices, USA).

All the data were analyzed with SPSS 15.0 for Windows (SPSS Inc., Chicago, USA) and described as mean ± SD. The illustrations were performed with SigmaPlot 10.0 (Systat Sofware, Inc., California, USA).

3. Results and discussion

3.1. Effect of I. pseudacorus L. on IPS and bEPS contents of M. aeruginosa
As shown in Fig. 1, the IPS and bEPS contents of M. aeruginosa co-cultivated with 20 g/L I. pseudacorus L. showed a trend of first rising and then declining, with a maximum of (1.84 ± 0.10) pg/cell and (1.11 ± 0.11) pg/cell, which were 1.12 and 1.98 times more than them of the control group during the same period. The contents of IPS and bEPS of M. aeruginosa co-cultivated with 40 g/L I. pseudacorus L. also showed a tendency to rise first and then decline, with the maximum values of (1.74 ± 0.17) pg/cell and (0.90 ± 0.12) pg/cell, respectively, which were 1.34 and 1.61 times more than them of the control group for the same period.

![Figure 1 IPS and bEPS contents of M. aeruginosa co-cultivated with different I. pseudacorus L. biomass](image)

The results showed that, the contents of IPS in M. aeruginosa cells co-cultivated with 20 g/L or 40 g/L of I. pseudacorus L. were 1.19 and 1.34 times more than them of the control group on day 6, which indicated that the production of PS in M. aeruginosa cells co-cultivated with I. pseudacorus L. was promoted significantly sin the early stages of cultivation. Some previous studies have proved that, the photosynthetic activity and some enzymes activities in M. aeruginosa cells co-cultivated with I. pseudacorus L. were also promoted in the early stages of cultivation [12, 13]. Therefore, it can be believed that, in the early stages of cultivation, the allelopathy effect of I. pseudacorus L. on M.
aeruginosa stimulated the defense system in M. aeruginosa cells, and the ability of PS synthesis in M. aeruginosa cells increased significantly. However, the contents of IPS in M. aeruginosa cells co-cultivated with 40 g/L of I. pseudacorus L. were reduced by 39.6% compared with the control group on day 15, which indicated that, with the extension of the cultivation time, I. pseudacorus L. showed allelopathy stress on M. aeruginosa and the production of PS in M. aeruginosa cells was inhibited significantly.

bEPS plays a very important role in the formation of M. aeruginosa colonies [17]. In our experiment, the contents of bEPS in M. aeruginosa cells co-cultivated with 20 g/L or 40 g/L of I. pseudacorus L. were 1.98 and 1.61 times more than them of the control group on day 6, indicating that in the early stages of cultivation, bEPS contents increased significantly. Some previous studies have shown that, the photoelectron transmission rates and photosynthesis efficiency in the colonial M. aeruginosa cells were higher than them in the unicellular cells [18]. Therefore, in the early stages of cultivation, the bEPS contents increased owing to the mass synthesis and secretion of PS in M. aeruginosa cells, which would promote the formation of M. aeruginosa colonies, thus maintaining a higher growth rate to adapt to the allelopathy effect of I. pseudacorus L. However, with the extension of the cultivation time, I. pseudacorus L. showed allelopathy stress on M. aeruginosa cells. The content of bEPS was significantly reduced, which was not conducive to the formation of M. aeruginosa colonies, thus resulting in a decrease in the ability of M. aeruginosa to resist I. pseudacorus L. stress. Previous studies have showed that, the oxidation damage of M. aeruginosa cells co-cultivated with I. pseudacorus L. was serious [12], chlorophyll decomposition and photosynthetic activity has also dropped sharply [13].

3.2. Effect of I. pseudacorus L. on the synthesis and release of MCs from M. aeruginosa

As shown in Fig. 2, the contents of intracellular and extracellular MC-LR in M. aeruginosa cells co-cultivated with 20 g/L of I. pseudacorus L. showed a trend of first rising and then declining, with a maximum values of (71.03±10.20) fg/cell and (0.66±0.07) fg/cell, which were 2.39 and 1.99 times more than them of the control group during the same period. The contents of intracellular and extracellular MC-LR in M. aeruginosa cells co-cultivated with 40 g/L of I. pseudacorus L. also showed a trend of first rising and then declining, with a maximum values of (70.33±8.62) fg/cell and (0.64±0.10) fg/cell, which were 2.09 and 2.34 times more than them of the control group during the same period (Fig. 2). The results indicated that, M. aeruginosa cells could synthesize more MCs in the early stage of cultivation in order to adapt to the allelopathy impact of I. pseudacorus L. Previous studies have also showed that, Lemna japonica could significantly increase the contents of intracellular and extracellular MCs in M. aeruginosa cells [19]. MCs play an important role in the resistance of M. aeruginosa to adverse environment and the acquisition of competitive advantage [1]. Therefore, under the allelopathy stress of I. pseudacorus L., M. aeruginosa cells responded to this environmental change by synthesizing and releasing more MCs. The higher the plant contents were, the more significant the exciting effect was.
The study also showed that, the change trend of MC-LR contents was consistent with that of PS contents in M. aeruginosa cells at the early stage of cultivation. For example, the contents of IPS, bEPS and intracellular MC-LR in M. aeruginosa cells co-cultivated with 40 g/L of I. pseudacorus L. reached the maximum values on the day 6, which was owing to the presence of appropriate concentrations of MCs activating some polysaccharide synthesis genes and promoting the synthesis of PS[10]. However, the contents of MC-LR decreased gradually with the extension of cultivation time, which was owing to that, under the continuously allelopathy stress of I. pseudacorus L., various physicochemical properties and photosynthetic system of M. aeruginosa were severely affected [12, 13], thus inhibiting the synthesis of MCs. In addition, when the allelopathy stress is intensified, the dead M. aeruginosa cells increased [12], and the intracellular MCs will be released in large quantities [20], resulting in a sharp decrease in the content of intracellular MCs.

![Figure 3 MC-LR contents in the roots of I. pseudacorus L. co-cultivated with M. aeruginosa](image)

The changes in the content of extracellular MCs depend on a variety of factors, including the release of intracellular MCs, adsorption of plants, and natural degradation. The physicochemical properties of MCs are relatively stable. The cultivation conditions are consistent throughout the cultivation process, and the cultivation time was short. Therefore, the reduction in MCs owing to natural degradation is basically negligible. The release of large amount of intracellular will cause a sharp increase in the content of extracellular MCs [21], which was not consistent with our research that the extracellular MC-LR content decreased gradually. The extracellular MC-LR contents of M. aeruginosa cells co-cultivated with 20 or 40 g/L of I. pseudacorus L. were only (0.47 ± 0.05) fg/cell and (0.24 ± 0.04) fg/cell on day 15, respectively. Therefore, the adsorption by I. pseudacorus L. was an important reason for the significant reduction of extracellular MC-LR content. Our results showed that, at the end of the experiment, the MC-LR contents were (8.37 ± 1.58) ng/g and (4.33 ± 1.31) ng/g in the roots of 20 and 40 g/L of I. pseudacorus L., respectively (Fig. 3). Studies have shown that, many aquatic plants could absorb MCs. For example, Vallisneria natans seedlings could absorb MC-RR and concentrated the MC-R in the roots [22]. The average concentration of MCs in Eichhornia crassipes was (5.95 ± 0.76) ng/g [23], and the amount of MCs accumulated in Lemna gibba can reach 2.24 µg/g [24]. The effects of plants on the content of extracellular MCs in M. aeruginosa cells vary with the change in the plant contents. The content of extracellular MC-LR of M. aeruginosa cells co-cultivated with 20 g/L of I. pseudacorus L. still maintained a high concentration on the day 15, which was 1.74 times more than that of the control group during the same period. However, the content of extracellular MC-LR of M. aeruginosa cells co-cultivated with 40 g/L of I. pseudacorus L. maintained a low concentration on the day 15, which was 86.8% of that in the control group during the same period. That was because that, the higher the content of plant was, the stronger the allelopathic stress was at the latter stage of the cultivation, thus reducing the synthesis of MCs. Although the death of algae cells and the increase in cell membrane permeability would lead to an increase in the release of MCs, the total MC-LR absorbed by plant would also increase, thus leading to a large decrease in the concentration of extracellular MCs.
4. Conclusions
In the early stages of cultivation, *M. aeruginosa* adapted to the allelopathy of *I. pseudacorus* L. through two strategies. Firstly, *M. aeruginosa* cells synthesized and secreted a large amount of PS, leading to an increase in the content of bEPS, which would promote the formation of *M. aeruginosa* colonies. Secondly, *M. aeruginosa* cells produced and released plenty of MCs to respond to this environmental change. The higher the plant contents were, the more significant the excitation effect was. However, with the extension of the cultivation time, *I. pseudacorus* L. showed allelopathic stress on *M. aeruginosa* cells, thus inhibiting the synthesis of PS and MCs. The content of bEPS was significantly reduced, which was not conducive to the formation of *M. aeruginosa* colonies, thus resulting in a decrease in the ability of *M. aeruginosa* L. stress. And, the adsorption by *I. pseudacorus* L. was one of the main reasons for the significant reduction of the extracellular MC-LR content.

5. References
[1] Yan H, Zhang SJ, Li C, Liu L and Zhang TT 2014 *J. Hyg. Res.* 43 290 (in Chinese)
[2] Pantelić D, Svirčev Z, Simeunović J, Vidović M and Trajković I 2013 *Chemosphere* 91 421
[3] Dittmann E, Erhard M, Kaebenick M, Scheler C, Neilan BA, von Döhren H and Börner T 2001 *Microbiology* 147 3113
[4] Zilliges Y, Kehr JC, Mikkat S, Bouchier C, Marsac NT de, Boerner T and Dittmann E. 2008 *J. Bacteriol.* 190 2871
[5] Ding Y, Gan NQ, Zheng LL and Song LR 2013 *Acta Hydrobiol. Sinica* 37 515 (in Chinese)
[6] Máté C, Beyer D and Erdödi F 2009 *Aquat. Toxicol.* 92 122
[7] Hu ZQ, Liu YD, Li DH and Dauta A 2005 *Hydrobiologia* 534 23
[8] Ren XX, Jiang H, Leng X and An SQ 2013 *Chin. J. Ecol.* 32 762 (in Chinese)
[9] Zhang YQ, Yang GJ, Qin BQ, Zhou J, Xu HP, Wang Y and Wu YL 2014 *J. Lake Sci.* 26 559 (in Chinese)
[10] Gan NQ, Xiao Y, Zhu L, Wu ZX, Liu J, Hu CL and Song LR 2012 *Environ. Microbiol.* 14 730
[11] Yuan SF, Wang WD, Dong HY, Qiang ZM, Li S, Wang ZQ, Yang JZ and Yin CQ 2013 *Acta Scien. Circum.* 33 1475 (in Chinese)
[12] Chen GY and Tang K 2014 *Chin. J. Environ. Eng.* 8 465 (in Chinese)
[13] Chen GY, Li QS, Xie PY and Chen YH 2015 *Chin. J. Environ. Eng.* 9 4145 (in Chinese)
[14] Stanier RY, Kunisawa R and Mandel M 1971 *Bacteriol. Rev.* 35 171
[15] Li DM, Kong FX, Yu Y, Zhang M and Shi XL 2011 *Acta Scien. Circum.* 31 292 (in Chinese)
[16] Yang Z, Kong FX, Shi XL, Zhang M, Xing P and Cao H 2008 *J. Phycol.* 44 716
[17] Dai XX, Zhu W and Li M 2013 *J. Lake Sci.* 25 277 (in Chinese)
[18] Wu ZX and Song LR 2008 *Phycologia* 47 98
[19] Jang MH, Ha K and Takamura N 2007 *Toxicon* 49 727
[20] Daly RJ, Ho L and Brookes JD 2007. *Environ. Sci. Technol.* 41 4447
[21] Fang L, Liu WQ, Zhao NJ, Duan JB, Wang ZG, Xiao X, Zhang YJ, Liu J, Yin GF and Shi CY 2013 *J. Saf. Environ.* 13 6 (in Chinese)
[22] Yin LY, Huang JQ, Li DH and Liu YD 2005 *Environ. Toxicol.* 20 308
[23] Zhou Q, Han SQ, Song W and Liu GF 2014 *Environ. Sci.* 35 597 (in Chinese)
[24] Saqrane S, El ghazali I, Ouahli Y, El Hassni M, El Hadrami I, Bouarab L, del Campo FF, Oudra B and Vasconcelos V 2007 *Aquat. Toxicol.* 83 284

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