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Effects of copper sulfate, hydrogen peroxide and N-phenyl-2-naphthylamine on oxidative stress and the expression of genes involved photosynthesis and microcystin disposition in *Microcystis aeruginosa*

Haifeng Qian, Shuqiong Yu, Zhengqi Sun, Xiucai Xie, Weiping Liu, Zhengwei Fu *

College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, Zhejiang, 310032, China

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ABSTRACT

Algal blooms have been increasing in prevalence all over the world, destroying ecosystems and placing other organisms at risk. Chemical remediation is one of most important methods of controlling algal bloom formation. The effects of copper sulfate, hydrogen peroxide (H_2O_2) and N-phenyl-2-naphthylamine on photosynthesis-related and microcystin-related gene transcription and physiological changes of *Microcystis aeruginosa* were analyzed. The results suggest that transcription of *psa*B, *psb*D1 and *rbc*L was inhibited by the three algaecides, which blocked the electron transport chain, significantly enhanced reactive oxygen species (ROS) accumulation and overwhelmed the antioxidant system. The increase in ROS destroyed pigment synthesis and membrane integrity, which inhibited or killed the algal cells. Furthermore, H_2O_2 treatment down-regulated *mcy*D transcription, which indicated a decrease in the microcystin level in the cells. Our results demonstrate that H_2O_2 has the greatest potential as an algaecide because it not only inhibits algae growth but may reduce microcystin synthesis.

1. Introduction

In recent decades, algal blooms have been occurring worldwide. In algal blooms, cyanobacteria are the most common group of algae, and they live in terrestrial, fresh, brackish or marine waters (Sinclair et al., 2008). These bacteria are usually too small to be seen, but when the cyanobacteria increase rapidly to form a bloom, the surface of the water becomes painted in colors of blue, bright green, brown or red. As algae of the cyanobacterial bloom die, the water can smell bad, which is a serious problem for drinking water supplies and recreational economic development.

Within the algal blooms, some cyanobacteria can produce toxins (Sivonen and Jones, 1999) that pose a risk to human health and can affect fishing and aquaculture (Malbrouck and Kestemont, 2006). *Microcystis aeruginosa* is the most common toxin-producing cyanobacterium. It produces hepatotoxic microcystins, which are the main type of cyanobacterial toxin (Rinehart et al., 1994) and are responsible for liver disease (Carmichael, 2001) and even liver and colon cancer (Ueno et al., 1996; Humpage et al., 2000).

Many methods for preventing blooms have been tested, including reducing nutrient input into water systems to prevent over-enrichment and altering the hydrophysical conditions to allow the environment to favour other phytoplankton over the cyanobacteria (Hrudey et al., 1999). However, these methods were not particularly successful. Chemical remediation utilizes chemical reagents to inhibit or kill algae, which may offer an alternative for bloom control (Barrington and Ghadouani, 2008). Copper sulfate ($CuSO_4$) is regarded as an economical, effective algaecide because it is considered to be generally safe for human health at the doses commonly used (WHO, 1996), while causing aggregation of DNA fibrils, rupture of the thylakoids and cell death in algae (Verhoeven and Eloff, 1979). Hydrogen peroxide (H_2O_2) is a strong oxidizing agent that is considered to be an effective and environmentally benign treatment for the inhibition of cyanobacterial growth (Drábková et al., 2007; Barrington and Ghadouani, 2008) because it is a natural photochemical product formed in waters under sunlight and can be quickly degraded into oxygen and water (Cooper and Zika, 1983). N-phenyl-2-naphthylamine (PNA) is a secondary metabolite produced by plants (Sultankhodzhaev and Tadzhibaev, 1976; Sun et al., 1993) and is a strong anti-algal allelochemical (Qian et al., 2009). Allelopathic compounds are considered to be less polluting than traditional herbicides because they are degraded rapidly (Macías et al., 1998).

The effects of potential algaecides on algae growth have been analyzed based on inhibition of algal growth (Nakai et al., 2000), pigment content and photosynthetic rate (Gouvêa et al., 2008), among other things. Recently, real-time PCR analysis has been applied to measure gene transcription during algal

* Corresponding author. Tel.: +86 571 8832 0599; fax: +86 571 8832 0599.
E-mail address: azwfu2003@yahoo.com.cn (Z. Fu).

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growth in *Chlorella vulgaris* (Qian et al., 2008a) and *Thermosynechococcus elongatus* (Kós et al., 2008). Photosynthesis is the principal mode of energy metabolism of algae. In this process, light energy is captured and used to synthesize sugar while generating oxygen and consuming carbon dioxide. Therefore, photosynthesis is an indispensable metabolic process. For this reason, we focused on photosynthesis-related gene transcription to analyze the effects of the three potential algaecides ($CuSO_4$, H_2O_2 and PNA) on photosynthesis of *M. aeruginosa*. These photosynthesis-related genes included (1) *rbc*L, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) in alga, (2) *psb*D1, which encodes the D2 protein that forms the reaction center of photosystem II (PSII), and (3) *psa*B, which encodes one of the reaction center subunits of photosystem I (PS I).

Since cyanotoxins are one of the main pollutants in algal blooms, changing the microcystin content has also been considered as a method to inhibit algal growth. Microcystins are synthesized in a mixed polyketide synthase/non-ribosomal peptide synthetase system called microcystin synthetase. Microcystin synthetase is encoded in two transcribed operons in *M. aeruginosa*, *mcy*A-C and *mcy*D-J (Tillett et al., 2000). *mcy*A belongs to the *mcy*A-C gene cluster and encodes microcystin synthetase; *mcy*D belongs to the *mcy*D-J gene cluster, and it encodes the modular polyketide synthase involved in the synthesis of the β-amino acid Adda that is responsible for the toxicity of the microcystins (Tillett et al., 2000). Moreover, *mcy*D expression is also essential for microcystin synthesis (Christiansen et al., 2006). *mcy*H is located upstream of *mcy*E, and bioinformatic and experimental data have shown that *mcy*H is an ABC transporter responsible for microcystin transport, and that it is intimately associated with the microcystin biosynthesis pathway (Pearson et al., 2004). For these reasons, we compared the inhibitory effect of three algaecides on the transcriptional levels of *mcy*A, *mcy*D and *mcy*H to select the best microcystin inhibitory agent.

In this study, we also investigated parameters to confirm the toxicological effects of the three potential algaecides at the physiological level, such as the activities of three antioxidant enzymes [superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)], the oxidant index [malondialdehyde (MDA) content] and chlorophyll content. The purpose of this research was to characterize the physiological and molecular effects of these three kind potential algaecides on *M. aeruginosa* growth, and to select the most valuable algaecide for controlling algal bloom formation and potential for microcystin synthesis.

2. Materials and methods

2.1. Algae strains and culture conditions

M. aeruginosa was obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences (Code: 905) and grown in BG-11 medium as batch cultures in 250 mL flasks. The cultures were maintained under cool-white fluorescent lights (4000 lx) with a daily cycle of 14 h of light and 10 h of dark. The cell density of culture was monitored spectrophotometrically at 685 nm (OD_{685}). The regression equation between the density of algal cells ($Y \times 10^5$/mL) and OD_{685} (X) was established as $Y = 34.11X + 0.73$ ($R^2 = 0.99$). Different concentrations of copper sulfate ($CuSO_4$, reagent grade, 99.0% purity; ZhengXin Chemical Co., China), hydrogen peroxide (product number 31642, Sigma) and N-phenyl-2-naphthylamine (product number 178055, Aldrich) in the culture medium were prepared. The relationship between the inhibitor concentration and algal cell growth rate was evaluated during acute toxicity (from 1 to 4 d) for *M. aeruginosa* (Fig. 1).

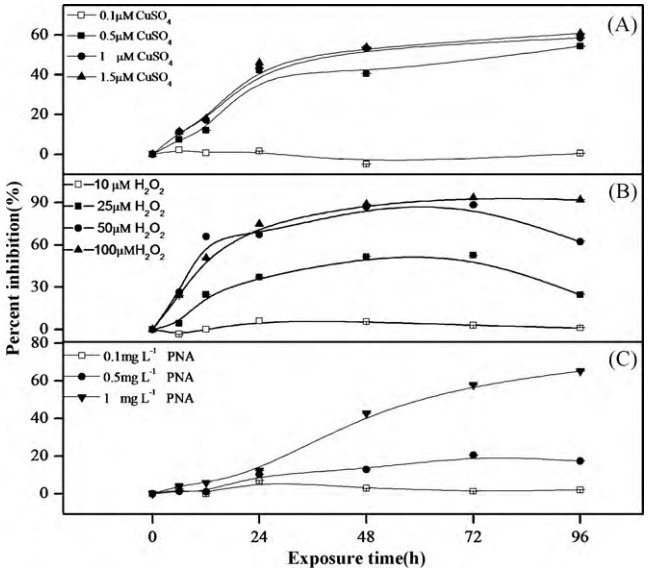

Fig. 1. Growth of *M. aeruginosa* cultured in different concentrations of and periods of exposure to (A) $CuSO_4$, (B) H_2O_2 and (C) PNA.

2.2. RNA extraction, reverse transcription and real-time analysis

Thirty milliliters of culture was centrifuged at 7000 *g* for 10 min at 4 °C. Cell pellets were frozen at −80 °C until RNA extraction. Total RNA was extracted using the RNAiso kit (TaKaRa Company, Dalian, China) following the manufacturer's instructions. For reverse transcription, 500 ng of total RNA was mixed with random primers and reverse transcriptase according to the instructions of the reverse transcriptase kit (Toyobo, Tokyo, Japan). Real-time PCR was carried out using an Eppendorf MasterCycler® ep RealPlex4 (Wesseling-Berzdorf, Germany). Primer pairs for *psa*B, *psb*D1, *rbc*L, *mcy*A, *mcy*D and *mcy*H are listed in Table 1. The following PCR protocol was used with two steps: one denaturation step at 95 °C for 1 min and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. 16S rRNA was used as a housekeeping gene to normalize the expression changes. The relative gene expression among the treatment groups was quantified by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3. Pigment and enzyme assays

Ten milliliters of each culture was collected and the cell pellet was resuspended in 1 mL of 0.5 mM K-phosphate (pH 7.0). Phycocyanobilin (PC), allophycocyanin (APC) and phycoerythrin (PE) were extracted by −80 °C freezing and thawing and absorbance at 565, 620 and 650 nm, respectively, was measured in order to estimate the phycobiliprotein contents according to a previous report (Li et al., 2008). Thirty milliliters of each culture was were collected for extracting enzyme, the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were measured by microplate Reader according to our previous report (Qian et al., 2008a). The activity of each enzyme was expressed on a protein concentration basis.

2.4. Oxidant evaluations

Thirty milliliters of each culture was collected for extracting product of lipid peroxidation. The lipid peroxidation level was determined from the MDA content according to Zhang and Kirkham (1994). ROS were measured using the DCFH-DA probe following the instructions of the ROS kit (Beyotime Institute of Biotechnology, Haimen, China). DCFH-DA reacts with ROS to form the fluorescent

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Table 1
Sequences of the primer pairs in *Microcystis aeruginosa* for real-time PCR.

16S rRNA	Forward 5′-GCCGCRAGGTGAAAMCTAA-3′ Reverse 5′-AATCCAAARACCTTCCTCCC-3′	140–369	U03402
*psa*B	Forward 5′-CGGTGACTGGGGTGTGTATG-3′ Reverse 5′-ACTCGGTTTGGGGATGGA-3′	348–460	GeneID: 5865589
*psb*D1	Forward 5′-TCTTCGGCATCGCTTTCTC-3′ Reverse 5′-CACCCACAGCACTCATCCA-3′	109–189	GeneID: 5864945
*rbc*L	Forward 5′-CGTTTCCCCGTCGCTTT-3′ Reverse 5′-CCGAGTTTGGGTTTGATGGT-3′	762–889	GeneID: 5865621
*mcy*D	Forward 5′-GGTTCGCCTGGTCAAAGTAA-3′ Reverse 5′-CCTCGCTAAAGAAGGGTTGA-3′	10,960–11,257	GeneID: 5864684
*mcy*A	Forward 5′-GCCGATGTTTGGCTGTAAAT-3′ Reverse 5′-ATCCAGCAGTTGAGCAAGC-3′	6149–6338	GeneID: 5864681
*mcy*H	Forward 5′-GGAATGCAGGAACTGGTTGTTT-3′ Reverse 5′-GTCATTTCACGGGTTGTTTTAGG-3′	1262–1391	GeneID: 5864688

product DCF, which is measured with a fluorescence plate reader (Bio-TEK, USA). An increase in fluorescence intensity indicates the content of ROS.

2.5. Data analysis

All Data are presented as mean ± standard error of the mean (SEM) and tested for statistical significance by analysis of variance (ANOVA) followed by the Dunnett's post hoc test using StatView 5.0 program. When the probability (p) was less than 0.05 or 0.01, the values were considered significantly different.

3. Results

3.1. Effects of $CuSO_4$, H_2O_2 and PNA on M. aeruginosa growth

Culture media containing five concentrations of $CuSO_4$ (0, 0.1, 0.5, 1 and 1.5 μM) and H_2O_2 (0, 10, 25, 50 and 100 μM) and four concentrations of PNA (0, 0.1, 0.5 and 1 mg L^{-1}) were prepared to evaluate their ability to inhibit growth of *M. aeruginosa*. The growth of *M. aeruginosa* was significantly inhibited during 6–96 h of exposure to $CuSO_4$ (Fig. 1). The percent inhibition showed dose- and time-dependent behaviors. The highest percent inhibition achieved was 60.8% after 96 h of exposure to 1.5 μM $CuSO_4$. We selected 0.1 and 0.5 μM $CuSO_4$ concentrations for subsequent exposure experiments. H_2O_2 inhibited algae growth in a dose-dependent manner, and the highest percent inhibition was 93.4% after 72 h of exposure to 100 μM H_2O_2. Algal growth recovered significantly after 96 h of H_2O_2 exposure, which indicated that H_2O_2 was readily degraded in the water system. We selected 25 and 50 μM H_2O_2 concentrations for subsequent exposure experiments. PNA also inhibited algal growth significantly in time- and dose-dependent manners similarly to $CuSO_4$. The highest percent inhibition was 65.3%, which was observed after 96 h of exposure to 1 mg L^{-1} PNA. We selected 0.5 and 1 mg L^{-1} PNA concentrations for subsequent exposure experiments.

3.2. Effects of $CuSO_4$, H_2O_2 and PNA on transcription of photosynthesis-related genes

The level of *psa*B was significantly reduced by treatment with $CuSO_4$ in a dose-dependent manner; 54.2% and 50.5% of the control at 0.1 μM and 11.2% and 42.1% of the control at 0.5 μM were observed after 48 and 96 h of exposure, respectively (Fig. 2A). Treatment with 25 μM H_2O_2 did not influence the transcription of *psa*B significantly; however, 50 μM H_2O_2 decreased the transcription of *psa*B to 29.3% and 24.0% of the control after 48 and 96 h of exposure, respectively (Fig. 2B). The effect of PNA on the transcription of *psa*B was quite different compared to both $CuSO_4$ and H_2O_2 exposure. The transcription of *psa*B was significantly lower after 48 h of PNA exposure but returned to the control level after 96 h of exposure (Fig. 2C).

The transcription of *psb*D1 decreased after $CuSO_4$ exposure, and the lowest level of *psb*D1 transcription was only 14.0% of the control after 48 h of exposure to 0.1 μM $CuSO_4$ (Fig. 2D). *psb*D1 transcription was not affected significantly after 48 h of exposure to H_2O_2, but it decreased to 46.7% after exposure to 50 μM H_2O_2 for 96 h (Fig. 2E). The effect of PNA on *psb*D1 transcription showed a similar pattern to H_2O_2 exposure. After 96 h of exposure to 0.5 and 1 mg L^{-1} PNA, the abundance of *psb*D1 decreased to 51.4% and 49.1% of the control, respectively (Fig. 2F).

The transcription of *rbc*L was inhibited significantly by these three algaecides (Fig. 2G–I). The lowest *rbc*L abundance was 17.1% of the control after 48 h of exposure to 0.5 μM $CuSO_4$. The lowest *rbc*L abundance after H_2O_2 treatment was 38.9% of the control, which was observed after 48 h of exposure to 50 μM H_2O_2. The lowest *rbc*L abundance after PNA treatment was 21.0% of the control, which was observed after 96 h of exposure to 1 mg L^{-1} PNA. In Fig. 2G–I, the results also show dose-dependent inhibition of *rbc*L transcription. The higher the concentration of inhibitor used, the stronger was the inhibition of *rbc*L transcription.

3.3. Effects of $CuSO_4$, H_2O_2 and PNA on transcription of microcystin-related genes

The three algaecides also greatly affected the transcription of toxin-related genes. Fig. 3A shows that 0.5 μM $CuSO_4$ stimulated the transcription of *mcy*A by 2.1-fold compared to the control after 48 h of exposure, but it did not affect its transcription after 96 h of exposure. H_2O_2 at 50 μM also stimulated *mcy*A transcription by 1.8-fold after 48 h of treatment, but it did not significantly influence transcription after 96 h of exposure (Fig. 3B). PNA did not change the transcription of *mcy*A after 48 h of treatment, but 1 mg L^{-1} PNA stimulated *mcy*A transcription by 3.6-fold after 96 h of exposure (Fig. 3C).

The transcription of *mcy*D was not affected by 0.1 or 0.5 μM $CuSO_4$ treatment after 48 h, but levels decreased to 59.8% and 46.5% of the control after 96 h of exposure, respectively (Fig. 3D). The pattern of H_2O_2 inhibition of *mcy*D transcription was similar to the pattern of $CuSO_4$. After 96 h of exposure, *mcy*D transcription decreased to 47.1% of the control with 50 μM H_2O_2 (Fig. 3E). PNA did not have a significant effect on *mcy*D transcription (Fig. 3F). Fig. 3G–I shows that *mcy*H transcription was not stimulated or inhibited by the three algaecides at the two time points analyzed, except for a decrease in expression after 96 h of exposure to 50 μM H_2O_2.

3.4. Effects of $CuSO_4$, H_2O_2 and PNA on antioxidant enzymes

To determine whether these three algaecides affect the antioxidant system, we examined the activities of antioxidant enzymes (Fig. 4A–I). SOD activity increased to 6.1- and 2.6-fold of the con-

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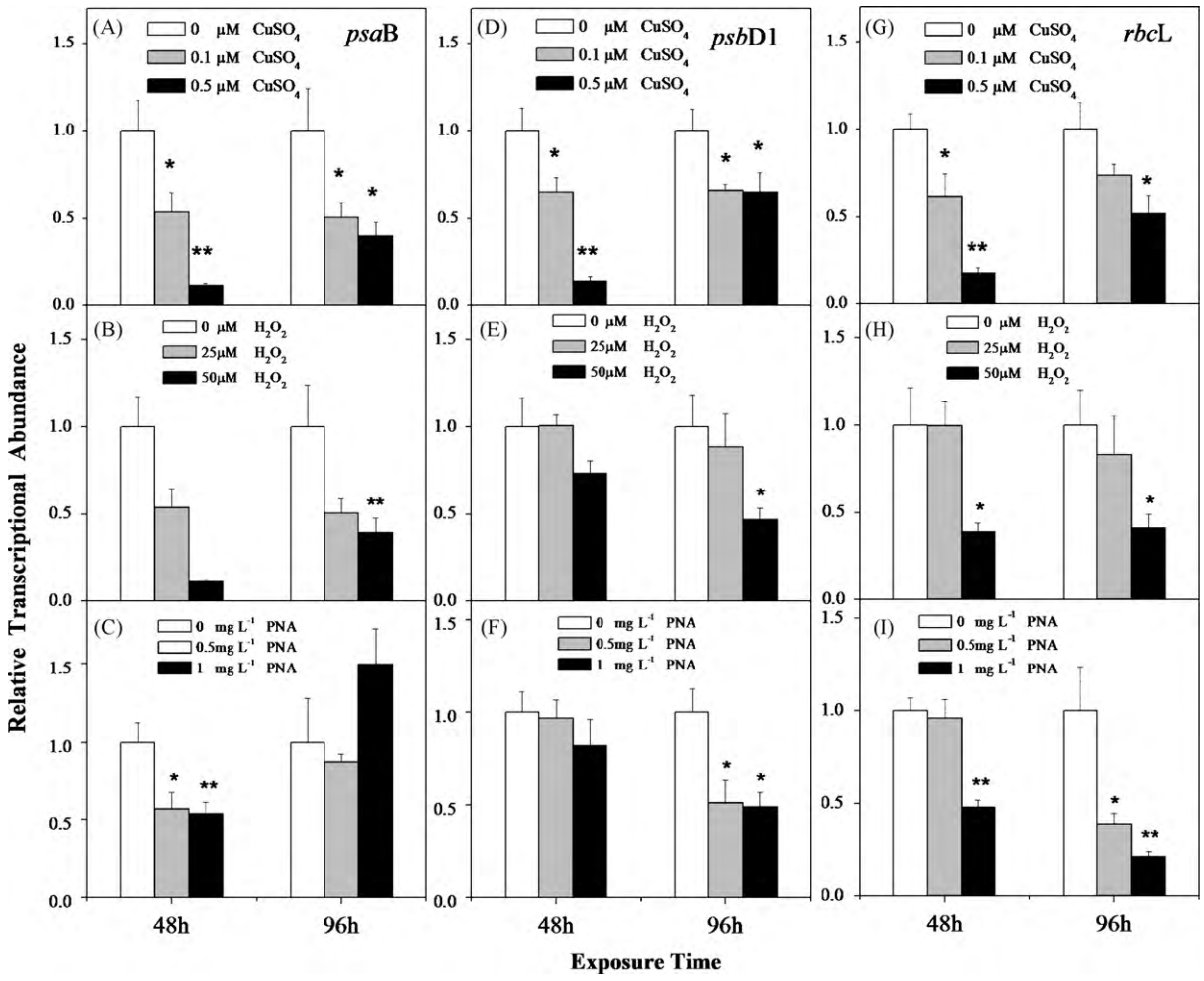

Fig. 2. Expression of *psa*B (A–C), *psb*D1 (D–F) and *rbc*L (G–I) in *M. aeruginosa* exposed to different concentrations of $CuSO_4$, H_2O_2 and PNA for 48 and 96 h. Values were normalized to levels of 16S rRNA, a housekeeping gene, and represent the mean mRNA expression value ± S.E.M. ($n = 3$) relative to the control. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

trol after 48 and 96 h of exposure to 0.5 μM $CuSO_4$, respectively (Fig. 4A). Higher concentrations of H_2O_2 also stimulated SOD activity, which increased to 3.6- and 1.4-fold of the control after 48 and 96 h of exposure (Fig. 4B). The two concentrations of PNA did not affect the activity of SOD significantly (Fig. 4C). Fig. 4D–F shows that the three algaecides induced significant increases in POD activity. $CuSO_4$ at 0.5 μM stimulated the activity of POD by 2.8-fold compared to the control after 48 h of exposure. H_2O_2 treatment also increased POD activity, and the highest POD activity observed was 1.6-fold of the control after 48 h of exposure to 25 μM H_2O_2. PNA did not increase POD activity after 48 h of treatment; however, the two concentrations of PNA stimulated POD activity to more than 5-fold of the control after 96 h of exposure. As shown in Fig. 4G–I, the three algaecides stimulated the activity of CAT to different degrees. The higher concentration of $CuSO_4$ stimulated CAT activity by 4.3- and 2.7-fold of the control after 48 and 96 h of exposure, respectively. H_2O_2 and PNA also showed similar effects on CAT activity as shown in Fig. 4G and I.

3.5. Effects of $CuSO_4$, H_2O_2 and PNA on pigment, MDA and ROS levels

Chlorophyll a (Chl a) and phycobiliproteins, including PE, PC and APC, are the primary light-harvesting chromoproteins in cyanobacteria and have an important role in algal photosynthesis. Chl a content decreased after exposure to the algaecides in a dose-dependent manner. The lowest level of Chl a was only 7.3% of the control after 96 h of exposure to 0.5 μM $CuSO_4$ (Fig. 5). The synthesis of phycobiliproteins was also inhibited by the three algaecides. The lowest levels of PE, PC and APC were 10.2%, 7.0% and 7.0% of the control, respectively (Fig. 6).

MDA, a by-product of lipid peroxidation, was quantified to ascertain the involvement of lipid peroxidation in the toxicity of these algaecides. Treatment with each algaecide had similar effects on MDA. After 0.5 μM $CuSO_4$ treatment, the MDA concentration was more than 4- and 5-fold higher than the control after 48 and 96 h of exposure (Table 2). H_2O_2 at 25 μM caused a nearly 2-fold increase in the MDA concentration relative to the control (Table 2). The highest level of MDA was 3.9-fold of the control after 96 h of exposure to 1 mg L^{-1} PNA.

ROS production was assessed quantitatively by fluorescence intensity. ROS production resembled an oxidative burst after exposure to 0.5 μM $CuSO_4$ (Fig. 7) and accumulated to levels 2.1- and 2.5-fold that of the control after 48 and 96 h of exposure, respectively. The change in ROS production induced by H_2O_2 was similar to $CuSO_4$ exposure. The two selected concentrations of H_2O_2 stimulated ROS production in a dose-dependent manner. The highest level of ROS production was 1.4-fold of the control after 48 h of exposure to 50 μM H_2O_2. The highest concentration of PNA also stimulated ROS formation significantly.

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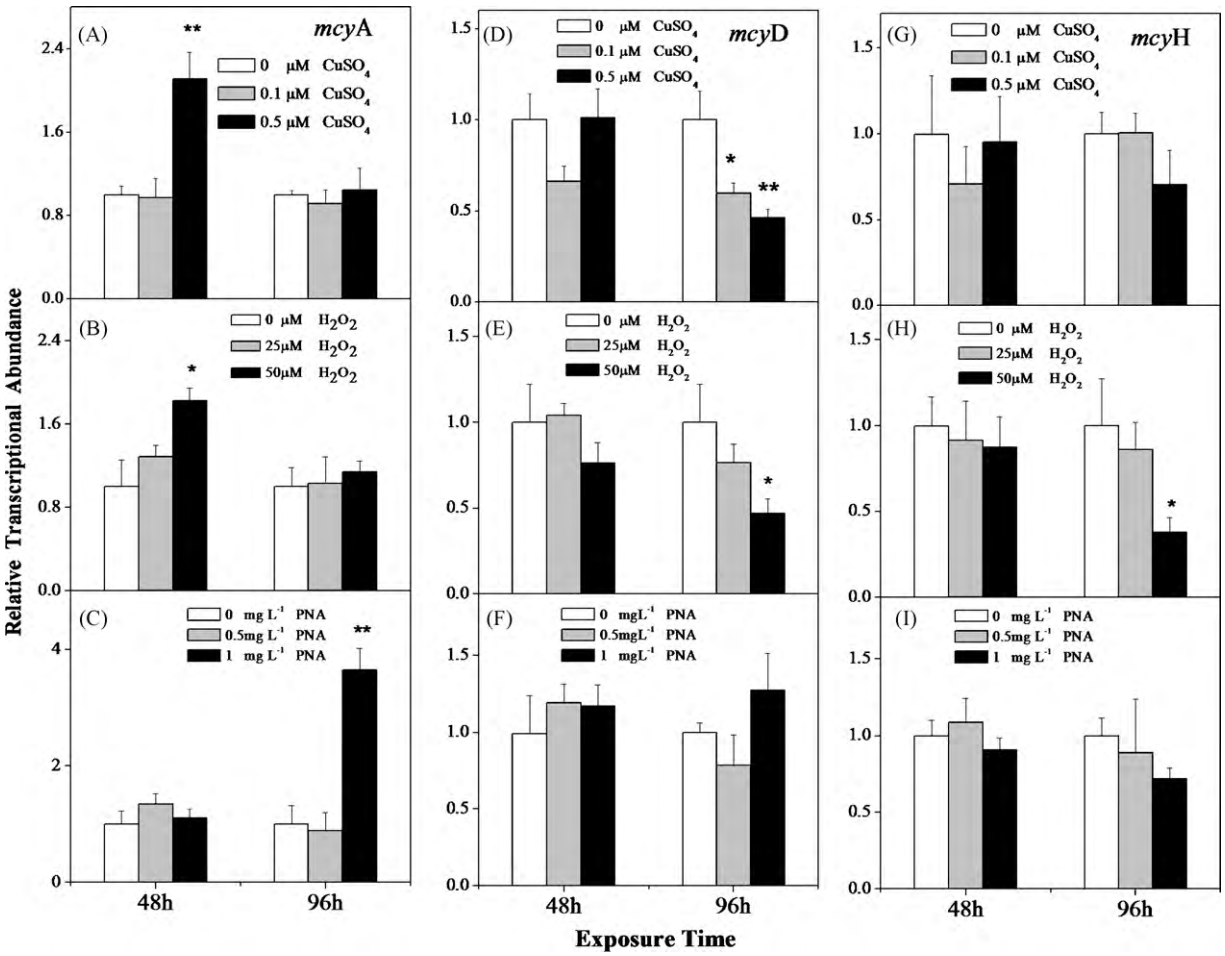

Fig. 3. Expression of *mcy*A (A–C), *mcy*D (D–F) and *mcy*H (G–I) in *M. aeruginosa* exposed to different concentrations of $CuSO_4$, H_2O_2 and PNA for 48 and 96 h. Values were normalized to levels of 16S rRNA, a housekeeping gene, and represent the mean mRNA expression value ± S.E.M. ($n = 3$) relative to the control. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

4. Discussion

There are few reports in the literature on the effects of chemical reagents on *M. aeruginosa*, and most of them only describe changes in algal mortality, chlorophyll content, cellular-soluble protein, photosynthetic activity and other physiological parameters (Drábková et al., 2007; Hong et al., 2008; Pan et al., 2008). In the present study, we investigated the effect of $CuSO_4$, H_2O_2 and PNA not only on the above-mentioned physiological parameters but also on the transcription of photosynthesis- and microcystin synthesis-related genes, *psa*B, *psb*D1 and *rbc*L, which are photosynthesis-related genes, encode key proteins in PSI, PSII and the carbon assimilation process, respectively. The results obtained in the present study showed that $CuSO_4$, H_2O_2 and PNA inhibited *psa*B and *psb*D1 transcription after 48 or 96 h of exposure. The decrease in photosynthesis-related gene transcription could block electron transport and decrease reducing equivalent production, which are necessary for the process of carbon assimilation. Therefore, the activity of rubisco, the rate-limiting enzyme in carbon assimilation, must be reduced, which agrees with the observed decrease in *rbc*L transcript abundance. We also analyzed the content of the photosynthetic pigments, chl a, PE, PC and APC and found that photosynthetic pigment levels decreased significantly. Because these pigments capture the light energy necessary for photosynthesis, the decrease in their abundance could also block photosynthesis. This phenomenon of inhibition of photosynthesis-related genes by algaecides was similar to our previous reports on *C. vulgaris* after herbicide exposure (Qian et al., 2008a; Qian et al., 2008b; Qian et al., 2009). ROS was produced by molecular oxygen combined with surplus electrons, which may also involved in the inhibition of the electron transport chain. The increased ROS production led to membrane deterioration, as indicated by the increase in the MDA level (Table 2).

Microcystins are another research hotspot because they cause serious health and environmental problems. Microcystin synthesis is catalyzed by microcystin synthetase, which includes two transcribed operons that encode the microcystin peptide synthetase and polyketide synthase genes. A few studies have focused specif-

Table 2
MDA levels after exposure to $CuSO_4$, H_2O_2 and PNA.

MDA (nmol/μg protein)		48 h	96 h
$CuSO_4$	0 μM	1.3 ± 0.1	1.7 ± 0.1
	0.1 μM	1.4 ± 0.0	2.0 ± 0.2
	0.5 μM	5.4 ± 0.5**	9.7 ± 0.6**
H_2O_2	0 μM	1.6 ± 0.1	3.9 ± 0.1
	25 μM	1.8 ± 0.1	4.3 ± 0.1
	50 μM	2.4 ± 0.3**	8.7 ± 0.7**
PNA	0 mg L^{-1}	2.7 ± 0.1	4.0 ± 1.2
	0.5 mg L^{-1}	4.0 ± 0.4	9.6 ± 1.5**
	1 mg L^{-1}	4.2 ± 0.7*	15.5 ± 0.4**

* Represents a statistically significant difference of $p < 0.05$ when compared to the control.
** Represents a statistically significant difference of $p < 0.01$.

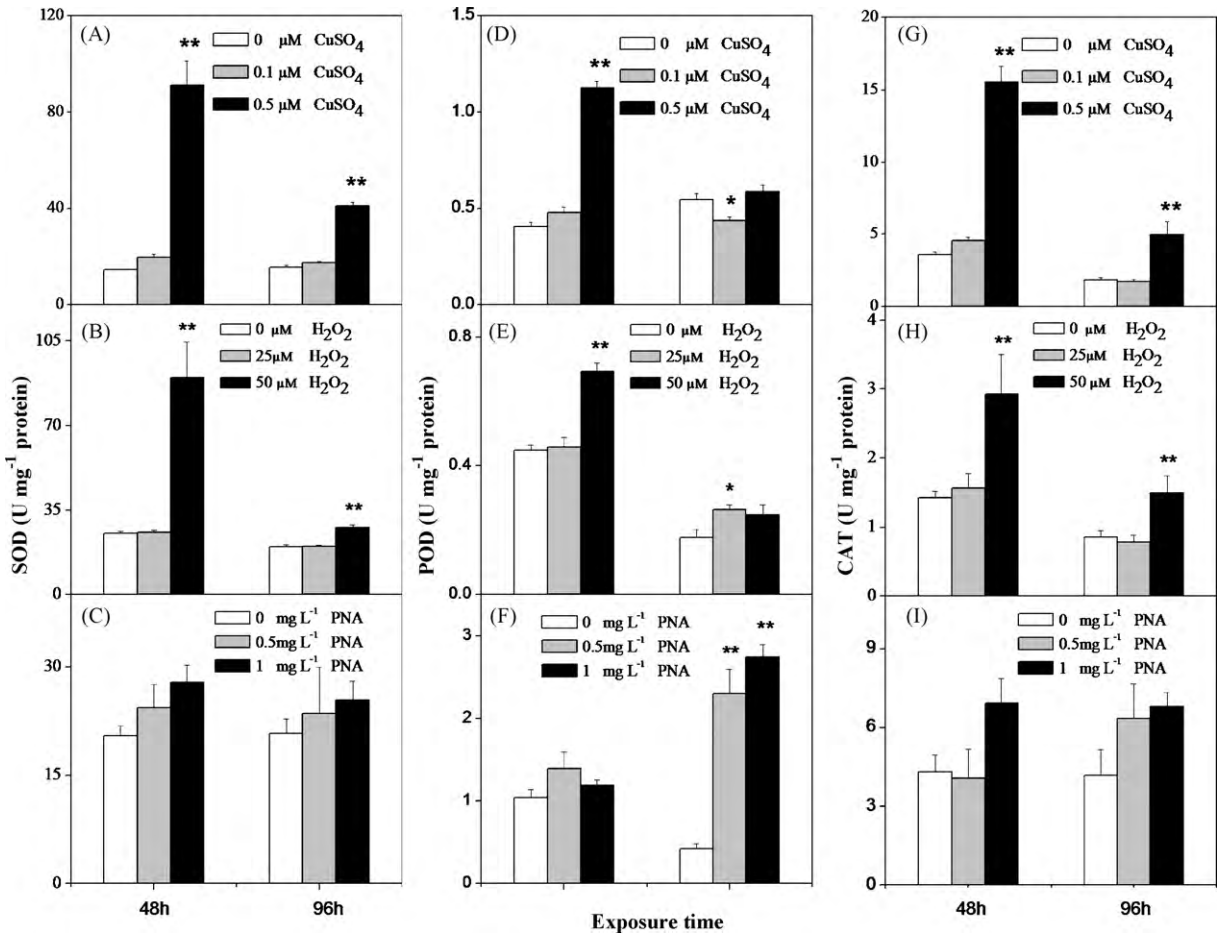

Fig. 4. Activities of superoxide dismutase (A–C), peroxidase (D–F) and catalase (G–I) in *M. aeruginosa* exposed to different concentrations of $CuSO_4$, H_2O_2 and PNA for 48 and 96 h. The *Y*-axis represents the activities of enzymes expressed as the mean ± SEM of three replicate cultures. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

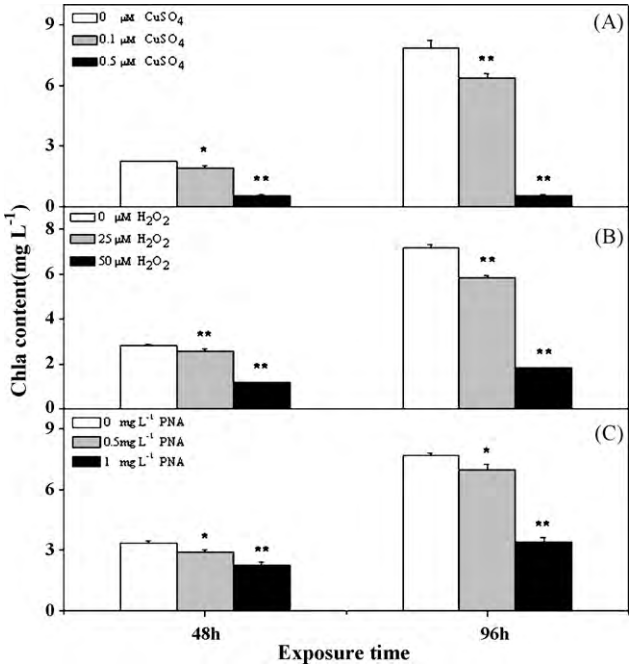

Fig. 5. Inhibitory effects of $CuSO_4$, H_2O_2 and PNA on chlorophyll a content in *M. aeruginosa* exposed to different inhibitor concentrations for 48 and 96 h. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

ically on the microcystin biosynthesis genes and shown the effects of single or combined factors on microcystin synthesis (Kaebernick et al., 2000; Sevilla et al., 2008; Shao et al., 2009). However, some of these results have been contradictory. In this study, we selected *mcy*A and *mcy*D as representatives of microcystin synthetase and used real-time PCR to analyze the transcript levels of these genes. The results showed that the abundance of *mcy*A was increased by $CuSO_4$, H_2O_2 and PNA exposure to different degrees, which may indiate an increase in microcystin peptide synthetase. In a previous report, only the *mcy*A gene was used as a target gene to quantify microcystin production in cyanobacteria by real-time PCR (Furukawa et al., 2006). This report showing that $CuSO_4$, H_2O_2 and PNA stimulated the transcription of the *mcy*A gene to increase the activity of microcystin peptide synthetase, which could increase microcystin production. This result agrees with Shao et al. (2009), who reported that the transcription of *mcy*B (which is regulated by the same operon as *mcy*A) was up-regulated by pyrogallol, another allelochemical.

*mcy*D is involved in synthesis of the β-amino acid Adda, which is responsible for the toxicity of the microcystins (Tillett et al., 2000). Moreover, *mcy*D expression is essential for microcystin synthesis, and lack of the protein results in the absence of microcystin synthesis (Kaebernick et al., 2002; Christiansen et al., 2006). Our results showed that *mcy*D transcript abundance was decreased by $CuSO_4$ and H_2O_2. It is particularly interesting that the regulation of *mcy*D was different from *mcy*A, which may ascribe that these two genes belong to two operons and have bidirectional promoters. Due to its involvement in the synthesis of the β-amino acid Adda, the

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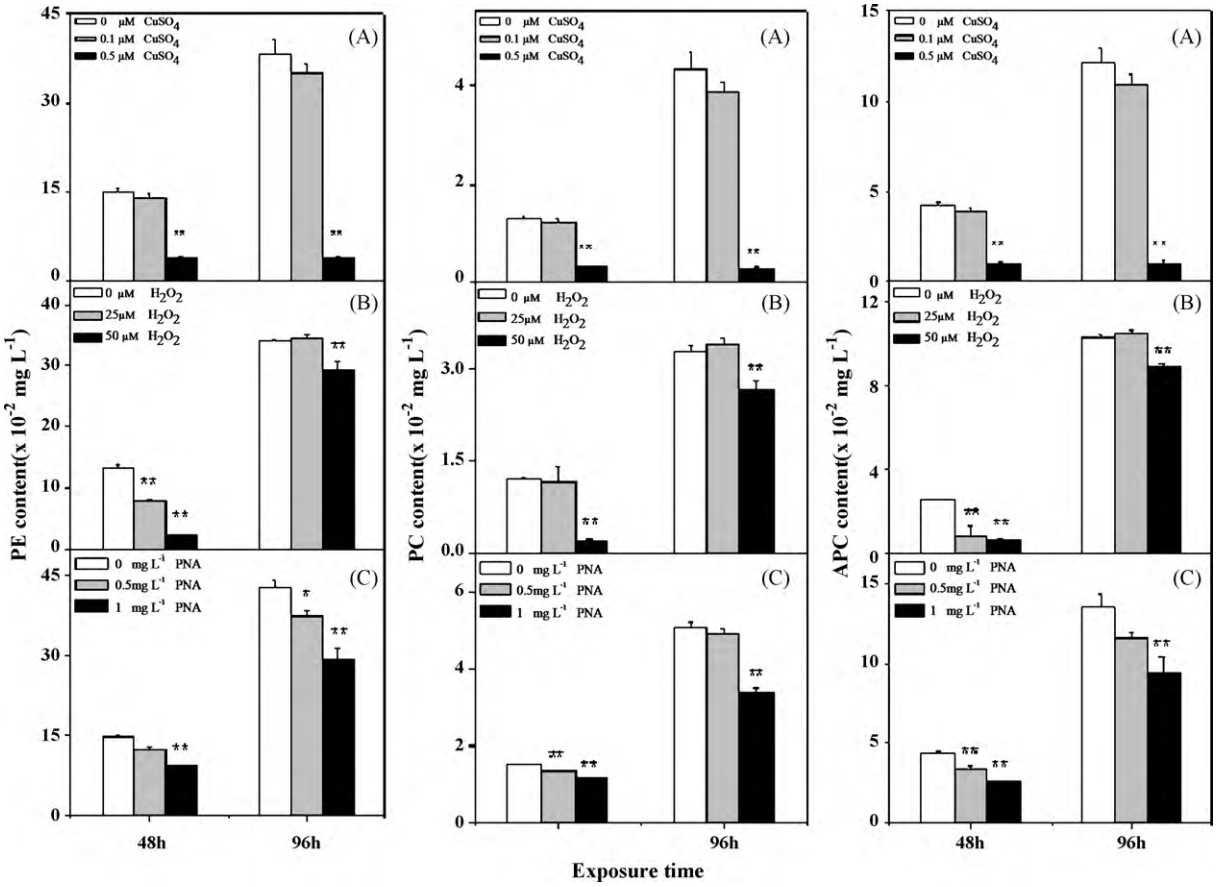

Fig. 6. Inhibitory effects of $CuSO_4$, H_2O_2 and PNA on PE (A–C), PC (D–F) and APC (G–I) levels in *M. aeruginosa* exposed to different inhibitor concentrations for 48 and 96 h. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

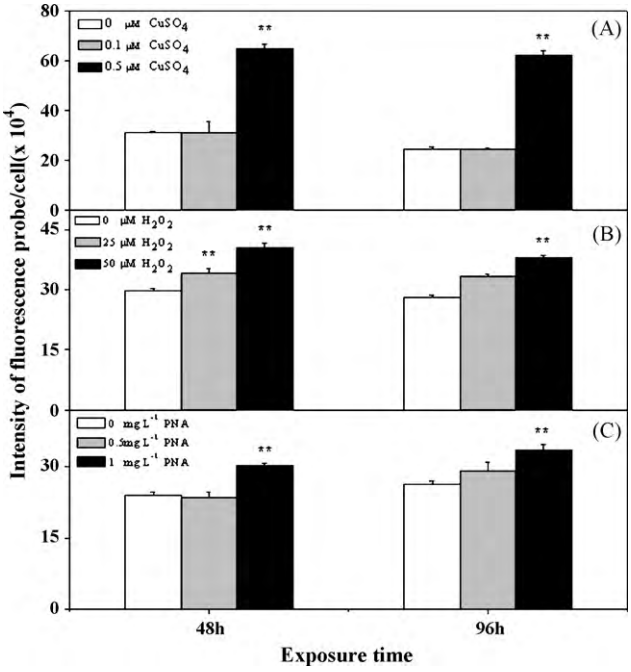

Fig. 7. Stimulatory effects of $CuSO_4$, H_2O_2 and PNA on ROS content in *M. aeruginosa* exposed to different inhibitor concentrations for 48 and 96 h. * represents a statistically significant difference of $p < 0.05$ when compared to the control, ** represents a statistically significant difference of $p < 0.01$.

*mcy*D protein directly determines the quantity of substrate available for microcystin synthesis. A decrease in *mcy*D transcription would block microcystin synthesis even though the amount of the microcystin peptide synthetase increased due to an increase in *mcy*A transcription. These results demonstrated that $CuSO_4$ and H_2O_2 are more suitable algaecide because they may inhibit microcystin synthesis.

5. Conclusion

Studies have demonstrated that the three algaecides inhibit transcription of photosynthesis-related genes, which may block the electron transport chain to form ROS. The increased level of ROS could destroy pigment synthesis and the integrity of membrane, resulting in algal cell death. However, copper compound is not biodegradable, and once it is released into the environment, it accumulates in organisms' bodies or sediments. Therefore, the broad application of $CuSO_4$ to inhibit harmful algae could result in metal compound secondary pollution. PNA is an effective new algaecide, but the toxicity of PNA (including its degradation products) to aquatic organisms, like fish, shellfish or plankton, has not been widely studied. Many more studies should be conducted before PNA is applied for algal bloom control. H_2O_2 inhibited *M. aeruginosa* growth by blocking transcription of photosynthesis-related genes or destroying photosynthetic pigments. More importantly, H_2O_2 was the only reagent of these three potential algaecides that decreased the transcription of the microcystin transport gene, which can prevent transport of microcystin into the water system. In view of these merits and its ability to be degraded easily, H_2O_2 has good activity and could potentially be used to control

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algal blooms. However, given easy degradation of H_2O_2 and complexity of natural water system, many studies are needed to reveal the persistence and applicable concentration of H_2O_2 under natural condition before it can be recommended as a common algicide.

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