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*

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**A B S T R A C T**

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 (H₂O₂) 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 *psbB, psbD1*, and *rbcL* 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, H₂O₂ treatment down-regulated *mcyD* transcription, which indicated a decrease in the microcystin level in the cells. Our results demonstrate that H₂O₂ has the greatest potential as an algaecide because it not only inhibits algal growth but may reduce microcystin synthesis.

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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 (CuSO₄) 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 Ellof, 1979). Hydrogen peroxide (H₂O₂) 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 (Macias et al., 1998).

The effects of potential algaecides on algal 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...
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 algaeicides (CuSO\(_4\), H\(_2\)O\(_2\) and PNA) on photosynthesis of M. aeruginosa. These photosynthesis-related genes included (1) rbcL, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) in alga, (2) psbD1, which encodes the D2 protein that forms the reaction center of photosystem II (PSII), and (3) psbB, which encodes one of the reaction center subunits of photosystem I (PSI).

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, mcyA-C and mcyD-J (Tillett et al., 2000). mcyA belongs to the mcyA-C gene cluster and encodes microcystin synthetase; mcyD belongs to the mcyD-J gene cluster, and it encodes the modular polyketide synthase involved in the synthesis of the \(\beta\)-amino acid Adda that is responsible for the toxicity of the microcystins (Tillett et al., 2000). Moreover, mcyD expression is also essential for microcystin synthesis (Christiansen et al., 2006). mcyE is located upstream of mcyE, and bioinformatic and experimental data have shown that mcyH 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 algaeicides on the transcriptional levels of mcyA, mcyD and mcyH to select the best microcystin inhibitory agent.

In this study, we also investigated parameters to confirm the toxicological effects of the three potential algaeicides 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 algaeicides on M. aeruginosa growth, and to select the most valuable algaeicide for controlling algal bloom formation and potential for microcystin synthesis.

### 2. Materials and methods

#### 2.1. Algae strains and culture conditions

*Microcystis 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 measured spectrophotometrically at 685 nm (OD\(_{685}\)). The regression equation between the density of algal cells (\(Y = 10^{2}\) \(\times\) mL) and OD\(_{685}\) (X) was established as \(Y = 34.11 + 0.73 (R^2 = 0.99)\). 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).

#### 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-Bergdorf, Germany). Primer pairs for *psbA*, *psbD1*, *rbcL*, *mcyA*, *mcyD* and *mcyH* 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 RNA was used as a housekeeping gene to normalize the expression changes. The relative gene expression among the treatment groups was quantified by the \(2^{-\Delta\Delta C_{T}}\) 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 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 (BeyoTechnology Institute of Biotechnology, Haimen, China). DCFH-DA reacts with ROS to form the fluorescent...
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 CuSO4, H2O2 and PNA on M. aeruginosa growth

Culture media containing five concentrations of CuSO4 (0, 0.1, 0.5, 1 and 1.5 μM) and H2O2 (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 CuSO4 (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 CuSO4. We selected 0.1 and 0.5 μM CuSO4 concentrations for subsequent exposure experiments. H2O2 inhibited algal growth in a dose-dependent manner, and the highest percent inhibition was 93.4% after 72 h of exposure to 100 μM H2O2. Algal growth recovered significantly after 96 h of H2O2 exposure, which indicated that H2O2 was readily degraded in the water system. We selected 25 and 50 μM H2O2 concentrations for subsequent exposure experiments. PNA also inhibited algal growth significantly in time- and dose-dependent manners similarly to CuSO4. 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 CuSO4, H2O2 and PNA on transcription of photosynthesis-related genes

The level of psaB was significantly reduced by treatment with CuSO4 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 H2O2 did not influence the transcription of psaB significantly; however, 50 μM H2O2 decreased the transcription of psaB 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 psaB was quite different compared to both CuSO4 and H2O2 exposure. The transcription of psaB 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 psbD1 decreased after CuSO4 exposure, and the lowest level of psbD1 transcription was only 14.0% of the control after 48 h of exposure to 0.1 μM CuSO4 (Fig. 2D), psbD1 transcription was not affected significantly after 48 h of exposure to H2O2, but it decreased to 46.7% after exposure to 50 μM H2O2 for 96 h (Fig. 2E). The effect of PNA on psbD1 transcription showed a similar pattern to H2O2 exposure. After 96 h of exposure to 0.5 and 1 mg L−1 PNA, the abundance of psbD1 decreased to 51.4% and 49.1% of the control, respectively (Fig. 2F).

The transcription of rbcL was inhibited significantly by these three algaecides (Fig. 2G–I). The lowest rbcL abundance was 17.1% of the control after 48 h of exposure to 0.5 μM CuSO4. The lowest rbcL abundance after H2O2 treatment was 38.9% of the control, which was observed after 48 h of exposure to 50 μM H2O2. The lowest rbcL 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 rbcL transcription. The higher the concentration of inhibitor used, the stronger was the inhibition of rbcL transcription.

3.3. Effects of CuSO4, H2O2 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 CuSO4 stimulated the transcription of mcyA 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. H2O2 at 50 μM also stimulated mcyA 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 mcyA after 48 h of treatment, but 1 mg L−1 PNA stimulated mcyA transcription by 3.6-fold after 96 h of exposure (Fig. 3C).

The transcription of mcyD was not affected by 0.1 or 0.5 μM CuSO4 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 H2O2 inhibition of mcyD transcription was similar to the pattern of CuSO4. After 96 h of exposure, mcyD transcription decreased to 47.1% of the control with 50 μM H2O2 (Fig. 3E). PNA did not have a significant effect on mcyD transcription (Fig. 3F).

The pattern of H2O2 inhibition of mcyH transcription was similar to the pattern of CuSO4. After 96 h of exposure, mcyH transcription decreased to 47.1% of the control with 50 μM H2O2 (Fig. 3E). PNA did not have a significant effect on mcyH transcription (Fig. 3F).

3.4. Effects of CuSO4, H2O2 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 2.5-fold of the con-
Fig. 2. Expression of psaB (A–C), psbD1 (D–F) and rbcL (G–I) in M. aeruginosa exposed to different concentrations of CuSO4, H2O2 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 \( p < 0.01 \).

3.5. Effects of CuSO4, H2O2 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 \( \mu \)M CuSO4 (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 \( \mu \)M CuSO4 treatment, the MDA concentration was more than 4- and 5-fold higher than the control after 48 and 96 h of exposure (Table 2). H2O2 at 25 \( \mu \)M 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 \( \mu \)M CuSO4 (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 H2O2 was similar to CuSO4 exposure. The two selected concentrations of H2O2 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 \( \mu \)M H2O2. The highest concentration of PNA also stimulated ROS formation significantly.
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 CuSO₄, H₂O₂ and PNA not only on the above-mentioned physiological parameters but also on the transcription of photosynthesis- and microcystin synthesis-related genes, *psaB*, *psbD1* and *rbcL*, 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 CuSO₄, H₂O₂ and PNA inhibited *psaB* and *psbD1* 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 *rbcL* 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-
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Fig. 6. Inhibitory effects of CuSO₄, H₂O₂ 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 CuSO₄, H₂O₂ 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 \( p < 0.01 \).

**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 CuSO₄ 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. H₂O₂ inhibited *M. aeruginosa* growth by blocking transcription of photosynthesis-related genes or destroying photosynthetic pigments. More importantly, H₂O₂ 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, H₂O₂ has good activity and could potentially be used to control *mcyD* protein directly determines the quantity of substrate available for microcystin synthesis. A decrease in *mcyD* transcription would block microcystin synthesis even though the amount of the microcystin peptide synthetase increased due to an increase in *mcyA* transcription. These results demonstrated that CuSO₄ and H₂O₂ are more suitable algaecide because they may inhibit microcystin synthesis.
algal blooms. However, given easy degradation of H₂O₂ and complexity of natural water system, many studies are needed to reveal the persistence and applicable concentration of H₂O₂ under natural condition before it can be recommended as a common algicide.

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