Species-dependent variation in sensitivity of Microcystis species to copper sulfate: implication in algal toxicity of copper and controls of blooms

Haiming Wu1, Gaojie Wei1, Xiao Tan2, Lin Li3 & Ming Li1,3,4

Copper sulfate is a frequently used reagent for Microcystis blooms control but almost all the previous works have used Microcystis aeruginosa as the target organism to determine dosages. The aim of this study was to evaluate interspecific differences in the responses of various Microcystis species to varying Cu²⁺ concentrations (0, 0.05, 0.10, 0.25, and 0.50 mg L⁻¹). The half maximal effective concentration values for M. aeruginosa, M. wesenbergii, M. flos-aquae, and M. viridis were 0.16, 0.09, 0.49, and 0.45 mg L⁻¹ Cu²⁺, respectively. This showed a species-dependent variation in the sensitivity of Microcystis species to copper sulfate. Malonaldehyde content did not decrease with increasing superoxide dismutase content induced by increasing Cu²⁺, suggesting that superoxide dismutase failed to reduce Cu²⁺ damage in Microcystis. Considering the risk of microcystin release when Microcystis membranes are destroyed as a result of Cu²⁺ treatment and the stimulation effects of a low level of Cu²⁺ on growth in various species, our results suggest that copper sulfate treatment for Microcystis control could be applied before midsummer when M. aeruginosa and M. viridis are not the dominant species and actual amount of Cu²⁺ used to control M. wesenbergii should be much greater than 0.10 mg L⁻¹.

Microcystis blooms are increasing in freshwater ecosystems as a result of eutrophication and global temperature increases, becoming a global environmental and ecological problem1,2. Besides the environmental consequences, such as unpleasant odor, loss of water transparency, and depletion of oxygen3, microcystin production is a serious problem resulting from Microcystis blooms that threatens public health through drinking water supply and fishing4.

Many methods, including biological5, chemical6, physical7, and nutrient restriction8, have been proposed and applied to blooms control by inhibiting Microcystis growth. However, most of these methods are impeded because they are expensive and slow to take effect, except for some chemical methods. Chemical methods can have serious negative effects on ecosystems, e.g., toxin release, persistence, and bioaccumulation5,10. Nevertheless, it is still considered both an effective emergency strategy and a last chance to control Microcystis blooms11.

Copper sulfate is a frequently-used, low-cost chemical reagent for blooms control and the copper sulfate toxicity mechanisms have been well studied12,13. Many lakes and reservoirs, e.g., Fairmont Lakes (USA)14, Myponga Reservoir (Australia)15, Omerli Reservoir (Turkey)16, and a prairie lake in Canada17, were treated with copper sulfate. A moderate dose of copper sulfate, not toxic to humans or aquatic animals, to control bloom-forming Microcystis was chosen to inhibit Microcystis growth. Hadjoudja et al.15 reported that the 24-h-EC₅₀ of Microcystis is 0.064 mg L⁻¹ Cu²⁺. Tsai18 reported that the minimum Cu²⁺ concentration required to inhibit M. aeruginosa growth is 0.160 mg L⁻¹. The 96-h-EC₅₀ of M. aeruginosa was reported as 1.02 mg L⁻¹ Cu²⁺ by Zhang et al.19. All of the above Cu²⁺ doses are considered safe according to the World Health Organization recommendation.
The medium was treated with varying amounts of copper sulfate and the Cu\textsuperscript{2+} BG-11 medium in a 250-mL conical flask under a 12:12-h light:dark cycle. All cultures were prepared in triplicate.

Organisms. Four Microcystis species were provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The identification numbers and origins of the four species are listed in Table 1. All strains were unicellular and purified through the dilution method and axenically cultivated in BG-11 medium for more than 3 months.

Table 1. Details of the four Microcystis species used in the current study.

| Species                  | Code  | Culture collection | Origin          | Morphology   |
|-------------------------|-------|--------------------|-----------------|--------------|
| M. aeruginosa           | 1343  | FACHB              | Lake Taihu, China | Unicellular  |
| M. wesenbergii          | 1324  | FACHB              | Lake Taihu, China | Unicellular  |
| M. flos-aquae           | 1272  | FACHB              | Lake Taihu, China | Unicellular  |
| M. viridis              | 1337  | FACHB              | Lake Dianchi, China | Unicellular |

Materials and Methods

Experimental design. Each strain was batch-cultured axenically in triplicate in 150 mL sterilized liquid BG-11 medium in a 250-mL conical flask under a 12:12-h light:dark cycle. All cultures were prepared in triplicate. The medium was treated with varying amounts of copper sulfate and the Cu\textsuperscript{2+} concentrations were 0.05, 0.10, 0.25, and 0.50 mg L\textsuperscript{-1}. The culture without copper sulfate treatment was used as the control. The initial cell density of Microcystis was 100 × 10\textsuperscript{4} cells mL\textsuperscript{-1}. The light intensity was 50 μmol photons m\textsuperscript{-2}s\textsuperscript{-1} and the culture time was 4 days. The cell density and efficiency of primary conversion of light energy of PS II (Fv:Fm) reduction, superoxide dismutase (SOD), and malonaldehyde (MDA) were analyzed to assess the physiological status of various Microcystis species to varying Cu\textsuperscript{2+} concentrations since the mechanisms by which Cu\textsuperscript{2+} inhibits growth of Microcystis had been well studied\textsuperscript{18,19,21}.

Data analysis. All of the data are presented as mean ± SD. The specific growth rate was calculated by equation (1):

$$\mu = \ln (D_t/D_0)/t$$  \hspace{1cm} (1)

where $D_t$ is the cell density at time $t$, $D_0$ is the cell density in the initial logarithmic growth phase, and $t$ is the duration of the logarithmic growth phase. In the current study, the value of $t$ was 4.

The half maximal effective concentration (EC\textsubscript{50}) was determined on day 4 at the 50% inhibition rate according to the relationship between inhibition rate and concentration of Cu\textsuperscript{2+}. The inhibition rate was calculated by equation (2):

$$\text{Inhibition rate} = (D_{4,0} - D_{4,t})/D_{4,0} \times 100\%$$  \hspace{1cm} (2)

Whereas, a recent study reported that a safe Cu\textsuperscript{2+} concentration (0.16–0.64 mg L\textsuperscript{-1}) would lyse Microcystis cells and release microcystins from the cells\textsuperscript{21,23,22}. Therefore, more care should be taken when recommending a safe Cu\textsuperscript{2+} dose and more information on the effects of Cu\textsuperscript{2+} on growth, physiology, and cell integrity of both toxic and non-toxic Microcystis species is required.

It is noteworthy that almost all of the previous studies used M. aeruginosa as the target organism to determine the Cu\textsuperscript{2+} dose for Microcystis control\textsuperscript{10–12}. However, several species have been recorded in the Microcystis genus\textsuperscript{23} and the growth, physiology, and toxicity of Microcystis species varies greatly\textsuperscript{24}. The effects of temperature, nutrients, and iron on growth of various Microcystis species differs significantly\textsuperscript{25,26}. Moreover, M. wesenbergii, M. flos-aquae, and M. viridis have been reported as the dominant species in lakes besides M. aeruginosa\textsuperscript{27,28}. Succession is always observed in these species in lakes\textsuperscript{29}. A serious and important question is whether or not we can use the Cu\textsuperscript{2+} dose determined from M. aeruginosa to control all Microcystis species in lakes.

Even though species-dependent variation in algal sensitivity to chemical compounds has been widely reported\textsuperscript{30,31}, significant differences could not be inferred because the species used in the previous study came from a different genus; however, we are talking about species in the same genus. The aim of this study was to evaluate interspecific differences in the responses of various Microcystis species to varying Cu\textsuperscript{2+} concentrations. The efficiency of primary conversion of light energy of PS II (Fv:Fm), cell viability (analyzed by 2,3,5-triphenyltetrazolium chloride (TTC) reduction), superoxide dismutase (SOD), and malonaldehyde (MDA) were analyzed.

Materials and Methods

Organisms. Four Microcystis species were provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The identification numbers and origins of the four species are listed in Table 1. All strains were unicellular and purified through the dilution method and axenically cultivated in BG-11 medium for more than 3 months.

Experimental design. Each strain was batch-cultured axenically in triplicate in 150 mL sterilized liquid BG-11 medium in a 250-mL conical flask under a 12:12-h light:dark cycle. All cultures were prepared in triplicate. The medium was treated with varying amounts of copper sulfate and the Cu\textsuperscript{2+} concentrations were 0.05, 0.10, 0.25, and 0.50 mg L\textsuperscript{-1}. The culture without copper sulfate treatment was used as the control. The initial cell density of Microcystis was 100 × 10\textsuperscript{4} cells mL\textsuperscript{-1}. The light intensity was 50 μmol photons m\textsuperscript{-2}s\textsuperscript{-1} and the culture time was 4 days. The cell density and efficiency of primary conversion of light energy of PS II (Fv:Fm) reduction, superoxide dismutase (SOD), and malonaldehyde (MDA) were analyzed to assess the physiological status of various Microcystis species to varying Cu\textsuperscript{2+} concentrations since the mechanisms by which Cu\textsuperscript{2+} inhibits growth of Microcystis had been well studied\textsuperscript{18,19,21}.

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$$\text{Inhibition rate} = (D_{4,0} - D_{4,t})/D_{4,0} \times 100\%$$  \hspace{1cm} (2)
where \( D_{4,0} \) is the cell density in the control on day 4, \( D_{4,c} \) is the cell density on day 4 treated with concentration \( c \) of Cu\(^{2+}\).

**Results**

**Microcystis growth.** Microcystis cell densities in the control increased with time and the maximum cell density of four species was in the order of \( M. aeruginosa > M. viridis > M. flos-aquae > M. wesenbergii \) (Fig. 1). The growth of these four Microcystis species was inhibited when treated with 0.50 mg L\(^{-1}\) Cu\(^{2+}\). It was also found that 0.05 mg L\(^{-1}\) and 0.10 mg L\(^{-1}\) Cu\(^{2+}\) promoted \( M. flos-aquae \) and \( M. viridis \) growth.

The specific growth rates were 0.57, 0.35, 0.45, and 0.50 day\(^{-1}\) in the control for \( M. aeruginosa \), \( M. wesenbergii \), \( M. flos-aquae \), and \( M. viridis \), respectively (Table 2). The specific growth rates of \( M. aeruginosa \) and \( M. wesenbergii \) decreased with increasing Cu\(^{2+}\) concentration. However, the specific growth rates of \( M. flos-aquae \) and \( M. viridis \) increased when treated with 0.05 mg L\(^{-1}\) and 0.10 mg L\(^{-1}\) Cu\(^{2+}\). Their growth rate was 0.27 and 0.25 day\(^{-1}\) in the treatment with the highest level of Cu\(^{2+}\). The EC\(_{50}\) values for \( M. aeruginosa \), \( M. wesenbergii \), \( M. flos-aquae \), and \( M. viridis \) were 0.16, 0.09, 0.49, and 0.45 mg L\(^{-1}\) Cu\(^{2+}\), respectively.

**Photosynthetic activity of Microcystis.** The initial Fv:Fm values for \( M. aeruginosa \), \( M. wesenbergii \), \( M. flos-aquae \), and \( M. viridis \) were 0.14, 0.26, 0.36, and 0.58, respectively. The Fv:Fm values decreased in all of the treatments on the first day and then increased, except for \( M. viridis \) (Fig. 2). There was no change in the \( M. viridis \) Fv:Fm value throughout the experiment. At the end of the experiment, Fv:Fm of both \( M. aeruginosa \) and \( M. wesenbergii \) decreased in the 0.50-mg L\(^{-1}\) Cu\(^{2+}\) treatment. The \( M. flos-aquae \) Fv:Fm value increased one-fold on day 4 in the 0.50-mg L\(^{-1}\) Cu\(^{2+}\) treatment compared with the control.

| Specific growth rate in treatments with various concentrations of Cu\(^{2+}\) (mg L\(^{-1}\)) | \( EC_{50} \) |
|---|---|
| Control | 0.05 | 0.10 | 0.25 | 0.50 |
| \( M. aeruginosa \) | 0.57 | 0.49 | 0.23 | 0.13 | 0.11 | 0.16 |
| \( M. wesenbergii \) | 0.35 | 0.17 | 0.03 | 0.02 | 0.00 | 0.09 |
| \( M. flos-aquae \) | 0.45 | 0.49 | 0.47 | 0.41 | 0.27 | 0.49 |
| \( M. viridis \) | 0.50 | 0.54 | 0.52 | 0.48 | 0.25 | 0.45 |

Table 2. Specific growth rate (day\(^{-1}\)) and EC\(_{50}\) (mg L\(^{-1}\)) in four Microcystis species exposed to varying Cu\(^{2+}\) concentrations.
Relative Microcystis TTC reduction. Relative TTC reduction in *M. aeruginosa* and *M. flos-aquae* significantly \((P < 0.05)\) decreased along with increasing \(\text{Cu}^{2+}\) concentration (Fig. 3). However, only very small changes were observed in *M. wesenbergii*. In the 0.05-mg L\(^{-1}\) Cu\(^{2+}\) treatment, the relative TTC reduction in *M. viridis* reached 160\% compared with 40\% in control.

Microcystis SOD and MDA content. In the control, SOD content in *M. wesenbergii* was greater than that in the other species (Fig. 4). With increasing \(\text{Cu}^{2+}\) concentration, SOD content significantly increased in all four species \((P < 0.05)\). In the highest \(\text{Cu}^{2+}\) treatment, SOD content was 9.0, 8.5, 5.0, and 4.1 U (10\(^8\) cells\(^{-1}\)), for *M. wesenbergii*, *M. aeruginosa*, *M. flos-aquae*, and *M. viridis*, respectively. MDA content also significantly increased with increasing \(\text{Cu}^{2+}\) concentration in all four species \((P < 0.05);\) Fig. 5). In the 0.50-mg L\(^{-1}\) Cu\(^{2+}\) treatment, MDA content was 10.8, 9.4, 7.8, and 3.4 \(\mu\)mol (10\(^8\) cells\(^{-1}\)) for *M. wesenbergii*, *M. flos-aquae*, *M. aeruginosa*, and *M. viridis*, respectively.

Figure 2. Variations in Fv:Fm in four *Microcystis* species in different treatments with varying \(\text{Cu}^{2+}\) concentrations.

Figure 3. Relative TTC reduction in four *Microcystis* species treated with varying \(\text{Cu}^{2+}\) concentrations for 4 days.
Discussion
Our results reveal species-dependent variation in the sensitivity of *Microcystis* species to copper sulfate. The species-dependent variation in algal sensitivity to copper was also recently reported by Tsai35. The 96-h-EC50 value in *Microcystis* species to Cu2+ was in the order of *M. flos-aquae* > *M. viridis* > *M. aeruginosa* > *M. wesenbergii* and ranged from 0.09 to 0.49 mg L−1. The decrease in Fv:Fm ratio in the highest Cu2+ treatment in the current study was *M. flos-aquae* < *M. viridis* < *M. aeruginosa* < *M. wesenbergii* (Fig. 2), which was consistent with the order of EC50.

It can be seen that Fv:Fm ratio of *M. aeruginosa* decreased with increase of Cu2+ concentration but Fv:Fm ratio of *M. flos-aquae* and *M. Wesenbergii* decreased in first 2 days of application of copper sulfate, and then increased as the days progress. For *M. viridis*, the ratio was at par with the control in all the treatments. These differences would be because of variations in sensitivity to Cu2+ among various species. Although, it was reported that high level Cu2+ reduced the electron transfer rate of the PS II system in *M. aeruginosa*22,36, inhibition of PS II system may not be the only way by which copper sulfate controls other *Microcystis* species. The decrease in the relative TTC reduction reflected cell damage exposed to Cu2+. Our results showed that cells of all the *Microcystis* species were damaged when exposed to Cu2+ except for *M. viridis* (Fig. 2). This result was consistent with the results of growth and Fv:Fm ratio.

SOD may be crucial to the growth inhibition of *Microcystis*37,38. *M. wesenbergii* had the highest SOD content in the control compared with the other *Microcystis* species (Fig. 4). However, the *M. wesenbergii* EC50 value was the lowest. Moreover, *M. aeruginosa*, *M. flos-aquae*, and *M. viridis* EC50 values varied greatly but their SOD contents were similar. The MDA content was considered an indicator of cell injury and increasing MDA indicated damage of cytomembrane39. In the current study, the values did not decrease with increasing SOD content induced by increasing Cu2+. All of the above results suggest that SOD failed to reduce Cu2+ damage in *Microcystis* in the current study. Both enzymatic and non-enzymatic antioxidants of *Microcystis* played important roles in tolerating oxidative damage37,38. Therefore, non-enzymatic antioxidants such as reduced glutathione (GSH) and ascorbic acid (AsA) would be important for *Microcystis* spp. to counteract the oxidative stress induced by Cu2+.

The initial *M. wesenbergii* SOD and MDA content was significantly higher than that of the other species. Temperature may have affected this, given that the optimal temperature for *M. wesenbergii* growth is approximately 30°C25 and the temperature in our experiment was much lower (25°C). Both SOD and MDA content increased with increasing concentrations of Cu2+ in the current study. Similar result was also reported by Chen et al.39 and Shao et al.40. However, it was considered that SOD and MDA had a rough inverse relationship38. It might be because that MDA was a continuously accumulating material but SOD varied against time41. As shown
in Fig. 2, damage from Cu\(^{2+}\) was highest on first day and then the physiological activity was improving later. Therefore, the relationship between SOD and MDA on day 4 was irregular.

Extracellular polysaccharide (EPS) release is another protective response against chemical compounds in algae including microcystins\(^{42}\), salt\(^{43}\), and heavy metals\(^{44–46}\). Li et al.\(^{47}\) suggested that EPS is an important strategy to reduce Cu\(^{2+}\) damage because –COO\(^-\) and some amino groups in EPS can absorb heavy metals effectively\(^{48}\). Xu et al.\(^{49}\) demonstrated that EPS content was significantly lower in \(M. \) wesenbergii than the other three \(Microcystis\) species under standard culture conditions similar to ours. It could be deduced from their results that \(M. \) wesenbergii was the most sensitive species. This conclusion is also supported by our results (Table 2). Therefore, the species-dependent variation in the sensitivity of \(Microcystis\) species to copper sulfate in the current study may have been the result of variations in EPS content in different \(Microcystis\) species. Forni et al.\(^{50}\) reported that the content of polysaccharide of \(M. \) viridis was much higher than other \(Microcystis\) species. This difference would cause variation in growth and physiology of \(Microcystis\) when treated with copper sulfate. It was noticed that the standard deviation of cell density obtained with \(M. \) viridis was much higher than other species and this result supported above deduction. In addition, it was also found that the standard deviation of TTO, SOD and MDA obtained with \(M. \) viridis was very high. Polysaccharide may be the main interfering substance for analysis of above enzyme activity. However, the error range was still within the equivalent range reported by some other researchers\(^{51,59,60}\).

The growth curves of \(M. \) flos-aquae and \(M. \) viridis exposed to 0.05 and 0.10 mg L\(^{-1}\) Cu\(^{2+}\) were higher even than control (Fig. 1). This was due to that Cu\(^{2+}\) are essential micronutrient for \(Microcystis\)\(^{51}\). Additionally, it has also been well documented that low-level contaminants promote \(Microcystis\) growth\(^{25–27}\). However, the sensitivity or tolerance to heavy metals varies amongst different algae and this variation caused that the beneficial concentration of Cu\(^{2+}\) for \(M. \) flos-aquae and \(M. \) viridis inhibits algal growth\(^{51}\). \(M. \) aeruginosa and \(M. \) wesenbergii. It was also noticed that 0.10 mg L\(^{-1}\) Cu\(^{2+}\), which promoted growth of \(M. \) flos-aquae and \(M. \) viridis, was higher than the \(M. \) wesenbergii EC\(_{50}\). This indicated that Cu\(^{2+}\) doses that control \(M. \) wesenbergii promote \(M. \) flos-aquae and \(M. \) viridis growth in lakes. In Fairmont Lakes (USA), 0.033–0.054 mg L\(^{-1}\) Cu\(^{2+}\) was used for over 58 years to control algal growth\(^{14}\). The recommended dose of Cu\(^{2+}\) to control cyanobacterial blooms in Canada was 0.05–0.125 mg L\(^{-1}\)\(^{51}\). These Cu\(^{2+}\) concentrations would stimulate \(M. \) flos-aquae and \(M. \) viridis growth. Therefore, the actual amount of Cu\(^{2+}\) used to control \(M. \) wesenbergii should be much greater than 0.10 mg L\(^{-1}\); while the correct dose for \(M. \) flos-aquae and \(M. \) viridis control requires investigation in lakes. In addition, the species of copper from different copper algaecides should also be considered\(^{51}\).

The risk of microcystin release when \(Microcystis\) membranes are destroyed by Cu\(^{2+}\) treatment is an important concern in the application of copper sulfate to control \(Microcystis\). Trace Cu\(^{2+}\) (0.16–0.50 mg L\(^{-1}\))\(^{10,11,21}\) can result in cell lysis and microcystin release. The most sensitive species, \(M. \) wesenbergii, potentially produces microcystins and other toxins\(^{57,58}\). This species always dominates in summer in lakes with high biomass\(^{51}\). Nevertheless, it has been reported as a non-microcystin production species in China and other countries\(^{29,60}\). Therefore, 0.10–0.16 mg L\(^{-1}\) Cu\(^{2+}\) would reduce \(M. \) wesenbergii growth without cell lysis and microcystin release. Additionally, this Cu\(^{2+}\) dose would not promote growth in other \(Microcystis\) species.

\(M. \) aeruginosa and \(M. \) viridis colonies may produce large amounts of microcystins\(^{58,61}\). The EC\(_{50}\) values of these two species to Cu\(^{2+}\) in this study were 0.16 and 0.45 mg L\(^{-1}\), respectively. These concentrations may induce cell lysis and microcystin release. Reports of toxin production are rare in \(M. \) flos-aquae (sometimes identified as \(M. \) ichthyoblabe)\(^{35,57}\). This species always dominates in lakes before early summer and the biomass is lower compared with \(M. \) wesenbergii in midsummer\(^{25}\). \(M. \) flos-aquae growth would be inhibited by 1 mg L\(^{-1}\) Cu\(^{2+}\), which is safe for drinking water and there is no risk of microcystin release. Therefore, copper sulfate treatment for \(Microcystis\) control could be applied before midsummer when \(M. \) aeruginosa and \(M. \) viridis are not the dominant species. The dose of copper sulfate should be evaluated according to the dominant \(Microcystis\) species.

The EC\(_{50}\) value is also affected by initial cell density\(^{63,64}\) and \(Microcystis\) phenotype\(^{66,67}\). In the current study, all of the strains were unicellular and the initial cell density was the same. The effects of initial cell density and \(Microcystis\) phenotype on EC\(_{50}\) were excluded. However, the EC\(_{50}\) values obtained in the current study should be re-evaluated because \(Microcystis\) always exists as colonies in lakes with varying cell densities\(^{63}\). The effects of temperature on heavy metal tolerance in \(Microcystis\) should also be considered\(^{68}\).

Conclusions

Our results reveal species-dependent variation in the sensitivity of \(Microcystis\) species to copper sulfate. The 96-h-EC\(_{50}\) value of \(Microcystis\) species to Cu\(^{2+}\) was in the order of \(M. \) flos-aquae > \(M. \) viridis > \(M. \) aeruginosa > \(M. \) wesenbergii and ranged from 0.09 to 0.49 mg L\(^{-1}\). MDA content did not decrease with increasing SOD content induced by increasing Cu\(^{2+}\), suggesting that SOD failed to reduce Cu\(^{2+}\) damage to \(Microcystis\) in the current study. However, the species-dependent variation in the sensitivity of \(Microcystis\) species to copper sulfate may have resulted from variations in EPS content in different \(Microcystis\) species. \(M. \) flos-aquae and \(M. \) viridis growth were promoted when exposed to 0.05 and 0.10 mg L\(^{-1}\) Cu\(^{2+}\). Our results suggest that copper sulfate treatment for \(Microcystis\) control could be applied before midsummer when \(M. \) aeruginosa and \(M. \) viridis are not the dominant species and the actual amount of Cu\(^{2+}\) used to control \(M. \) wesenbergii should be much greater than 0.10 mg L\(^{-1}\), while \(M. \) flos-aquae and \(M. \) viridis control in lakes requires further investigation.

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