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Original Research

Corresponding Responses of *Microcystis aeruginosa* to *Eichhornia crassipes* (Mart.) Solms Stress

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Abstract

Eichhornia crassipes (Mart.) Solms has been applied widely to assimilate nutrients and coexisted with blooming cyanobacteria (mainly *Microcystis aeruginosa*) in many eutrophic lakes. But the effect of *E. crassipes* on hepatotoxic microcystins production in cyanobacteria still needed to be made clear. In this study, the effect of *E. crassipes* on DNA damage and transcription of microcystin biosynthetic genes in *M. aeruginosa* were first investigated and the links between microcystins production and other biochemical characteristics in *M. aeruginosa* were also explored. Results showed that the relative transcript levels of *mcyA* and *mcyB* in *M. aeruginosa* under stress of *E. crassipes* were stimulated briefly and then soon inhibited, while that of *mcyD* was motivated fleetingly and then kept at same level as controls. The accumulation of DNA damage in *M. aeruginosa* was exacerbated at state of energy limitation resulted from stress of *E. crassipes* and then triggered death via apoptosis. The transcription of microcystin biosynthetic genes in *M. aeruginosa* was not completely regulated by oxidative stress. It needed active photosynthetic electron transfer which could be blocked by *E. crassipe*s. Furthermore, poor repair of oxidative DNA damage in *M. aeruginosa* inhibited the expression of microcystin biosynthetic genes under exposure of *E. crassipe*s.

Keywords: *Eichhornia crassipes*, *Microcystis aeruginosa*, microcystins production, DNA damage, transcription, microcystin biosynthetic genes

Introduction

As global climate changes and water eutrophication are aggravated, cyanobacterial blooms has become a worldwide ecological problem and poses risks to public health [1]. *Microcystis aeruginosa* may originate from the African continent and has depersed into Asia, Europe and America [2]. Toxic *Microcystis aeruginosa* is the most primary bloom-forming cyanobacteria in eutrophic freshwaters [3-4]. It produces microcystins that can lead to poisoning, cancer and even death of humans and animals [5]. The Toledo drinking water crisis in USA, the Wuxi drinking water crisis in China and the recreational water risk in Portugal have caused public health concerns [6-8]. Many methods for controlling cyanobacterial blooms have been

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tested. Mechanical salvage is an emergency method to alleviate cyanobacterial blooms [9]. Chemical methods are alternatives to kill cyanobacteria [10]. But phytoremediation technology is still more attractive because of its low-cost, effective assimilation of nutrients and emission of algicidals [11]. *Eichhornia crassipes* (Mart.) Solms is an invasive aquatic plant. If growing naturally without control, it will block water channel and cause ecological problems. However, due to its superior ability in nitrogen and phosphorus removal, it is also considered as a powerful tool to remediate eutrophic waters [12]. Because the eradication of *E. crassipes* is difficult to achieve at present, there have been attempts to make good use of it. When confined growth, mechanized harvesting and utilization have been realized and all these processes are effectively linked, *E. crassipes* are applied in Lake Dianchi and Lake Taihu (China) on a large scale [13]. Due to the eutrophic water status, *E. crassipes* always coexits with *M. aeruginosa* blooms, whether it is artificially confined in special aera of Lake Dianchi (China) or spread naturally in Guadiana River (Spain) [14-15]. Moreover, *E. crassipes* has a special advantage of being able to grow well in waters full of thick cyanobacterial scum where many other aquatic macrophytes cannot survive [14]. The interactions between *E. crassipes* and *M. aeruginosa* is worth paying attention to.

Prior researches have shown that blooming *Microcystis* could elevate the nitrogen and phosphorus bioaccumulation in *E. crassipes*, while the allelopathic substances secreted from *E. crassipes* have influences on the biomass, pigments, cell structure, antioxidant enzymes, membrane lipid peroxidation product of *M. aeruginosa* [16-18]. Whether there is the potential risk of elevated microcystin production in *M. aeruginosa* under the effect of *E. crassipes* needs to be assessed. Our previous work attempted to explore the intracellular microcystin productivity of *M. aeruginosa* influenced by *E. crassipes* using enzyme-linked immunosorbent assay (ELISA) [19]. However, the determination of toxins using ELISA are now considered as a prelimminary screening method, because of the false positive reactions and relatively high variability [20]. Moreover, toxic microcystins are found to bind to some proteins in algae cells so that insufficient disruption of microcystin-protein complexes may make the toxins be lost in the discarded protein fraction [21]. These conjugated microcystins in algae cells may be underestimated by traditional methods like LC-MS [22]. With the microcystin biosynthetic gene cluster from *M. aeruginosa* PCC7806 characterized, it is possible to investigate microcystin production at a genetic level using real-time RT-PCR technique [23]. Therefore, the effect of *E. crassipes* on microcystins production will be clearer based on the transcriptional response of microcystin biosynthetic gene. Furthermore, microcystins are not only toxins for human, animals and aquatic organisims but also metabolites performing physiological functions for algae [24]. The impact of *E. crassipes* on the microcystin production is not definitely direct. The intertwined relations between microcystin production and other physiological characteristics in cyanobacteria under the stress of *E. crassipes* are still unknown.

The aims of this study were to explore the potential risk of *E. crassipes* to microcystin production in *M. aeruginosa* and characterize how *E. crassipes* affect the toxins production. In this work, the effect of *E. crassipes* on the transcription of microcystin biosynthetic genes in *M. aeruginosa* are first investigated. Furthermore, their relations with growth rate, cellular energy levels, photosynthetic potential, oxidative stress and damaged DNA levels are explored.

Materials and Methods

Strains and Culturing Conditions

The microcystin-producing strain *M. aeruginosa* (FACHB-912) was obtained from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). This strain was axenically grown in batch cultures using 1/10 modified Hoagland's medium (pH 7.0, adjusted with NaOH) in incubator. *E. crassipes* was collected from a concrete breeding pool at Jiangsu Academy of Agricultural Sciences (Nanjing, China). Selected individuals were cleaned of debris, rinsed with tap-water and then immersed in 70% ethanol, 10% sodium hypochloride solution and sterile water in turn according to the method described by Jang et al [25]. Each sterilized plant individual was axenically cultured using 1/10 modified Hoagland's medium in incubators for 1 month. These pretreated plants were then used for coexistence experiments. 1/10 modified Hoagland's medium was sterilized at 121ºC for 20 min. The incubator was maintained at 28℃ with a constant relative humidity of 75% and illuminated by cool-white fluorescent lamps (40 μmol photons·s^{-1}·m^{-2}) in 12-hour diurnal cycles.

Coexistence Experiments

*M. aerugi*nosa was axenically amplified in 10 L of 1/10 modified Hoagland's medium in flat-bottomed glass containers with 10% (v/v) inoculum. The incubator was also maintained at the same conditions as above. Then, pretreated *E. crassipes* (about 110 g) was co-cultivated in these exponentially growing cultures of *M. aeruginosa* (OD650 = 0.251). Controls were prepared also in these exponentially growing cultures of *M. aeruginosa* without *E. crassipes*. During the co-existence period of 5 days, samples were collected for analysis of algal density, algal physiological parameters and relative transcriptional level of microcystin biosynthetic genes at 1-day interval. All co-existence experiments were conducted with three independent

replicates in incubator. The culture solutions were stirred once a day by magnetic force.

Measurement of Algal Growth

Algae cells were counted in a hemocytometer using a microscope (XS-213; Jiangnan Optical Instrument Factory, Nanjing, China), after stained with Lugol's iodine solution. Algal growth rate was then calculated according to the division time method [26].

Chlorophyll A Fluorescence Parameters Measurements

The determination of chlorophyll a fluorescence at room temperature was done by Phyto-PAM phytoplankton analyzer (Walz, Germany). Algal suspension (3 mL) of every sample was collected and dark-adapted for 30 min before each measurement. The initial fluorescence F_0, the maximal fluorescence *Fm* and the variable fluorescence *Fv* were got. Then the photosystem II maximal photochemical efficiency (*Fv*/*Fm*) and the photosystem II potential photochemical efficiency (Fv/F_0) were calculated [27].

Detections of ATP and Hydrogen Peroxide (H_2O_2)

For the detection of intracellular ATP and H_2O_2, algae cells of each sample were harvested by centrifugation at 8,000 g for 10 min at 4ºC and stored at -20ºC until analysis. Intracellular ATP and H_2O_2 level were detected following the instruction of commercial ATP assay kit and H_2O_2 assay kit (Nanjing Jiancheng Bioengineering Inc., China). Protein concentration was determined by the Bradford method [28] using BSA as a standard.

Apurinic/Apyrimidinic (AP) Sites Assay

For the assay of AP sites, algae cells of each sample were harvested by centrifugation at 8,000 g for 10 min at 4ºC and stored at -20ºC until analysis. Genomic DNA of algae was extracted and purified using PowerWater® Sterivex™ DNA isolation kit (MOBIO Laboratories, USA). The quantity and quality of purified DNA were examined using spectrophotometry and electrophoresis. Then the number of AP sites was determined using commercial DNA damage quantification kit (Dojindo Molecular Technology, Japan).

RNA Isolation, cDNA Preparation and Real-time PCR

For the detection of transcriptional level of microcystin biosynthetic genes, algae cells of each sample were harvested by centrifugation at 8,000 g for 10 min at 4ºC, rapidly frozen in liquid nitrogen and then stored at -80ºC until RNA isolation.

Total RNA was isolated and purified using PowerWater® RNA isolation kit (MOBIO Laboratories, USA). The quantity and quality of purified RNA were examined by spectrophotometry and electrophoresis. First-strand cDNA was synthesized from intact RNA (1 μg) using Q RT SuperMix kit for qPCR (Biouniquer Technology CO., LTD., China). The resulting cDNA samples were then aliquoted and stored at -20ºC for further use.

Quantitative PCR was performed using Realtime PCR Master Mix (SYBR® Green I) kit (Biouniquer Technology CO., LTD., China). The sample cDNA (1 μL, if necessary diluted 1:10 -1:1000 in DEPC water, v/v) was used in PCR reactions in a 25 μL final reaction volume. Primers were listed in Table 1. The PCR amplification was performed on ABI 7500 (Applied Biosystems, USA) and the parameters were set as an initial temperature at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 5 s, annealing at the indicated temperature (Table 1) for 34 s, then a disassociation stage at 95ºC for 15 s, 60ºC for 30 s and 95ºC for 15 s as instructed by the user's manual.

Relative transcriptional levels were calculated by the relative standard curve method [29]. A standard curve was obtained using the cycle threshold value from ten-fold serial dilutions of *M. aeruginosa* genomic DNA (90 ng). The relative amount of target gene was then

Table 1. Primers for real-time PCR.

Target Gene	Primer	Sequence (5' to 3')	Annealing temperature (ºC)	Reference
16S rDNA	MC16S-F	GGACGGGTGAGTAACGCGTA	60	[30]
	MC16S-R	CCCATTGCGGAAAATTCCCC		
mcyD	mcyD-F	GGTTCGCCTGGTCAAAGTAA	60	[31]
	mcyD-R	CCTCGCTAAAGAAGGGTTGA		
mcyB	mcyB2959-F	TGGGAAGATGTTCTTCAGGTATCCAA	57	[32]
	mcyB3278-R	AGAGTGGAAACAATATGATAAGCTAC		
mcyA	mcyAcd-F	AAAATTAAAAGCCGTATCAAA	48	[33]
	mcyAcd-R	AAAAGTGTTTTATTAGCGGCTCAT		

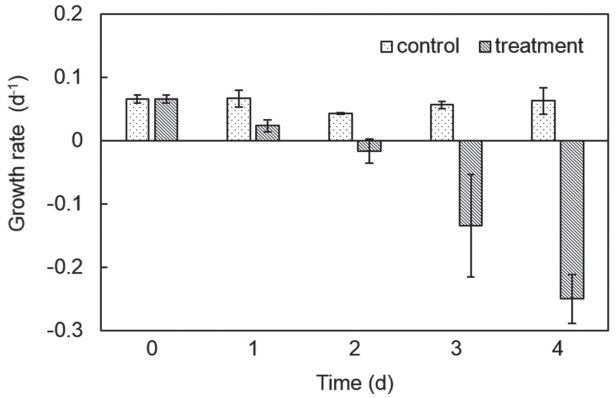

Fig. 1. Effect of *E. crassipes* on the growth of *M. aeruginosa*.

calculated from each standard curve and normalized to the corresponding 16S rRNA gene.

Statistical Analysis

Data were expressed as mean±standard deviation (SD). Pearson correlation analysis was used to analyze the correlation among parameters. Furthermore, linear regression analysis was used to test the relationship. Significant differences between controls and treatments were determined by one-way ANOVA. Differences were considered to be significant at $p<0.05$. All statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM SPSS Inc., USA).

Results

Effect of *E. crassipes* on the Growth of *M. aeruginosa*

As shown in Fig. 1, the growth of *M. aeruginosa* was strongly inhibited by *E. crassipes* and its death was then promoted. Compared to the controls, the growth rate of *M. aeruginosa* declined significantly after one day of co-culture with *E. crassipes* and gradually became negative two days later.

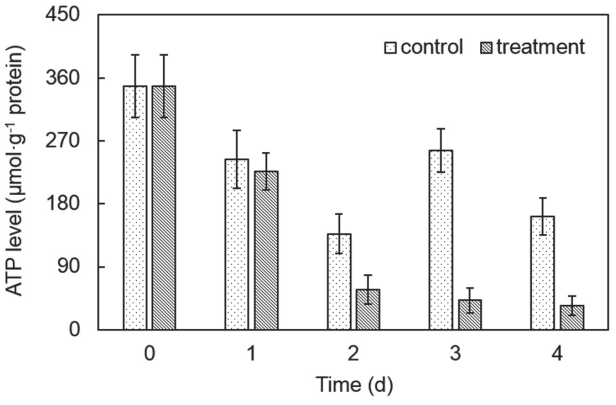

Fig. 2. Effect of *E. crassipes* on the ATP pool of *M. aeruginosa*.

Effect of *E. crassipes* on ATP Pool of *M. aeruginosa*

As presented in Fig. 2, the concentrations of algal ATP in controls were maintained at a dynamic balance, but those under the effect of *E. crassipes* declined gradually without recovery and were remarkably lower than in controls after two days. The concentrations of algal ATP in controls did not vary with growth rate, while those in treatment had markedly positive linear correlation with algal growth rate over time ($r = 0.747$, $p<0.05$).

Effects of *E. crassipes* on Photosynthetic Activity of *M. aeruginosa*

As shown in Fig. 3, *Fv*/*Fm* ratio of *M. aeruginosa* under the stress of *E. crassipes* decreased significantly compared to the control after 3-day co-culture, while Fv/F_0 ratio of *M. aeruginosa* during the exposure to *E. crassipes* declined significantly compared to the control only after 2-day co-culture. Fv/F_0 ratio of *M. aeruginosa* appeared to be more sensitive to the stress of *E. crassipes* than *Fv*/*Fm* ratio. Correlation analysis revealed that Fv/F_0 ratio in algae controls did not change with growth rate, while that in treatment had notably positive linear correlation with algal growth rate ($r = 0.913$, $p<0.05$) and ATP concentration ($r = 0.814$, $p<0.05$) over time.

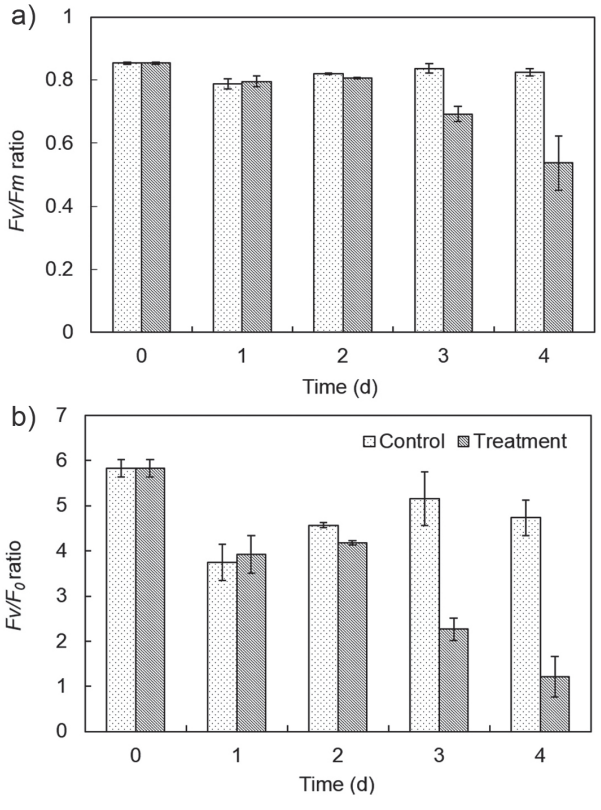

Fig. 3. Effects of *E. crassipes* on *Fv*/*Fm* ratio a) and Fv/F_0 ratio b) of *M. aeruginosa*.

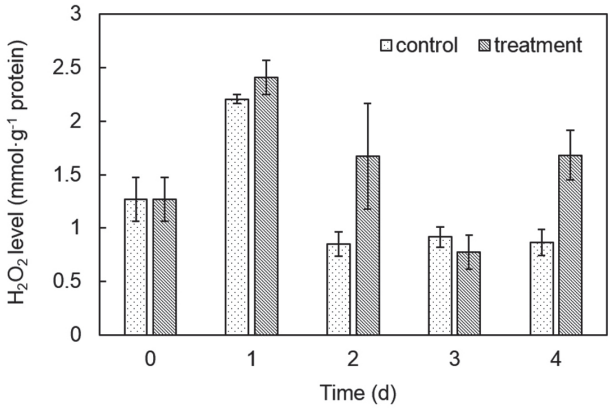

Fig. 4. Effect of *E. crassipes* on H_2O_2 production of *M. aeruginosa*.

Effect of *E. crassipes* on H_2O_2 Production of *M. aeruginosa*

Algal H_2O_2 levels were significantly increased at day 1, 2 and 4 after exposure to *E. crassipes* compared to the control (Fig. 4). It was clear that the endogenous oxidative stress of *M. aeruginosa* could be elevated by *E. crassipes*. Correlation analysis showed that there was notably negaitive linear correlation between algal H_2O_2 levels and *Fv*/*Fm* ratio (r = -0.595, p<0.05) in controls. But this corelation was not significant in treatments under the influence of *E. crassipes*.

Effect of *E. crassipes* on DNA Damage of *M. aeruginosa*

AP sites are common lesions in DNA. As presented in Fig. 5, Number of AP sites in *M. aeruginosa* was greatly elevated at day 1, 3 and 4 after exposure to *E. crassipes* compared with the control. *M. aeruginosa* was suffered DNA damage from *E. crassipes*. Correlation analysis indicated that number of AP sites in *M. aeruginosa* controls had significantly negative linear correlation with algal ATP levels over time (r = -0.814, p<0.05). In treatment, number of AP sites in *M. aeruginosa* had significantly negative linear correlation with growth rate (r = -0.691, p<0.05), algal ATP levels (r = -0.674, p<0.05) and Fv/F_0 ratio (r = -0.806, p<0.05) over time.

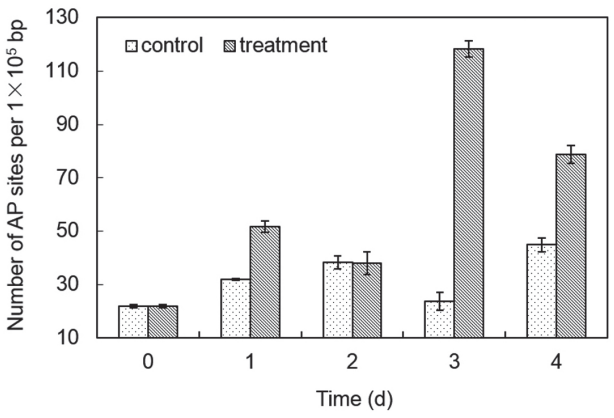

Fig. 5. Effect of *E. crassipes* on DNA damage of *M. aeruginosa.*

Effects of *E. crassipes* on the Transcription of Microcystin Biosynthetic Genes

Gene *mcyA*, *mcyB* and *mcyD* are important constituents of microcystin synthetase gene clusters. Gene *mcyA* and *mcyB* belong to one transcribed operon that encode the microcystin peptide synthetase, while *mcyD* belongs to another transcribed operon that encode the polyketide synthase. As shown in Fig. 5, the relative amounts of *mcyA*, *mcyB* and *mcyD* transcripts

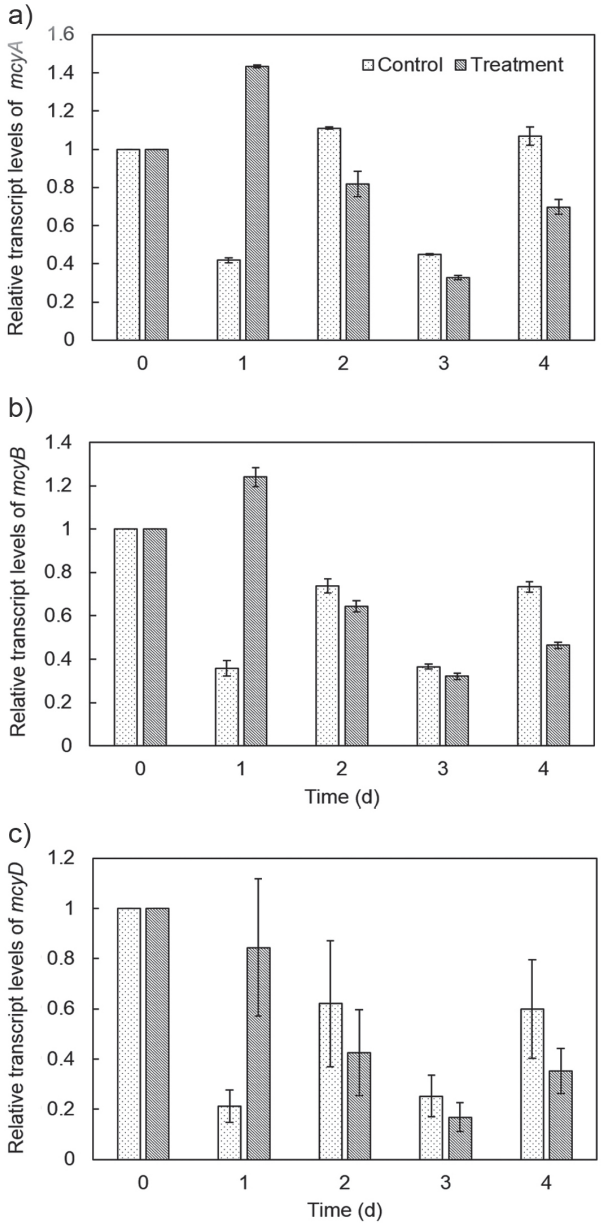

Fig. 6. Effects of *E. crassipes* on the transcription of *mcyA* a), *mcyB* b) and *mcyD* c). The starting value at 0 day are set at 1 for *mcyA*, *mcyB* and *mcyD*.

of *M. aeruginosa* in treatment were significantly higher than in controls at the first day. But during the following days, the relative transcript levels of *mcyA*, and *mcyB* in treatments were markedly lower than in controls, while the relative transcript levels of *mcyD* in treatments declined to the same level as controls. Correlation analysis revealed that there existed significantly positive linear correlation among *mcyA*, *mcyB* and *mcyD* transcripts no matter in controls or treatments. Furthermore, Fv/F_0 ratio in algae control had significantly positive linear correlation with the relative transcript level of *mcyB* (r = 0.569, p<0.05) and *mcyD* (r = 0.556, p<0.05). Algal H_2O_2 levels in controls had notably negative linear correlation with the relative transcript level of *mcyA* (r = -0.577, p<0.05). Due to the exposure to *E. crassipes*, many indicators appeared significant correlation with *mcyA*, *mcyB* and *mcyD* transcripts too. Fv/F_0 ratio in treatment also had remarkably positive linear correlation with the relative transcript level of *mcyA* (r = 0.535, p<0.05), *mcyB* (r = 0.691 p<0.05) and *mcyD* (r = 0.751, p<0.05). The number of AP sites in treatment had significantly negative linear correlation with the relative transcript level of *mcyA* (r = -0.700, p<0.05), *mcyB* (r = -0.712, p<0.05) and *mcyD* (r = -0.742, p <0.05). Algal ATP levels in treatment had notably positive linear correlation with the relative transcript level of *mcyA* (r = 0.659, p<0.05), *mcyB* (r = 0.808, p<0.05) and *mcyD* (r = 0.878, p<0.05). In addition, algal H_2O_2 levels in treatment had significantly positive linear correlation with the relative transcript level of *mcyA* (r = 0.793, p<0.05) and *mcyB* (r = 0.638, p<0.05).

Discussion

Under the stress of *E. crassipe*s, the growth rate, DNA damage level, intracellular H_2O_2 level and the transcription level of gene *mcyA*, *mcyB* and *mcyD* in *M. aeruginosa* firstly reflected the difference from the control, while the changes of ATP level and photosynthetic activity in *M. aeruginosa* were relatively delayed.

N-phenyl-naphthylamine is an important algaecidal compound from *E. crassipe*s. N-phenyl-1-naphthylamine and N-phenyl -2-naphthylamine are two conformations. They could bind to the hydrophibic regions of cell membranes [34]. After comparison, it was found that N-phenyl-naphthylamine were easily oxidized. This characteristic was similar with some allelopathic substances from submerged macrophytes, such as pyrogallic acid. N-phenyl-2-naphthylamine could be transferred to electrophilic *p*-quinone [35], while pyrogallic acid could be transferred to electrophilic *o*-quinone. Pyrogallic acid was reported to induce futile redox cycling and excessive intracellular reactive oxygen species (ROS) in *M. aeruginosa* because of the formation of *o*-quinone [36]. Our results indicated that the endogenous H_2O_2 level of *M. aeruginosa* was soon elevated due to the stress of *E. crassipes* and the oxidative damage level in DNA of *M. aeruginosa* was also boosted soon. Thus, it was speculated that futile redox cycling might occur in *M. aeruginosa* due to algaecidal compounds from *E. crassipe*s.

Previous studies showed that the growth rate of some heterotrophic bacteria (such as *Escherichia coli* and *Phaeobacter inhibens*) were determined by ATP production only under the status of energetic limitation [37-38]. This phenomenon also occured in *M. aeruginosa* in our study. The decline of Fv/F_0 index in our data reflected that the structural integrity of the photosystem II reaction centers in *M. aeruginosa* was interfered by *E. crassipe*s. It has been demonstrated that the interference of electron transport in cyanobacteria photosystem II would decrease the intracellular ATP level [39]. As a result, *M. aeruginosa* might be gradually induced to be in the state of energy limitation by *E. crassipe*s. In the defense strategies of cyanobacteria, it was found that the repair of photosystem II needed ATP [39], while some repair enzymes of damaged DNA were also ATP-dependent [40]. Our data showed that more damaged DNA of *M. aeruginosa* in treatment was produced and the growth rate value gradually appeared negative. Therefore, it was reasonable to assume that the accumulation of DNA damage in *M. aeruginosa* was exacerbated at the state of energy limitation resulted from the exposure of *E. crassipe*s and then triggered death via apoptosis.

Many regulating factors, such as light, temperature, nutrients, pH, iron, plants, bacteria and zooplankton, have been reported to influence microcystin synthesis. *Lemna japonica* was revealed to increase microcystin production of *M. aeruginosa* [25]. *Sphingomonas* spp., a microcystin-degrading bacterium, was detected to upregulate *mcyD* expression in *Microcystis* spp. [41]. *Diphylleia rotans*, a zooplankton, was also found to elevate microcystin production of *M. aeruginosa* [42]. But our data showed that the expression of microcystin biosynthetic genes in *M. aeruginosa* was only promoted in a short term and then soon lowered by exposure to *E. crassipe*s. The speculation that microcystins were induced as allelochemicals by *M. aeruginosa* might be not universally applicable. Microcystins have also been reported to bind to some proteins and play protein-modulating role to protect *Microcystis* against oxidative stress [43]. But the relations between oxidative stress and transcription of microcystin biosynthetic genes were not consistent. Gene *mcyD* transcript in *M. aeruginosa* was found to be elevated as ROS stress increased by sulfate [44]. Meanwhile, *mcyD* transcript was showed to be lowered as H_2O_2 stress increased [45]. Our data also showed an interesting result that the relationship between H_2O_2 stress and *mcyA* transcript in controls was opposite to that in treatments, while there was no significant relation between H_2O_2 stress and *mcyD* transcript. Therefore, the expression of microcystin biosynthetic genes did not seem to be completely regulated by internal oxidative stress. The binding

sites of ferric uptake regulator Fur and global nitrogen regulator NtcA are present in *mcyA* and *mcyD* promoter region [46]. Regulator Fur has been reported to be involved in response to many factors such as oxidative stress, acidic stress, iron metabolism and so on [47]. In our study, the positive correlation of Fv/F_0 ratio with *mcyA*, *mcyB* or *mcyD* in *M. aeruginosa* might indicate that the transcription of microcystin biosynthetic genes needed active photosynthetic electron transfer. Furthermore, the negative correlation of the demaged DNA level with *mcyA*, *mcyB* or *mcyD* in *M. aeruginosa* might show that poor repair of oxidative DNA damage in *M. aeruginosa* could inhibit the expression of microcystin biosynthetic genes under the exposure of *E. crassipe*s. Therefore, it was obvious that a variety of changes in *M. aeruginosa* might be induced by *E. crassipe*s and then resulted in the combined inhibitory effect on the expression of microcystin biosynthetic genes. On the other hand, the fast response of *mcyA*, *mcyB* and *mcyD* transcript in *M. aeruginosa* to *E. crassipe*s and the multiple relations between *mcyA*, *mcyB* or *mcyD* transcript and other quotas in our study might support the idea that microcystins were also regulatory molecules linked to many different algal cell processes [48].

Conclusions

The growth of *M. aeruginosa* could be strongly inhibited by *E. crassipes*. The accumulation of DNA damage in *M. aeruginosa* was exacerbated at state of energy limitation resulted from stress of *E. crassipes* and then triggered death via apoptosis. Poor repair of oxidative DNA damage in *M. aeruginosa* inhibited the expression of microcystin biosynthetic genes under the exposure of *E. crassipe*s. The transcription of microcystin biosynthetic genes in *M. aeruginosa* was not completely regulated by oxidative stress. It needed active photosynthetic electron transfer which could be blocked by *E. crassipe*s. A variety of changes in *M. aeruginosa* might be induced by *E. crassipe*s and then resulted in the combined inhibitory effect on the expression of microcystin biosynthetic genes.

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Conflict of Interest

The authors declare no conflict of interest.

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