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Photosynthetic and biochemical responses of the freshwater green algae *Closterium ehrenbergii* Meneghini (Conjugatophyceae) exposed to the metal coppers and its implication for toxicity testing

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The freshwater green algae *Closterium* is sensitive to water quality, and hence has been suggested as ideal organisms for toxicity testing. In the present study, we evaluated the photosynthetic and biochemical responses of *C. ehrenbergii* to the common contaminants, coppers. The 72 h median effective concentrations (EC_{50}) of $CuSO_4$ and $CuCl_2$ on the test organism were calculated to be 0.202 mg/L and 0.245 mg/L, respectively. Exposure to both coppers considerably decreased pigment levels and photosynthetic efficiency, while inducing the generation of reactive oxygen species (ROS) in cells with increased exposure time. Moreover, the coppers significantly increased the levels of lipid peroxidation and superoxide dismutase (SOD) activity, even at relatively lower concentrations. These suggest that copper contaminants may exert deleterious effects on the photosynthesis and cellular oxidative stress of *C. ehrenbergii*, representing its powerful potential in aquatic toxicity assessments.

Keywords: *Closterium ehrenbergii*, copper contaminant, pigment levels, photosynthetic efficiency, lipid peroxidation, antioxidant enzyme

Introduction

Microalgae constitute an important group of photosynthetic organisms in the aquatic environment, and are key players in primary productivity and biogeochemical cycles (Sarthou *et al*., 2005). These organisms are highly diverse and sensitively respond to environmental changes. Some microalgae are grow well and are relatively simple to culture under laboratory conditions. These characteristics make microalgae an ideal model for monitoring both short- and long-term environmental changes, as well as performing ecotoxicology assessments (Suzelei *et al*., 2012).

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Aquatic ecosystems are easily influenced by environmental factors such as thermal shock, UV irradiation, and inflow of toxic contaminants that are hazardous to living organisms (Lee *et al*., 2015). Such environmental stressors can stimulate reactive oxygen species (ROS) formation inside microalgal cells (Marshall and Newman, 2002). Low or moderate levels of ROS serve as an important second messenger in intracellular signalling for altering gene expression and modulating the activities of stress-related proteins (Sharma *et al*., 2012), whereas high concentrations of ROS lead to lipid peroxidation, protein oxidation, and DNA damage (Mishra *et al*., 2011). In fact, microalgae have developed a wide range of defence mechanisms involving enzymatic (antioxidants) and/or non-enzymatic antioxidant systems (e.g. low-molecular-weight compounds, such as glutathione, tocopherols, phenolics, carotenoids, and flavonoids). These defence mechanisms can efficiently alleviate cellular oxidative damage by scavenging or detoxifying excess ROS (Sharma *et al*., 2012). Particularly, superoxide dismutase (SOD) acts as the first line of defence against ROS within cells (Ken *et al*., 2005). Other antioxidant enzymes, such as catalase (CAT), peroxidase (POD), and/or glutathione peroxidase (GPX), are reported to counteract oxidative stress in both higher plants and microalgae (Wang *et al*., 2012).

Copper (Cu) is an essential micronutrient for plants and microalgae in numerous physiological processes at low concentrations, but it can also be toxic at higher concentrations (Gaetke and Chow, 2003). High concentrations of copper exert toxic effects on the metabolic machinery of microalgae through the enhanced formation of ROS (Knauert and Knauer, 2008). Cu-induced ROS can oxidize a large variety of biological macromolecules (Mittler, 2002), and cause subsequent damage to DNA, lipids, proteins, and other biomolecules, eventually leading to cell death (Mittler, 2002). Previous studies reported that microalgal production of glutathione, thiols, or SOD can be stimulated by copper, and enzymatic activity may be a defensive response against excess copper exposure (Ebenezer *et al*., 2014). Considering that excess copper induces severe cellular stress to generate high amounts of ROS, evaluating antioxidant enzyme activity might provide insights into the mode of action of this toxic metal (Ebenezer *et al*., 2014). In context of environmental pollution, these antioxidant enzymes have been suggested for use as biochemical markers to assess environmental toxicity.

In the present study, we evaluated the toxicological effects of copper contaminants on the physiological and biochemical activity of the green algae *C. ehrenbergii*, with an emphasis on variations in levels of pigments, ROS, and anti-

oxidant enzymes. Then, based on the median effective concentrations (EC_{50}) of copper, we accessed the potential use of *C. ehrenbergii* as an aquatic toxicity testing model. Our test species *C. ehrenbergii* is a unicellular green algae that is distributed in freshwater ecosystems (Ichimura and Kasai, 1984). The morphological characteristics of this species include crescent-shaped cells (long, curved, and tapered at both ends) and much larger cell body size (250–300 μm in length) than most other microalgae (Lee *et al*., 2015); thus, it is relatively easy to observe morphological and cellular changes in *C. ehrenbergii* cells microscopically. Indeed, this species has been used as a testing species for algal sexual reproduction and cell cycles for several years (Fukumoto *et al*., 1997). Recently, it has been recommended as a potential model for ecotoxicology testing to detect the presence of hazardous substances in aquatic systems (Kim *et al*., 1998; Sathasivam *et al*., 2016; Wang *et al*., 2017).

Materials and Methods

Algae culture and test chemicals

A strain of *C. ehrenbergii* (Ce-01) was obtained from Environmental Bio Inc. (Seoul, Korea). It was cultured in C medium (Watanabe *et al*., 2000) at 20 ± 1°C, with 12:12 h light-dark cycle and a photon flux density of ~ 65 μmol photons/m^2/sec.

Copper sulfate ($CuSO_4$, Cat. No. C1297, Sigma) and copper chloride ($CuCl_2$, Cat. No. 222011, Sigma) were obtained commercially and prepared for standard stock solutions. To assess the toxicity effects of copper ($CuSO_4$ and $CuCl_2$) on *C. ehrenbergii*, the concentrations tested were the following: $CuSO_4$ (0.1, 0.5, 1.0, 2.5, and 5.0 mg/L) and $CuCl_2$ (0.1, 0.5, 1.0, 2.5, and 5.0 mg/L). All dilutions were prepared from standard stock solutions and all exposures were repeated in triplicate independent experiments.

Doses of copper adjusted according to copper demand, as described above, were added to 200 ml of *C. ehrenbergii* culture at the exponential phase in triplicates. The initial cell concentration was $3.0 \pm 0.1 \times 10^4$ cells/ml. The samples were drawn for growth-based assays and biochemical assays at 0, 6, 12, 24, and 72 h.

Cell observations and median effective concentration (EC_{50})

Cell counts in each test flask were determined using a plankton-counting chamber (HMA-S6117, Matsunami Glass) and were plotted against time of exposure to copper.

Biocidal efficiency was calculated based on percent inhibition, following the recommendation by OECD (2011). The percent inhibition was calculated based on the following equation: % I = (μC-μT/μC) × 100, where % I = percent inhibition, μC = mean value of growth in the control, and μT = mean value of growth rate in the treated samples.

Levels of chlorophyll *a* (Chl*a*) and carotenoids (CAR) were determined using a DU730 Life Science UV/Vis spectrophotometer (Beckman Coulter). Briefly, 10 ml of the treated culture was filtered using Whatman GF/C filter paper and a vacuum pump filtration unit. The pigments were extracted from the filter paper in dark using 90% acetone and their concentrations were estimated spectrophotometrically, following Parsons *et al*. (1984).

The percentile inhibition and 72 h median effective concentration (EC_{50}-72 h) were calculated as recommended in OECD testing guidelines (OECD, 2011). The values of EC_{50}-72 h were estimated using a sigmoidal dose-response curve and plotted using Origin v8.5 (MicroCal Software Inc.) based on the four parameter equation (Teisseyre and Mozrzymas, 2006).

Measurements of photosynthetic efficiency

Chlorophyll fluorescence is an effective way to measure the efficiency of photosynthetic apparatus *in situ* and photosynthetic responses to various stresses (Schreiber *et al*., 1995). The photosynthetic efficiency (F_v/F_m) was measured by using a handy Plant Efficiency Analyser fluorometer (Handy PEA, Hansatech Instruments Ltd). To determine the photosynthetic efficiency of *C. ehrenbergii*, each 2 ml of per sample were collected into specimen cup in triplicate. The fluorescence parameters F_o (Minimal fluorescence in the dark-adapted state), F_m (Maximal fluorescence in the dark-dapted state), and F_v (Variable fluorescence, $F_v=F_m-F_o$) were measured at 0 h, 12 h, 24 h, and 48 h after 1.0 mg/L $CuSO_4$ exposure. Values of *Fv* and maximal quantum efficiency of PSII photochemistry (F_v/F_m) were calculated from F_o and F_m.

ROS measurement

Dihydroxyrhodamine 123 (DHR123-D1054; Sigma) staining was used to measure the production of ROS. DHR123 can be oxidized by ROS and emits green fluorescence (Qin *et al*., 2008). Cells were treated with copper after 12, 24, and 48 h incubation. The cells were stained with DHR123 for 1 h at a final concentration of 20 μM, then harvested by centrifugation and washed twice with fresh C medium. The cultures were resuspended in fresh C medium, and mounted onto a slide and sealed. The cultures were observed using a fluorescence microscope (Carl Zeiss Axioskop) to determine the ROS production in cells. The relative ROS levels were quantified with ImageJ software (NIH) from the fluorescence microscopic images.

Cell death imaging

We determined the cytotoxic effect of $CuSO_4$ *in vitro*, measuring cell viability by propidium iodine (PI) staining using the Cellometer imaging cytometry. In brief, we harvested the cells treated with copper, washed, and re-suspended in 1 ml of C medium. Five microliters of propidium iodide (PI) was added to 100 μl of cell suspension taken out from each sample. Samples were gently mixed and incubated for 20 min at room temperature in dark. Samples were mixed again and 20 μl of each sample was loaded into the cellometer counting chamber. Samples in the cellometer counting chamber were analyzed by the Cellometer Vision (Nexcelom, Biosciences).

Lipid peroxidation and SOD assays

Lipid peroxidation was measured according to the method of Heath and Packer (1968). The cells were harvested by centrifugation at 4,217 × g for 10 min, and then 2 ml of 10%

Table 1. EC_{10}, EC_{20}, and EC_{50} values after 72 h $CuSO_4$ and $CuCl_2$ exposure to *C. ehrenbergii*

Chemicals	EC_{10} (mg/L)	EC_{20} (mg/L)	EC_{50} (mg/L)
$CuSO_4$	0.091 ± ND[a]	0.118 ± ND[a]	0.202 ± 0.022
$CuCl_2$	0.079 ± ND[a]	0.112 ± ND[a]	0.245 ± 0.033

[a] ND, not determined.

trichloroacetic acid (TCA) was added to the pellet. The cells were homogenized using a Teflon pestle tissue homogenizer in ice. The tube was then placed in a water bath at 40°C for 5 min, a modification of the method described by Soto *et al.* (2011). The mixture was centrifuged at 4,217 × g for 10 min. An equal volume of 0.25% thiobarbituric acid freshly prepared in 10% TCA solution was added to the supernatant. The tube was heated at 95°C for 30 min in a water bath. The mixture was then cooled to room temperature and centrifuged for 10 min at 4,217 × g. The absorbance of the solution was measured at 532 and 600 nm. Results were expressed as micromoles of malondialdehyde (MDA) per 10^4 cells.

SOD was assayed according to the method of Beauchamp and Fridovich (1971). Algal cells were harvested by centrifugation at 4,217 × g for 10 min, and then 5 ml of 100 mM dihydrogen phosphate buffer was added to the algal cell pellet. The cells were homogenized using a Teflon pestle tissue homogenizer in ice. Next, the tube was placed in a water bath at 40°C for 5 min using a method modified from Soto *et al.* (2011). The mixture was centrifuged at 4,217 × g for 10 min. To the supernatant, 2.6 ml of the reaction mixture (0.05 M phosphate buffer, 130 mM methionine, 750 µM nitroblue tetrazolium, 100 µM Na_2EDTA, and 20 µM riboflavin) were added. The tubes were incubated in light (65 µmol photons/m^2/sec) for 30 min. The absorbance was read at 560 nm. One unit of SOD (U) was defined as the amount of enzyme resulting in 50% inhibition of photochemical reduction of nitroblue tetrazolium (NBT). SOD levels were represented as U per 10^4 cells (U/10^4 cells).

Data analysis

All data are presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) with post hoc Dunnet's test using Graphpad InStat (Graphpad Software, Inc.) was used for comparisons between untreated and treated cultures. $P < 0.05$ was accepted as significant.

Results

Toxic effects of copper solutions on cell growth and pigment levels

C. ehrenbergii cells were exposed to different concentrations (0.1, 0.5, 1.0, 2.5, and 5.0 mg/L) of copper compounds, including $CuSO_4$ and $CuCl_2$. Cell counts, growth rate, and cell division rate decreased in a dose- and time-dependent manner after 6 h and 72 h of exposure to copper compounds (data not shown). The EC_{50} values were calculated using sigmoidal dose-response curves that were estimated from cell counts after 72 h exposure (Table 1). The EC_{50} value at 72 h for $CuSO_4$ (0.202 mg/L) was slightly lower than the value for $CuCl_2$ (0.245 mg/L). As for the threshold effect parameter, we calculated EC_{10} and EC_{20} values, which represented the

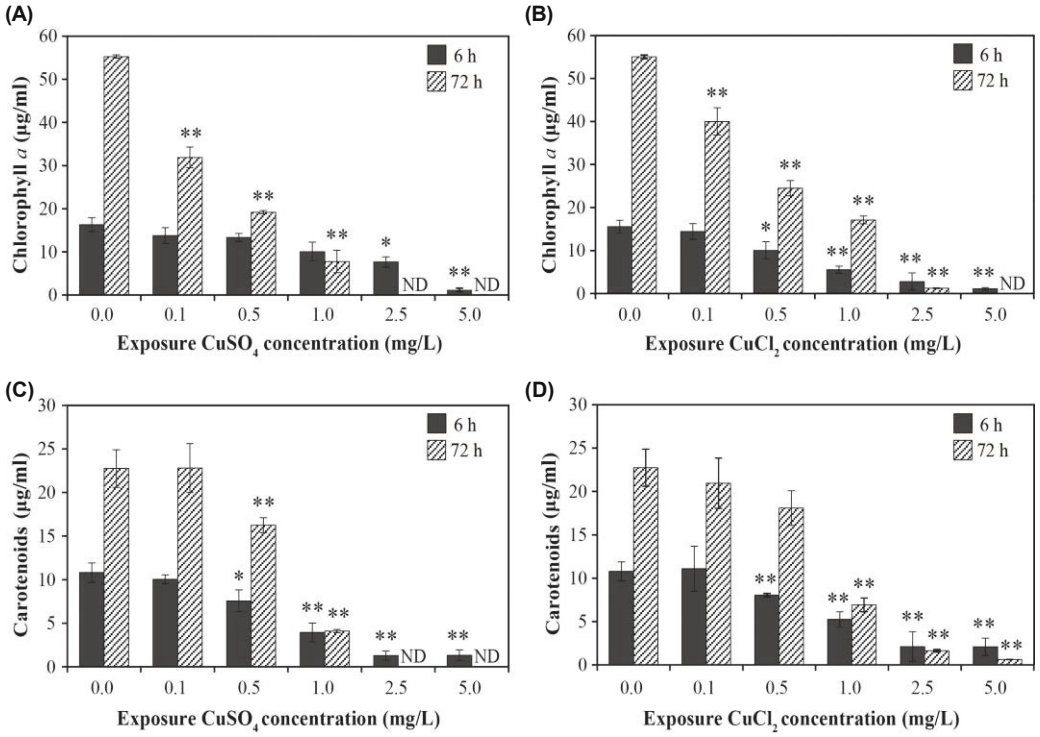

Fig. 1. Chlorophyll *a* and carotenoids levels after 6 and 72 h exposure of *C. ehrenbergii* to copper sulphate and copper chloride. Significant differences between the control and treated sample, as determined by one-way ANOVA, are highlighted *$P < 0.05$; **$P < 0.01$. ND, not detected due to lack of viable cells.

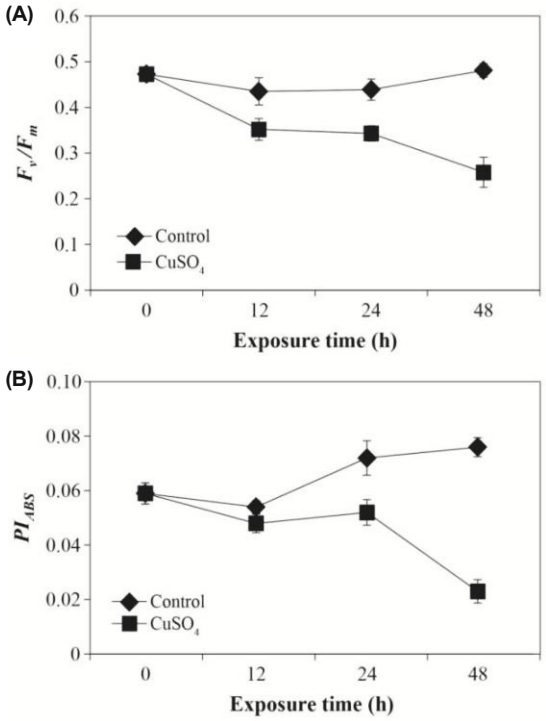

Fig. 2. Effect of copper on photosynthetic efficiency of *C. ehrenbergii* cells. (A) Variation in *Fv/Fm* after treated to 1.0 mg/L $CuSO_4$ (B) Variation in PI_{ABS} after treated to 1.0 mg/L $CuSO_4$.

initial concentration of the test chemical that triggers an effect on the test microalgae.

In addition, chlorophyll *a* (Chl*a*) and carotenoid (CAR) levels were significantly reduced ($P < 0.05$) after 6 h of copper exposure (Fig. 1). For Chl*a* contents, we found reductions of 18.1% and 35.4% at 0.5 mg/L, 38.3% and 64.3% at 1.0 mg/L, 52.9% and 82.1% at 2.5 mg/L, 92.5% and 92.3% at 5.0 mg/L for $CuSO_4$ and $CuCl_2$, respectively, when compared with the untreated control. CAR levels showed a similar descending trend as that observed for Chl*a* after 6 h of copper treatment. After 72 h of exposure to copper, Chl*a* and CAR levels reduced considerably by 18.1–97.2% for the test doses. Moreover, cells died upon exposure to high copper concentrations after 72 h.

Photosynthetic efficiency, cell death, and ROS production in *C. ehrenbergii*

To access the effects of Cu^{2+} on the photosynthetic systems of *C. ehrenbergii*, we measured chlorophyll fluorescence. Considering the EC_{50} and pigment results, we measured the photosynthetic efficiency of *C. ehrenbergii* upon exposure to 1.0 mg/L $CuSO_4$. In the present study, the photosynthetic efficiency of *C. ehrenbergii* was affected by copper toxicity, showing a significant decline as time increased. This indicates that the Chl*a* content, or the photosynthetic electron transport, was inhibited in some algal cells. Specifically, *Fv/Fm* values were slightly decreased to a similar degree after 12

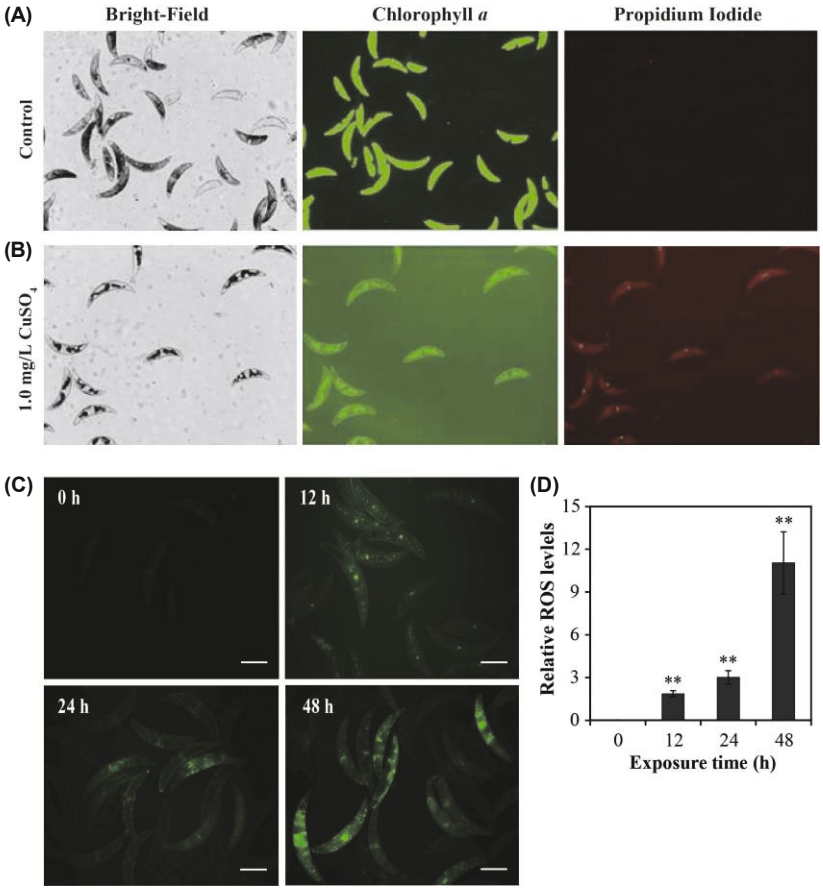

Fig. 3. Chlorophyll *a*, cell death observation (5X), ROS production and relative ROS level of *C. ehrenbergii* exposed to 1.0 mg/L $CuSO_4$. (A) Chlorophyll *a* (B) Cell death observation (C) ROS production (D) Relative ROS level. They were measured by the cellometer imaging cytometry (Nexcelom, Biosciences). Significant differences between the control and treated sample, as determined by one-way ANOVA, are highlighted $^*P < 0.05$; $^{**}P < 0.01$. Scale bars represent: 100 µm.

and 24 h exposure, whereas the values showed significant decreases ($P < 0.01$) after 48 h of exposure to $CuSO_4$ (Fig. 2). The *Fv/Fm* values showed a decrease of 80.9%, 78.1%, and 53.6% at 12, 24, and 48 h after 1.0 mg/L $CuSO_4$ exposure, respectively, when compared to the controls. Moreover, PI_{ABS} showed a similar trend as time increased.

Excess copper exposure may be involved in cell death. In the present study, cell death of *C. ehrenbergii* was examined by using propidium iodide staining and the Cellometer imaging cytometry (Fig. 3A and B). Compared with untreated samples, both cell counts and Chl*a* decreased when *C. ehrenbergii* cells were exposed to 1.0 mg/L of $CuSO_4$.

To evaluate oxidative stress, ROS production was measured using DHR123 and relative ROS levels were quantified (Fig. 3C and D). A time-dependent increase in relative ROS levels was observed. Thus, copper treatment provoked ROS generation in *C. ehrenbergii*.

Effects of copper on MDA level and SOD activity

Lipid peroxidation (MDA) was used to measure oxidative damage in the test algae. Cu-induced lipid peroxidation increased dose-dependently. The changes in the MDA levels with respect to concentrations of copper used are depicted in Fig. 4. There was a significant ($P < 0.01$) increase in lipid peroxidation at relatively lower doses of $CuSO_4$ (0.5 and 1.0 mg/L) after 6 h of exposure, and a similar trend was also observed at 72 h (Fig. 4A). After 6 h of $CuSO_4$ exposure, MDA levels increased from 0.574 to 1.082 mM/10^4 cells. However, the lipid peroxidation was not significantly increased ($P < 0.05$) in comparison to the control cells at higher concentrations of copper (2.5 and 5.0 mg/L). In addition, after 72 h of exposure at the high $CuSO_4$ concentration, MDA levels were not detectable, possibly owing to cell death. Interestingly, the levels of MDA in *C. ehrenbergii* exposed to $CuCl_2$ showed a trend considerably similar to that observed upon $CuSO_4$ treatment (Fig. 4B). At 1.0 mg/L of $CuCl_2$ exposure, MDA levels increased to 1.002 and 0.904 mM/10^4 cells after 6 and 72 h, respectively.

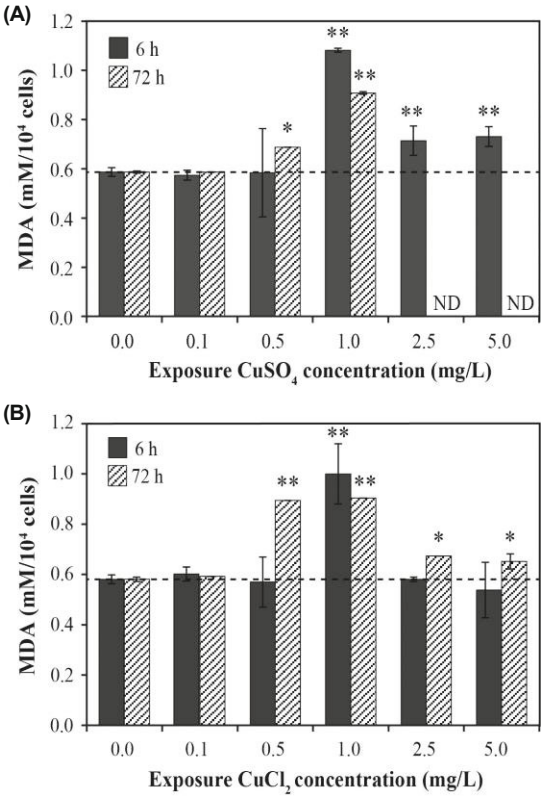

Fig. 4. Effect of copper sulphate and copper chloride on the lipid peroxidation levels of *C. ehrenbergii* cells after 6 and 72 h exposure. Significant differences between the control and treated sample, as determined by one-way ANOVA, are highlighted *$P < 0.05$; **$P < 0.01$. Dot lines represent mean values of controls at 6 h and 72 h. ND, not detected due to lack of viable cells.

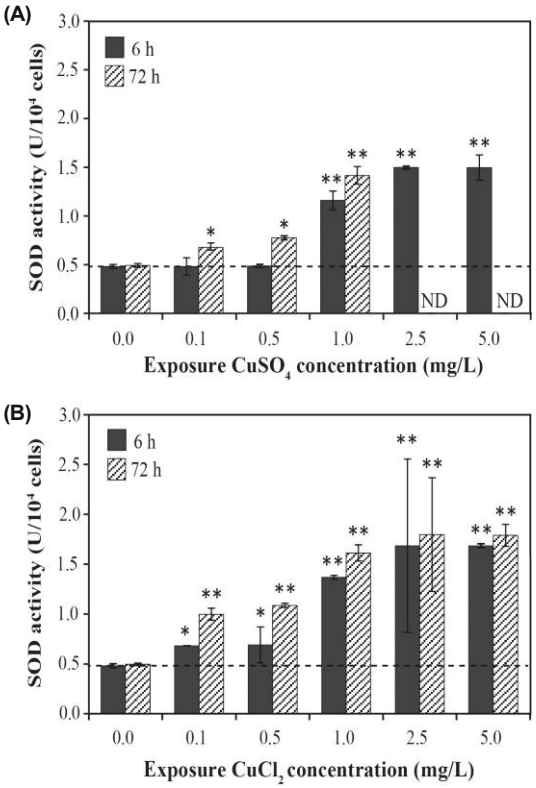

Fig. 5. Effect of copper sulphate and copper chloride on the SOD activity of *C. ehrenbergii* cells after 6 and 72 h exposure. Significant differences between the control and treated sample, as determined by one-way ANOVA, are highlighted *$P < 0.05$; **$P < 0.01$. Dot lines represent mean values of controls at 6 h and 72 h. ND, not detected due to lack of viable cells.

In addition, SOD activity in *C. ehrenbergii* was significantly correlated with copper concentration (Fig. 5). After 6 h of exposure (Fig. 5A), at lower $CuSO_4$ concentrations (0.1 and 0.5 mg/L) SOD activity gradually increased, whereas at higher concentrations SOD activity was drastically increased approximately 4-fold ($P < 0.01$) compared to the unexposed controls. Moreover, after 72 h, SOD activity in cells treated with $CuSO_4$ showed a similar increase and was particularly high when the copper concentration in the growth medium was 1.0–5.0 mg/L. For $CuCl_2$ treatment, SOD activity was increased after 6 and 72 h exposure, even at low copper concentrations (Fig. 5B).

Discussion

Copper compounds are one of the most persistent contaminants in aquatic environments. Large quantities of copper contaminants have been discharged into freshwater and marine ecosystems owing to their widespread industrial use, and their levels have risen substantially worldwide over the past several decades (Penuelas and Filella, 2002). The toxicity of copper contaminants in organisms is mainly due to the free ions (Knauer *et al*., 1997). Thus, copper may affect photosynthesis in microalgae, causing serious damage to the macromolecules; however, copper sensitivity varies among microalgae species (Soldo and Behra, 2000). In the present study, we evaluated the toxic effects of excess copper on cells of the freshwater algae *C. ehrenbergii* by measuring various cellular parameters such as growth inhibition, photosynthetic pigment levels, oxidative stress, lipid peroxidation, and SOD activity.

EC_{50} values are useful for determining a range of environmental pollutions (such as heavy metals) that cause growth inhibition in an algal population. In the present study, the EC_{50} values for $CuSO_4$ (0.202 mg/L) and $CuCl_2$ (0.245 mg/L) were very close to each other (Table 1). In general, the bioavailability (or susceptibility) of metal salts is increased when they occur as inorganic salts (Gibbon-Walsh *et al*., 2012). In marine water, the toxicity of copper compounds ($CuSO_4$ and $CuCl_2$) may be the result of the interaction of the metal cation (Cu^{2+}) and secondary subsidiary chemical species (e.g. SO_4^{2-} or Cl^-), which differentially affect toxicity in the marine diatom *Ditylum brightwellii* (Lee *et al*., 2014). However, the present result showed that copper may play a major role in the cellular toxicity of green algae, suggesting that the Cu^{2+} toxicity may not be affected by its conjugated anions in freshwater.

The high toxicity of excess copper has been well-documented by previous studies (Danilov and Ekelund, 2001). Overall, most results showed that freshwater green algae cells exhibited relative sensitivity when exposed to copper. Upon comparing the EC_{50} values reported so far (Table 2), the smallest was 0.200 mg/L in the green algae *Chlorella vulgaris* (Blaylock *et al*., 1985) and *Closterium lunula* (Yan and Pan, 2002), while the largest was 0.774 mg/L in the green algae *Staurastrum chaetoceras* (Ivorra *et al*., 1995). On the contrary, the freshwater diatoms (e.g. *Aulacoseira granulata*, *Ditylum brightwellii*, *Navicula incerta*, and *Planothidium lanceolatum*) showed relatively higher copper tolerance than green algae (Table 2), and thus, the EC_{50} values were much higher than those observed in green algae, ranging from 0.406 to 10.429 mg/L (Rachlin *et al*., 1983; Viana and Rocha, 2005; Sbihi *et al*., 2012). As for marine microalgae, copper EC_{50} values in the dinoflagellate *Cochlodinium polykrikoides* and green algae *Tetraselmis suecica* were 12.7 mg/L and 43.0 mg/L, respectively (Ebenezer and Ki, 2012, 2013). Upon comparing these results and other toxicity data to results in algae (Table 2), we found that freshwater microalgae were much more sensitive to copper than marine microalgae. Furthermore, based on these kinds of EC_{50} values, our tested *C. ehrenbergii* might be more sensitive to copper compounds than some of other algae (Wang *et al*., 2017).

Chlorophyll pigments have often been used as indicators to monitor environmental stressors in plants including algae. With increasing copper concentration, we found a significant reduction in Chl*a* and CAR content in *C. ehrenbergii* (Fig. 1). Similar to the present study, several reports (Chen *et al*., 2012; Devi and Mehta, 2014; Kebeish *et al*., 2014) showed the inhibition of algae pigment content by copper contaminants. Chen *et al*. (2012) and Kebeish *et al*. (2014) reported that the Chl*a* contents of the green algae *Scenedesmus obliquus* and *Chlorella vulgaris* declined after exposure to copper, and the trend was linked with increased copper concentrations (2–10 and 1.5–4.5 μM, respectively). When compared to the exposure concentrations in other algae, our tested species *C. ehrenbergii* exhibited much higher sensitive to copper compound (Table 2). In addition, in the present study, a remarkable reduction in CAR content was observed in a copper dose- and time-dependent manner, which agreed with the result reported by Ebenezer *et al*. (2014). However, the decrease in CAR content in *C. ehrenbergii* seems surprising, as earlier studies reported that CAR has a protective role in restoring photosynthesis and membrane stability (Pinto *et*

Table 2. **EC_{50} values of copper for different microalgae, obtained by different works**

Species	Type of organism	Chemical	Result (mg/L)	Reference
Chlorella ellipsoidea	Green algae	$CuSO_4{\cdot}5H_2O$	0.489	Magdaleno *et al*. (2013)
Chlorella saccharophila	Green algae	$CuCl_2$	0.550	Rachlin *et al*. (1983)
Chlorella vulgaris	Green algae	$CuCl_2$	0.200	Blaylock *et al*. (1985)
Closterium lunula	Green algae	$CuCl_2$	0.200	Yan and Pan (2002)
Pseudokirchneriella subcapitata	Green algae	$CuCl_2$	0.435	Cypaite *et al*. (2014)
Selenastrum capricornutum	Green algae	$CuCl_2$	0.400	Blaylock *et al*. (1985)
Staurastrum chaetoceras	Green algae	$CuCl_2$	0.774	Ivorra *et al*. (1995)
Staurastrum manfeldtii	Green algae	$CuCl_2$	0.429	Ivorra *et al*. (1995)
Tetraselmis suecica[a]	Green algae	$CuSO_4{\cdot}5H_2O$	43.0	Ebenezer and Ki (2013)
Aulacoseira granulata	Diatom	$CuSO_4$	2.580	Viana and Rocha (2005)
Navicula incerta	Diatom	$CuCl_2$	10.429	Rachlin *et al*. (1983)
Planothidium lanceolatum	Diatom	$CuSO_4{\cdot}5H_2O$	0.620	Sbihi *et al*. (2012)
Ditylum brightwellii[a]	Diatom	$CuCl_2$	1.455	Lee *et al*. (2014)
Ditylum brightwellii[a]	Diatom	$CuSO_4$	0.406	Lee *et al*. (2014)
Cochlodinium polykrikoides[a]	Dinoflagellate	$CuSO_4{\cdot}5H_2O$	12.7	Ebenezer and Ki (2012)

[a] Marine organism

al., 2011; Kebeish *et al*., 2014). Many organisms tend to increase their CAR content under various stresses (Kobayashi *et al*., 1997) and it is commonly regarded as one of the antioxidants that counteracts stress in organisms (Devi and Mehta, 2014). Thus, the decrease in CAR contents we observed upon copper treatment might be due to the Cu-induced damaging effects or interaction with biosynthetic processes of these pigments.

In addition, our finding of inhibited photosynthetic efficiency by copper is in accordance with those observed by Mamboya *et al.* (1999) and Guo *et al.* (2016). Knauert and Knauer (2008) reported that photosynthetic efficiency decreased with exposure time when *Chlorella vulgaris* was treated with 1.0 mg/L copper. Thus, the results of this study showed a relative reduction in algal growth rate and cell density at 1.0 mg/L $CuSO_4$ (Fig. 3). Such a growth retardation effect is similar to effects observed in *Isochrysis galbana* (Kagalou *et al*., 2002), *Scenedesmus subspicatus* 86.81 SAG (Ma *et al*., 2003), and *Chlorella vulgaris* (Kebeish *et al*., 2014). A possible reason for this reduced growth might be the binding of Cu^{2+} to sulfhydryl groups, which are important for regulating cell division and leads to inhibition of activity or cell structure disruption (Assche and Clijsters, 1990).

It is well known that Cu^{2+} can affect membrane properties by oxidizing membrane lipids, which can be determined from measuring increased MDA levels (one of the lipid peroxidation products), thereby destroying cell membranes (Azooz *et al*., 2012). Recently, lipid peroxidation has been used to measure oxidative damage in test algae (Sathasivam *et al*., 2016). Present results showed that MDA level significantly increased after 6 and 72 h of treatment with 1.0 mg/L $CuSO_4$ and $CuCl_2$, and then decreased (Fig. 4), which agrees with earlier studies by Karimi *et al.* (2012) and Devi and Mehta (2014). Taken together, these results indicated that alkoxyl and peroxyl radicals are formed by the action of Cu-generated free radicals, and this collective ROS production may lead to cell membrane damage, as determined by fluorescence microscopy (Fig. 3). Copper-induced lipid peroxidation is completely blocked by treatment with free radical scavengers (e.g. taurine) when lipid peroxidation reaches saturation (Spickett *et al*., 2000). The decrease in lipid peroxidation activity at high $CuSO_4$ concentrations observed in the present study could be due to increased free radical production.

When acclimating to increased oxidative stress, SOD concentrations in plants and microalgae typically increase with the degree of stress (Peng *et al*., 2013). In the present study, we observed that SOD activity markedly increased with increasing copper concentration and exposure time (Fig. 5), which is consistent with increased ROS levels in the algae cells following copper exposure (Fig. 3). A previous study by Li *et al.* (2006) reported increased SOD activity when the marine algae *Pavlova viridis* was exposed to copper (0.2–3.0 mg/L). Similarly, when the green microalgae *Chlorella vulgaris* was exposed to copper (0.5–4.5 μM) there was a significant increase in antioxidative enzyme activity (CAT, POD, PPO, and SOD) (Kebeish *et al*., 2014). In the present study, SOD activity was significantly increased at the lowest concentration of copper (0.1 mg/L), but when other algae were exposed to this concentration, there was no significant increase in SOD activity. From these results, it is clearly shown that *C. ehrenbergii* was much more sensitive to copper than other algae, and this species can be used a biochemical marker for monitoring environmental pollution in freshwater.

Fig. 6 shows a schematic view of the oxidative stress caused by copper and the counteraction of antioxidative enzymes, as observed in the present study. It is well known that when copper is in excess, it catalyses the formation of ROS, particularly, highly toxic hydroxyl radicals (HO) from the Haber-Weiss reaction, leading to increased MDA levels (Karimi *et al*., 2012). Enhanced ROS levels cause oxidative damage to DNA, proteins, and lipids, consequently altering intrinsic

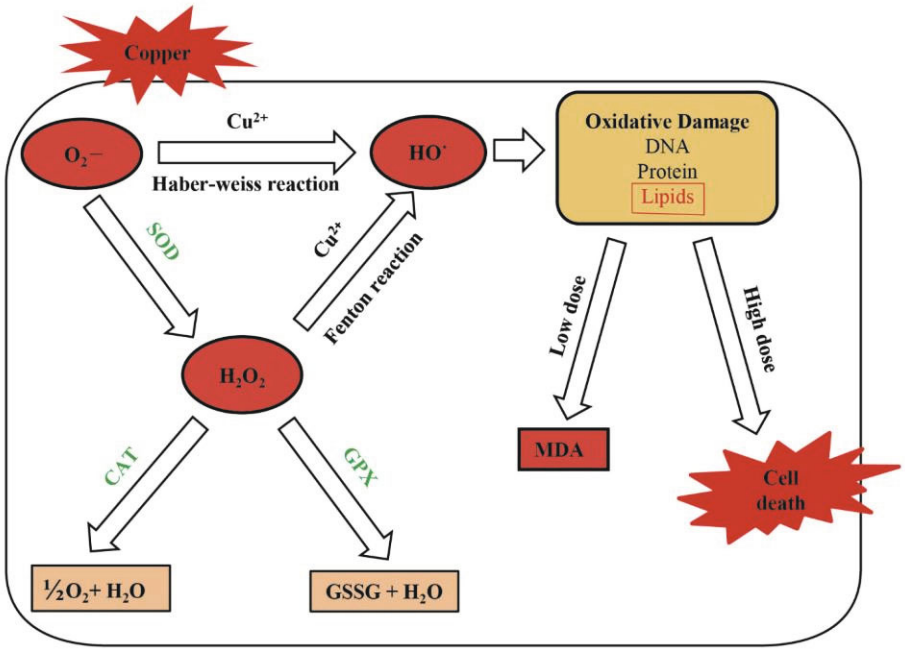

Fig. 6. A schematic view of variation in lipid peroxidation and SOD activity in the green algae *C. ehrenbergii* treated with copper. It is the general pathway reported elsewhere.

membrane properties like fluidity, ion transport, loss of enzyme activity, inhibition of protein synthesis, and eventual cell death (Sharma *et al*., 2012). Therefore, in response to the presence of excessive copper levels, microalgae should enhance the production of antioxidants due to increased ROS formation. Furthermore, excess copper in plants and microalgae elicits the expression of defence genes encoding antioxidant enzymes including SOD, CAT, and GPX, which aids in the removal of excess ROS.

In summary, exposure to the metal copper considerably reduced the growth rate and pigment (Chl*a* and CAR) levels of the green algae *C. ehrenbergii*. Upon comparing EC_{50} values, we found that *C. ehrenbergii* might exhibit much higher sensitivity to copper than other algae. Toxic effects of copper were reflected by inhibited photosynthetic efficiency and ROS production in *C. ehrenbergii*. The stimulatory effect of copper on the MDA biosynthesis and antioxidant SOD activity may play an important role in protecting cells at relatively lower levels. These results suggest that copper contaminants may exert deleterious effects on the photosynthetic machinery and cellular oxidative status, causing subsequent cell death, even at low concentrations. In addition, this species is very susceptible to other metal and inorganic contaminants (Sathasivam *et al*., 2016), suggesting that it can be a reliable model in aquatic toxicity assessments.

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Conflict of Interest

The authors declare that they have no competing interest.

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