Toxicity Effects of Copper and Zinc on the Photosynthetic Efficiency and Oxidative Stress-Related Parameters of the Green Alga *Chlorella vulgaris* Beijerinck

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ABSTRACT

Microalgae are widely used as models for ecotoxicological assays. The present study investigated the physiological responses of *Chlorella vulgaris* to five days of exposure to copper and zinc at different concentrations (control, 125, 250, 500 and 1,000 µM). Both heavy metals showed dose-dependent cellular accumulation. Decreased maximum quantum efficiency of photosystem II (Fv/Fm) was influenced by both heavy metal concentration and time of exposure. A reduction in Fv/Fm indicated that photodamage occurred starting from day 3 of exposure. Other toxicity symptoms included chlorophyll degradation and an increase in reactive oxygen species (ROS). While exposure to both heavy metals resulted in a decrease in chlorophyll *a* content to a similar extent, an increase in ROS was detected only for 1,000 µM copper, suggesting stronger toxicity effects of copper compared to zinc. Nevertheless, an increase in lipid peroxidation was not detected, indicating that ROS produced by 1,000 µM copper was not sufficient to induce disintegration of membrane lipids via the oxidation process. Proline, an amino acid with various putative protective functions against stress, exhibited a rapid increase with increases in heavy metal concentration and time of exposure. These results provide a set of effective biomarkers for heavy metal contamination using *C. vulgaris* as a bioindicator.

Keywords: *Chlorella vulgaris*, Heavy metal, Physiology, Toxicology

INTRODUCTION

Aquatic environments today are facing contamination by various heavy metals from anthropogenic activities such as agricultural practices and small to large industries (Veenstra *et al*., 1999; Terry and Stone, 2002; Fathi *et al*., 2008). These heavy metals can be taken up by the flora and fauna and exert effects on their cellular processes, growth and survival (Chekroun and Baghour, 2013).

Copper (Cu) and Zinc (Zn) are among the heavy metals commonly found in water bodies, and their contamination is often associated with wastewater discharges (Terry and Stone, 2002; Ullah *et al*., 2015). Cu and Zn are essential nutrient elements for all photosynthetic organisms and play important roles in a number of physiological processes such as enzymatic reactions, photosynthesis, cellular respiration and other oxidation-reduction reactions (Hafeez *et al*., 2013). However, when present at high concentration, these heavy metals can inhibit the functionality of key enzymes and metabolisms, leading to disturbance of cellular homeostasis (Singh *et al*., 2010; Oves *et al*., 2016). Previous studies have shown that copper reacts with various components of light reactions such as

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quinone B (Q_B) and oxygen evolving complex (OEC), impeding photosynthetic electron transport (Oukarroum *et al*., 2012; Ravet and Pilon, 2013). In addition, excessive Cu and Zn can promote oxidative stress by increasing reactive oxygen species (ROS), e.g., superoxide anion, hydrogen peroxide and hydroxyl radicals. These ROS are extremely harmful to many components of the cell such as proteins, lipids and nucleic acids and such damage may lead to modification of the structure of the cell or mutagenesis (Halliwell and Gutteridge, 1999), an inhibition of photosynthesis (Schutzendubel and Polle, 2003; Kaciene *et al*., 2015) and a disturbance of the activity of mitochondria (Pinto *et al*., 2003). These phytotoxic effects can eventually reduce growth and development, and may result in death (Lu *et al*., 2015).

Chlorella vulgaris Beijerinck is one of the green microalgae widely used as a model to investigate cellular mechanisms (Kowalewska, 1999; Wang *et al*., 2010). When cultivated in wastewater contaminated with heavy metals, they can take up the metals rapidly and exhibit fast physiological responses (Rodrigues *et al*., 2012). It has been reported that heavy metals including Cu and Zn caused a decrease in pigment content (Kebeish *et al*., 2014; Kumar *et al*., 2016; Zeraarkar *et al*., 2016) and photosynthetic rates in *C. vulgaris* after 96 hours of exposure (Stiborova, 1988; Saavedra *et al*., 2018). Induced oxidative stress under excessive exposure to metals has also been observed in *C. vulgaris* (Saavedra *et al*., 2018). Nevertheless, it has been reported that under sub-lethal concentration of heavy metals, *C. vulgaris* induced defense mechanisms such as antioxidant enzymes (Filippis and Pallaghy, 1976; Alam *et al*., 2014) as well as sequestration mechanisms which safely translocate metals into the vacuole (Saradhi, 1991; Ashraf and Foolad, 2007). Another mechanism which has been proposed to play a role in tolerance to heavy metals in *C. vulgaris* is an accumulation of proline (Saradhi, 1991; Ashraf and Foolad, 2007). Proline is a proteinogenic amino acid synthesized from L-glutamate (Hayat *et al*., 2012). It has been suggested that proline maintains homoeostasis of the cells through osmoregulation, apoptosis control (Mehta and Gaur, 1999) and production of glutathione (GSH), which is an important component for heavy metal detoxification (Liang *et al*., 2013). Wu *et al*. (1998a) reported that proline supply can reduce the Cu uptake in the green alga *Chlorella* sp. (strain 2350), thus protecting the alga from metal toxicity.

This study investigates the physiological responses of *Chlorella vulgaris* to sub-lethal exposure to Cu and Zn. We aim to elucidate the toxicity mechanisms of the two heavy metals in *C. vulgaris* as well as search for potential tolerance mechanisms. The information gained may be helpful in developing a set of markers for detection of heavy metal pollution and advancing the phytoremediation technology by microalgae.

MATERIALS AND METHODS

Material preparation and experimental set-up

Chlorella vulgaris Beijerinck (NIES) was provided by the laboratory of the Nation Institute for Environmental Studies (NIES, Japan) and cultured in Jaworski's medium (JM). The pH of the growth media was 7–8.5. All the culture medium used was autoclaved at 121 ºC for 20 minutes (Autoclave: HG50, Hirayama, Japan) prior to use. The microalga was cultured in 80 ml of JM culture medium in a 250 ml of volumetric flask under photosynthetically active radiation (PAR) of 25 µmole photons $m^{-2} \cdot s^{-1}$ with 16:8 light: dark cycle. The cultures were continuously shaken at 120 rpm in an air-conditioned room (24–29 ºC). Copper and zinc were prepared as stock solutions of 1 M $CuSO_4 \cdot 5H_2O$ and $ZnCl_2$ in de-ionized water, respectively.

Copper and zinc were introduced into the medium during the exponential phase of algal growth (Day 7 after culture initiation, OD_{665} = 0.50). The experiment included nine treatments: control (JM medium); 125 µM, 250 µM, 500 µM and 1,000 µM of Cu; and 125 µM, 250 µM, 500 µM and 1,000 µM of Zn. The experiment lasted for 5 days after heavy metal addition and each treatment used three replicate flasks. Heavy metal contents in the cell were assessed at the end of the experiment, i.e., day 5 after heavy metal addition. Photosynthetic parameters (maximum quantum

yield, Fv/Fm) and proline level were measured at 0, 6, 12 h, 3 and 5 days after treatments. Chlorophyll *a* content and oxidative stress, as parameter indicated by reactive oxygen species (ROS) and malondialdehyde (MDA) content were measured on days 0 and 5. The biochemical assays and physiological techniques used are described below.

Determination of heavy metals in the cells of C. vulgaris

The methods were modified from Franklin *et al*. (2003) and Grima *et al*. (2003). Algal biomass of 80 ml was collected by centrifugation at 3,000 rpm for 10 minutes (Rotofix 32 A, Hettich, Germany). After that, the pellet was mixed with 0.90 M of nitric acid (KNO_3) and 0.02 M of EDTA and digested overnight. The concentrations of Cu and Zn were determined using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 7400, Thermo Fisher Scientific, USA) and normalized against chlorophyll *a* content.

Photosynthetic activity

A Pulsed Amplitude Modulate Fluorometer (Mini-PAM, Walz, Germany) was used to assess the maximum quantum yield of photosystem II (Fv/Fm). An algal culture of 700 µl was collected from each flask and transferred to a 2 ml microcentrifuge tube. The samples were kept in darkness for 20 minutes (dark adaptation) and Fv/Fm was measured thereafter from the same distance (0.5 cm) and angle (90 degrees).

Determination of total chlorophyll a content

Chlorophyll *a* was extracted from the 5-ml of the algal culture. First, the samples were centrifuged at 3,000 rpm for 10 minutes to collect the cells. The algal cells were then re-suspended in 90% of acetone and incubated overnight. After centrifugation at 3,000 rpm for 10 minutes (Rotofix 32 A, Hettich, Germany), the supernatant was collected and quantitative determination of chlorophyll *a* content was performed by measuring the absorbance at 630, 645, 665 and 750 nm following Wintermans and De Mots (1965) and Saijo (1975) (Spectrophotometer V1710, Yoke, China). Chlorophyll *a* content was calculated as follows and was expressed as mg of chlorophyll *a* per liter of sample.

$$\text{Chlorophyll } a \text{ content } (\text{mg}\cdot\text{l}^{-1}) = \frac{([11.6(\text{OD665})-0.14(\text{OD630})-1.31(\text{OD645})]\times 1000\ (\text{ml}))}{(\text{volume of sample (ml)})}$$

Determination of total reactive oxygen species (ROS)

Total reactive oxygen species (ROS) was estimated using 5 ml of algal culture from each flask. The samples were first centrifuged at 3,000 rpm for 10 minutes to collect the cells (Rotofix 32 A, Hettich, Germany). The algal cells were then sonicated at a frequency of 3 kHz (Benchtop Ultrasonic Cleaners, Crest, USA) in 2 ml of 50 mM Tris-HCl buffer (pH 7), followed by 10 minutes centrifugation at 3,000 rpm. The supernatant 800 µl was collected, mixed with 200 µl of 0.2 M 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich, USA) and left in the dark for 20 minutes. The ROS content was estimated from the fluorescence (Spectrofluorometer FP-8200, Jasco, USA) signal emitted by the mixture (excitation at 488 nm and emission at 525 nm) (Sun *et al*., 2014). Total ROS was normalized against chlorophyll *a* content.

Determination of lipid peroxidation (LPO)

Lipid peroxidation was estimated from the content of malondialdehyde (MDA), the product of the oxidation of lipid, using a protocol modified from Senevirathne *et al*. (2006). A 10-ml volume of algal culture was collected from each flask, sonicated at a frequency of 3 kHz for 15 minutes (Benchtop Ultrasonic Cleaners, Crest, USA), and then centrifuged at 3,000 rpm for 10 minutes (Rotofix 32 A, Hettich, Germany). Subsequently, 2 ml of 1% trichloroacetic acid was added to 2 ml of the supernatant. After 5 minutes, it was mixed with 2 ml of 0.1% solution of TBA and 0.05% solution of butylated hydroxytoluene and the mixture was heated at 95 ºC for 45 minutes (Water Bath GP 20, Thermo Scientific Precision, USA), then placed on ice immediately. The absorbance of the mixture at 532

nm and 600 nm was measured (Spectrophotometer V1710, Yoke, China). The concentration of MDA was calculated as follows and normalized against chlorophyll *a* content.

$$\text{The MDA content } (\mu mol \cdot l^{-1}) = \frac{[OD532 - OD600]}{115x10^3}$$

Determination of proline content

The protocol was modified from Bates *et al*. (1973). A 5-ml volume of algal cultures was collected from each flask. The samples were centrifuged at 3,000 rpm for 10 minutes to collect the cells (Rotofix 32 A, Hettich, Germany). The algal cells were then sonicated at a frequency of 3 kHz (Benchtop Ultrasonic Cleaners, Crest, USA) in 3 ml of 3% of sulfosalicylic acid. The mixture was centrifuged at 3,000 rpm for 10 minutes and then 5 ml of the supernatant was collected. Three ml of 10% ninhydrin mixture was added to the supernatant and heated at 85 ºC for 1 hour (Water Bath GP 20, Thermo Scientific Precision, USA) before placing on ice for 15 minutes. Subsequently, the solution was left at room temperature for 20 minutes before mixing with 5 ml of toluene (Sigma-Aldrich, USA). The pink layer was collected and the absorbance at 520 nm was measured (Spectrophotometer V1710, Yoke, China), and finally the concentration was calculated based on the standard curve of proline (Sigma-Aldrich, USA).

Statistical analysis

Statistical analyses were performed using STATISTICA Academic software (StatSoft, 2011). Assumptions regarding normality and homogeneity of variance were confirmed before the analyses when necessary. The effects of heavy metal concentration and time of exposure on Fv/Fm and proline content were tested using a test to detect any overall differences between related means (repeated ANOVA) and a one-way ANOVA to incorporate a covariate (ANCOVA), repeated ANOVA and ANCOVA (Woodrow, 2014). The effects of heavy metal concentration on heavy metal content in the cells, chlorophyll *a*, ROS and LPO were tested using one-way ANOVA.

RESULTS AND DISCUSSION

Chlorella vulgaris showed an accumulation of copper (Cu) and zinc (Zn), which was dependent on the given dose ($p<0.05$, one-way ANOVA, Figure 1). A significant increase in Cu content was detected when the alga was exposed to at least 500 µM of Cu ($p<0.05$, LSD test). Copper exposure did not affect algal Zn uptake as shown by no difference in Zn accumulation among Cu treatments (Figure 1a). Similarly, a significant increase in Zn content was detected when the alga was exposed to at least 500 µM of Zn ($p<0.05$, LSD test). However, Zn treatments seemed to affect Cu uptake as the alga exposed to 1,000 µM Zn also accumulated higher Cu content (Figure 1b) ($p<0.05$, LSD test). Both Cu and Zn enrichment resulted in dose-dependent accumulation of heavy metals in the cells of *Chlorella vulgaris* harvested at the end of the experiment. The results are in line with previous studies which reported that algae can accumulate heavy metals in the different cell components and organelles such as the phospholipid membrane, chloroplast, endoplasmic reticulum, peroxisome and mitochondria (Metha and Guar, 1999; Johnson *et al*. 2007; Rodrigues *et al*., 2012). Although Cu and Zn share a family of transporters, specifically, the COPT (Copper transporter) family as a channel for cellular uptake, it appeared in our experiments that when one was present in excess, it did not inhibit the uptake of the other, but instead Zn addition seemed to promote Cu uptake. However, there are many transporters present in microalgal cells which play a common role in Cu and Zn uptake such as the ZIP family (Zinc transporter; Burleigh *et al*., 2003), CDF family (Cation diffusion facilitation), MTP group (Metal tolerance proteins) (Vert *et al*., 2002) and HMA (Heavy metal ATPase) (Grotz and Guerinot, 2006). These transporters also play similar role in higher plants (Penarrubia *et al*., 2015) and interactions between them in maintaining heavy metal homoeostasis remain to be further elucidated. Our results show that heavy metal content in *C. vulgaris* can be used as an effective biomarker for Cu and Zn availability in the environment. This has been found to be species-specific as different species of algae have different uptake capacities for different metals (Harris and Ramlow, 1989).

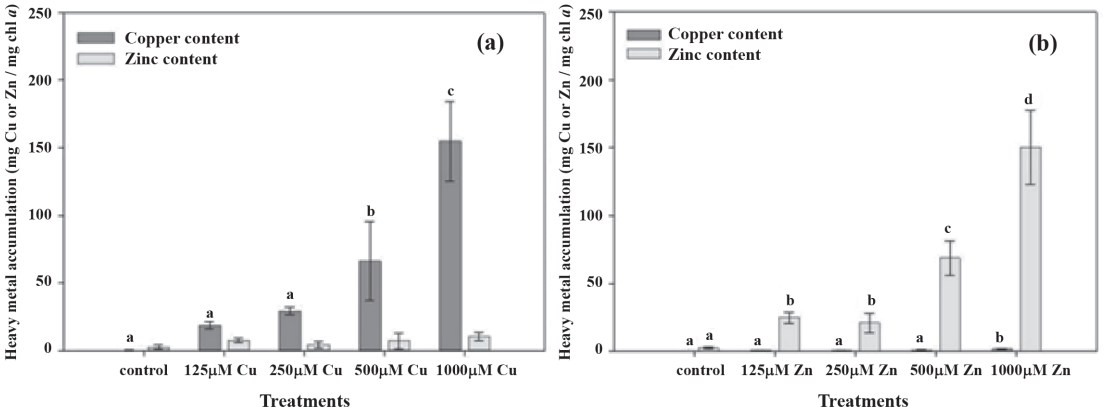

Figure 1. Heavy metal accumulation in *Chlorella vulgaris* treated at different concentrations of Cu (a) and Zn (b) at the end of the experiment (n=3, error bars show standard deviation). Bars with different letters are significantly different (LSD test, $p < 0.05$).

Interaction between time of exposure and heavy metal concentration was found to affect the maximum quantum yield of photosystem II (Fv/Fm) ($p<0.05$, repeated ANOVA, Figure 2). Although, ANCOVA showed no statistical difference among treatments, a clear trend of decreasing Fv/Fm in heavy metal-treated algae was observed. On day 3 a significant decrease in Fv/Fm compared to the control was detected in 500 and 1,000 µM Cu ($p<0.05$, LSD test) whereas on day 5, all the Cu treatments exhibited lowered Fv/Fm compared to control ($p<0.05$, LSD test). At the end of the experiment, algae treated with 500 and 1,000 µM Cu showed the lowest Fv/Fm (approximately 55% of control). Although, Zn addition induced slightly less inhibiton on Fv/Fm, decreased Fv/Fm compared to the control was detected from day 3 onward in all Zn treatments. At the end of the experiment, there was no difference in Fv/Fm among Zn-treatments (approximately 57% of control). Heavy metal addition resulted in a decrease in Fv/Fm, indicating that photodamage occurred (Salt *et al*., 1995; Tripathi *et al*., 2006; Gilroy *et al*., 2014). However the impact of Cu seemed slightly faster than Zn. This may be due to a higher number of target sites for copper toxicity in the photosynthetic machinery (Oukarroum *et al*., 2012; Penarrubia *et al*., 2015).

Similarly, chlorophyll *a* content decreased in both heavy metal treatments, with a steeper decreased observed in Cu-treated algae (Sun *et al*., 2014) (Figure 3). Heavy metal exposure induced chlorophyll *a* degradation in a dose-dependent manner in *C. vulgaris* ($p<0.05$, one-way ANOVA). Chlorophyll *a* content of the control was 195 ± 33% at the end of the experiment, indicating growth. Although both heavy metal treatments showed lower chlorophyll *a* compared to the control, algae exposed to 500 µM and 1,000 µM of Cu or Zn exhibited more detrimental impacts than the lower concentrations ($p<0.05$, LSD test). This phenomenon has previously been observed, and it was suggested that both metals can substitute magnesium in the chlorophyll molecules, leading to a loss in their function (Sharma and Chopra, 1987; Dobermann and Fairhurst, 2000; Enany and Issa, 2001), which may partly contribute to the decrease in photosynthetic activity observed here. In our study, a decrease in chlorophyll *a* may also indicate a decrease in biomass as chlorophyll *a* has been used as a proxy for growth in many microalgae (Enany and Issa, 2001). Both Cu and Zn enrichments were found to reduce growth and alter the structure of algae such as *Chlorella pyrenoidosa* and *Scenedesmus obliquus,* as well as promote chlorophyll degradation (Zhou *et al*., 2012).

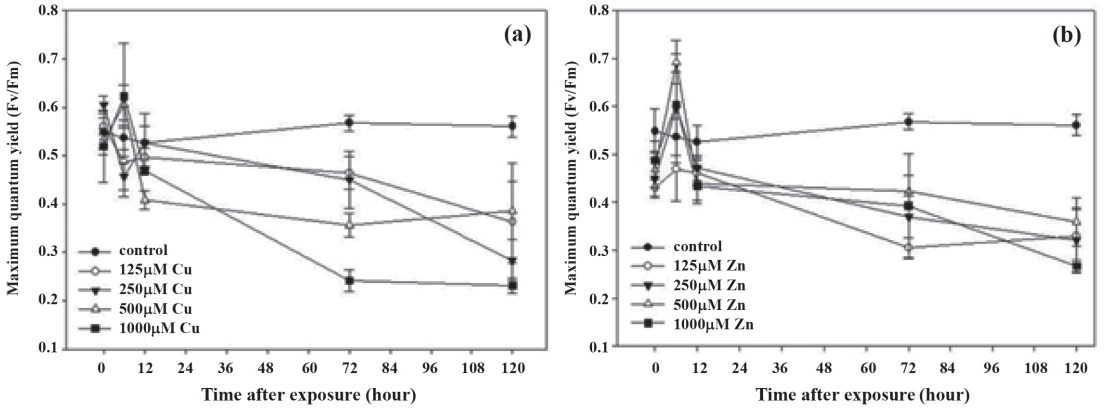

Figure 2. Maximum quantum yield (Fv/Fm) of *Chlorella vulgaris* treated with different concentrations of a) Cu and b) Zn at 0, 6, 12 hours, 3 and 5 days after heavy metal addition (n=3, error bars show standard deviation).

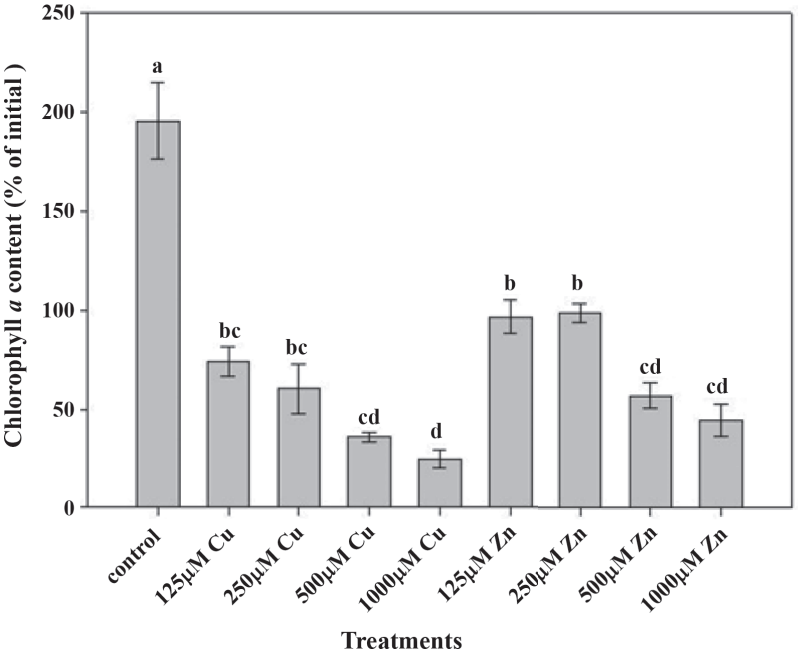

Figure 3. Chlorophyll *a* content, presented as percentage of initial (day 0), in *Chlorella vulgaris* treated with different concentrations of Cu and Zn at the end of the experiment (n=3, error bars show standard deviation). Bars with different letters are significantly different ($p < 0.05$).

Heavy metals including Cu and Zn are known to induce oxidative stress (Sokolnik *et al*., 2009; Sharma *et al*., 2012). Total reactive oxygen species (ROS) content in *C. vulgaris* was affected by heavy metal concentration ($p<0.05$, one-way ANOVA, Figure 4). However, only Cu levels at 1,000 µM induced ROS production while an increase in ROS was not observed in Zn-treated algae. Similar responses have been observed in *C. pyrenoidosa* and *Microcystis aeruginosa* (1-15 µM Cu, Lu *et al*., 2015), *Scenedesmus* sp. (2.5-40 µM Cu and 5-100 µM Zn, Tripathi and Gaur, 2004) and *Arthrospira* (*Spirulina*) *platensis* (approximately 1-300 µM Cu and Zn, Choudhary *et al*., 2007) treated with various concentrations of Cu and Zn, although the concentrations used in our experiment were higher. Similar to photosynthetic inhibition and chlorophyll degradation, Cu toxicity appeared to be more detrimental compared to Zn when considering the level of ROS for the present algal species. This may be due to the redox status of Cu in the cell. Cu is redox-active and is able to promote ROS production directly whereas zinc is redox-inactive and binds with certain proteins and enzymes, leading to slower production of ROS (Pinto *et al*., 2003). Tripathi *et al*. (2006) also demonstrated that different heavy metals are able to induce varying levels of oxidative stress via different pathways in different organelles. Lipid peroxidation exhibited a similar trend. An increase in MDA in comparison to the control was detected at 250, 500 and 1,000 µM Cu and 500 and 1,000 µM Zn ($p<0.05$, one-way ANOVA, Figure 5). This indicates that heavy metal enrichment may have induced disintegration of membrane lipids via the oxidation process (Choudhary *et al*., 2007; Elbaz *et al*., 2014). Similar to ROS production, Cu exerted a toxicity effect at lower concentration than Zn.

An interesting response which was also found to be dose and time-dependent is an increase in proline accumulation. Both heavy metal

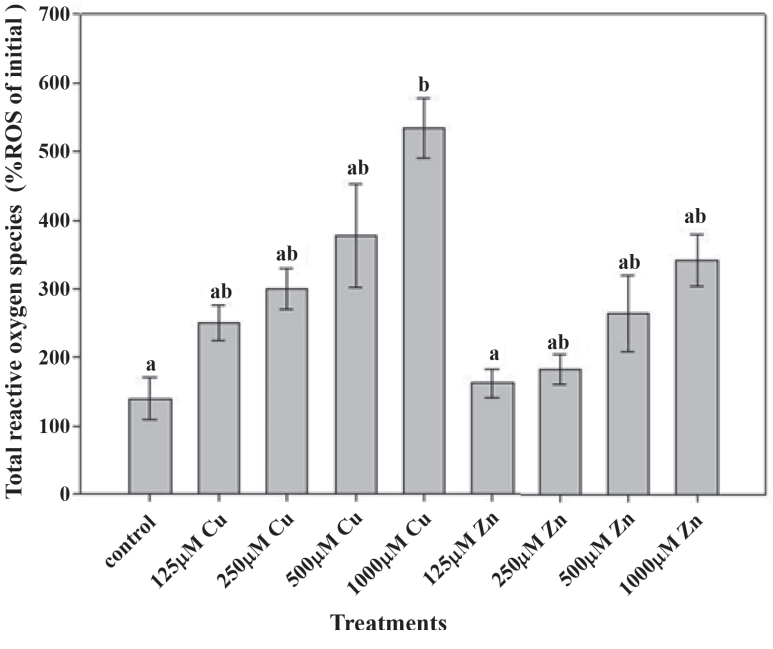

Figure 4. Total reactive oxygen species (ROS), presented as percentage ROS of initial (day 0), in *Chlorella vulgaris* treated with different concentrations of Cu and Zn at the end of the experiment (n=3, error bars show standard deviation). Bars with different letters are significantly different ($p < 0.05$).

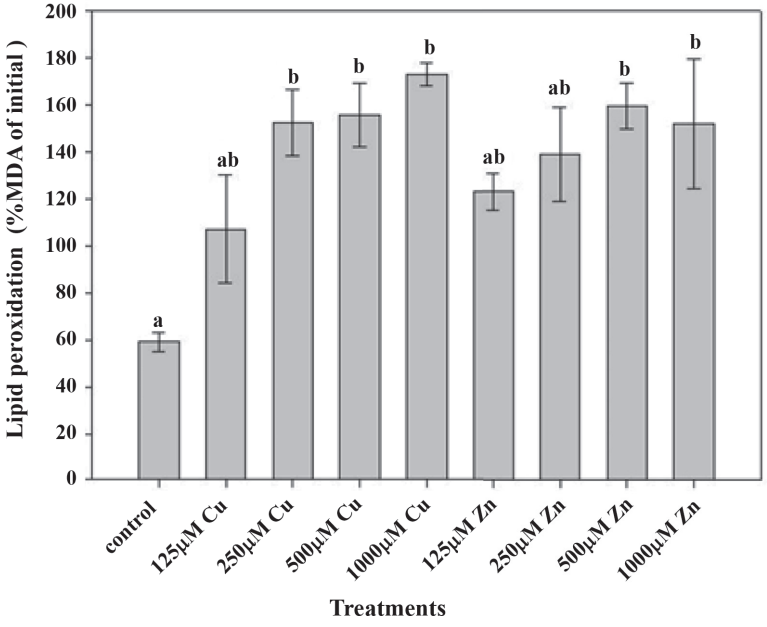

Figure 5. Lipid peroxidation, presented as percentage MDA of initial (day 0), in *Chlorella vulgaris* treated with different concentrations of Cu and Zn at the end of the experiment (n=3, error bars show standard deviation). Bars with different letters are significantly different ($p < 0.05$).

concentrations, time of exposure and interaction between the two factors were found to significantly effect proline content in the cells of *C. vulgaris* ($p<0.05$, repeated ANOVA). There was a significant variation in proline content in controls ($p<0.05$, LSD test) (Figure 6). However, the functional relationships between proline accumulation and time after exposure differed among given heavy metal concentrations ($p<0.05$, ANCOVA). The stimulating effect was more pronounced in Cu treatments than in Zn treatments. Cu at all concentrations induced proline production starting from hour 6 of exposure ($p<0.05$, LSD test) whereas increased proline production was observed only in 1,000 µM Zn at hour 6 of exposure ($p<0.05$, LSD test). Nevertheless, all the heavy metal-treated algae had accumulated a higher level of proline than the control at the end of the experiment ($p<0.05$, LSD test), and despite a steeper increase in Cu-treated alga, the highest proline accumulations, found for 500 and 1,000 µM of both Cu and Zn was did not differ at the same level. Previous investigations showed a positive relationship between the dose of heavy metals and the concentration of proline in algae at the low to medium levels of heavy metals, suggesting protective a role of this amino acid against heavy metal toxicity (Mehta and Gaur, 1999; Enany and Issa, 2001; Choudhary *et a*l., 2007). It has been proposed that proline plays a role in osmoregulation in both algae and plants under stress conditions, thus maintaining cellular homoeostasis (Saradhi, 1991; Smironoff and Cumbes, 1989; Schat *et al*., 1997; Ambikapathy *et al*., 2002; Ashraf and Foolad, 2007), while preventing heavy metal toxicity by reducing uptake of heavy metals (Wu *et al*., 1998a,b). Choudhary *et al*. (2007) reported that proline content in the cyanobacteria *Arthrospira platensis*, increased upon copper and zinc exposure alongside an increase in lipid peroxidation and superoxide dismutase activity. Other heavy metals such as nickel, cadmium and lead were also found to induce proline accumulation in *Scenedesmus armatus* (Enany and Issa, 2001). Molecular manipulation experiment showed that increasing the expression of the proline biosynthetic gene, pyrroline-5-carboxylate synthetase (*P5CS*), in

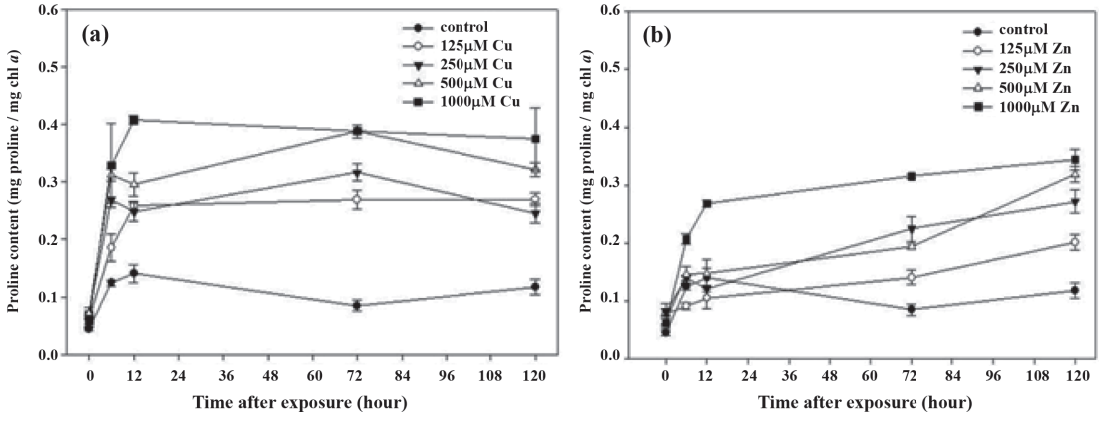

Figure 6. Proline content of *Chlorella vulgaris* treated with different concentrations of a) Cu and b) Zn at 0, 6, 12 hours, 3 and 5 days after heavy metal addition (n=3, error bars show standard deviation).

Chlamydomonas reinhardtii increased proline production and subsequently increased tolerance of *C. reinhardtii* to cadmium (Siripornadulsil *et al*., 2002). This work also suggested that proline is associated with glutathione, which is an important antioxidant and a precursor for biosynthesis of phytochelation, a peptide with a role in heavy metal detoxification and sequestration (Siripornadulsil *et al*., 2002). An increase in proline in our work may have been triggered by an increase in ROS, which are also signaling molecules under stress conditions (Costa and Morel, 1994; Enany and Issa, 2001). We suggest that the role of proline in heavy metal tolerance should be further investigated.

CONCLUSION

Our study demonstrates that Cu and Zn exposure lead to a number of responses at the physiological level in *C. vulgaris*, some of which are time and dose-dependent. The concentrations of Cu and Zn at 500 μM and 1,000 μM imposed clear toxicity effects. We propose that *C. vulgaris* can be used as a bioindicator for Cu and Zn contamination in aquatic systems and as a biomarker suite which includes heavy metal content in the cells, the maximum quantum yield by chlorophyll fluorescence, ROS, MDA and proline levels.

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