Environmental Science and Pollution Research (2021) 28:32475–32492
https://doi.org/10.1007/s11356-021-12950-6

RESEARCH ARTICLE

Effects of zinc and mercury on ROS-mediated oxidative stress-induced physiological impairments and antioxidant responses in the microalga *Chlorella vulgaris*

Vayampully Ajitha[1,2] · Chandrasekharan Parvathi Sreevidya[1] · Manomi Sarasan[3] · Jun Chul Park[4] · Ambat Mohandas[1] · Isaac Sarojini Bright Singh[1] · Jayesh Puthumana[1] · Jae-Seong Lee[4]

Received: 24 April 2020 / Accepted: 9 February 2021 / Published online: 24 February 2021

Abstract

The rapid growth of industrialization and urbanization results in deterioration of freshwater systems around the world, rescinding the ecological balance. Among many factors that lead to adverse effects in aquatic ecology, metals are frequently discharged into aquatic ecosystems from natural and anthropogenic sources. Metals are highly persistent and toxic substances in trace amounts and can potentially induce severe oxidative stress in aquatic organisms. In this study, adverse effects of the two metal elements zinc (maximum concentration of 167.25 mg/L) and mercury (104.2 mg/L) were examined using *Chlorella vulgaris* under acute and chronic exposure period (48 h and 7 days, respectively). The metal-induced adverse effects have been analyzed through photosynthetic pigment content, total protein content, reactive oxygen species (ROS) generation, antioxidant enzymatic activities, namely catalase and superoxide dismutase (SOD) along with morphological changes in *C. vulgaris*. Photosynthetic pigments were gradually reduced (~32–100% reduction) in a dose-dependent manner. Protein content was initially increased during acute (~8–12%) and chronic (~57–80%) exposure and decreased (~44–56%) at higher concentration of the two metals (80%). Under the two metal exposures, 5- to 7-fold increase in ROS generation indicated the induction of oxidative stress and subsequent modulations in antioxidant activities. SOD activity was varied with an initial increase (58–129%) followed by a gradual reduction (~3.7–79%), while ~1- to 12-fold difference in CAT activity was observed in all experimental condition (~83 to 1605%). A significant difference was observed in combined toxic exposure (Zn+Hg), while comparing the toxic endpoint data of individual metal exposure (Zn and Hg alone). Through this work, lethal effects caused by single and combined toxicity of zinc and mercury were assessed, representing the significance of appropriate monitoring system to trim down the release of metal contaminants into the aquatic ecosystems.

Keywords Metal pollution · Acute and chronic exposure · Photosynthetic pigment content · Single and combined effects · Antioxidant enzymes · ROS generation

Responsible Editor: Philippe Garrigues

✉ Jayesh Puthumana
jayesh@cusat.ac.in

✉ Jae-Seong Lee
jslee2@skku.edu

[1] National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi, Kerala 16, India

[2] School of Environmental Studies, Cochin University of Science and Technology, Kochi, Kerala 22, India

[3] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala 16, India

[4] Department of Biological Sciences, College of Science, Sungkyunkwan University, Suwon 16419, South Korea

Introduction

Pollution of water bodies through metal discharges from industries greatly affects the ecosystem. Because metals are highly persistent pollutants in the aquatic ecosystem, they can cause alteration of growth, development, morphology, physiological, and biochemical metabolism in aquatic organism (Assche and Clijsters 1990; Bidar et al. 2007; Ajitha et al. 2019). Metals enter the aquatic system and primarily act on small organisms, algae, which are ubiquitously distributed throughout the aquatic environment; and such metal contaminants are widely distributed out to and among various organisms due to unavoidable presence in the aquatic food chain system (Liu et al. 2008). Among various animal taxa,

extensive studies on the adverse effects of metal contamination in animals including aquatic vertebrates and invertebrates are available; however, relatively little attention has been given on the importance of primary producers, microalgae.

Microalgae are known to be sensitive to the alterations in the environment and often used as biological indicators for assessing the toxic effects of metals (Chouteau et al. 2004; Durrieu et al. 2011; Kumar et al. 2015), as they are extensively prevalent in lakes and seas (Chen et al. 2012) (Table 1). Among microalgae, *Chlorella vulgaris* is a well-known photosynthetic freshwater microalga and generally used for toxicity tests due to its high sensitivity to xenobiotics (Ajitha et al. 2019). In fact, metals having characteristics of non-biodegradability, biomagnification, and high toxicity are of great threat to the aquatic ecosystem, resulting in a significant reduction in algal diversity and productivity, which all contribute to changes in algal composition (Harding and Whitton 1976; Foster 1982; Shehata and Whitton 1982; Takamura et al. 1989; Gupta and Chandra 1994; Bajguz 2000; Mallick 2004). Furthermore, consequences of metal stress in algae include detrimental effects on growth, cell division, photosynthesis, and destruction of primary metabolites (Pokora and Tukaj 2010; Tukaj and Tukaj 2010; Wang et al. 2011).

Among various metal pollutants present in aquatic ecosystem, Zn is considered to be an essential microelement; however, at higher concentration, Zn is strongly phytotoxic and leads to the obstruction of algal growth, while the non-essential element Hg becomes highly toxic in metallic, ionic, and organic forms which is deleterious to aquatic fauna and flora (Ouyang et al. 2012; Dinesh Kumar et al. 2014). Also, Hg pollution is of great concern due to its high toxicity and resistance to biodegradability, and potential for bioaccumulation through trophic chains. Growth inhibition of *Chlorella* by Hg has been widely acknowledged (Hutchinson and Stokes 1975; Gipps and Biro 1978). Moreover, studies have evaluated Hg toxicity in aquatic organisms, highly focusing on the bioaccumulation and trophic transfer as well as lethal and sub-lethal toxicity to fish (Boening 2000). In addition, few studies have explored Hg toxicity to aquatic plants and larval stages of insects (Azevedo-Pereira and Soares 2010; Dirilgen 2011). Several findings revealed that Hg causes a significant reduction of plant growth and biomass (Godbold 1991; Israr et al. 2006; Cargnelutti et al. 2006; Zhou et al. 2007) and generates oxidative stress by the generation of reactive oxygen species (ROS) (Cargnelutti et al. 2006; Zhou et al. 2007).

To date, many toxicity tests have been performed based on individual toxicity; however, due to the potential combined effects, toxicity exerted by the combinations of various metals is likely more serious and threatening (Zeb et al. 2017). Also, literature on the assessment of the combined toxicity of metals

Table 1 Review on the metal exposure performed to the microalgae and the endpoints and responses assessed

Metals	Species	Endpoints/responses	References
Zn, Hg, CH_3Hg^+	*Chlorella vulgaris*	Specific growth rate, pH, phosphate, calcium, magnesium, total chlorophyll, and carotenoid content	Rai et al. 1981a
$ZnCl_2$, $HgCl_2$		Survival, growth measurement, pigments, protein content, CO_2 fixation, O_2evolution, ATP content, nutrient uptake, nitrate reductase activity	Rai et al. 1991
Cu		Lipid peroxidation, proline content, carotenoid content, protein content, GSH, GR, APX, CAT,SOD	Mallick 2004
Cu		Dose-dependent increase in ROS (50 and 250 nM)	Knauert and Knauer 2008
Cu, Cd		Significant increase in CAT activity and peroxidase under single exposure of Cu and mixture of Cd (1.5 μM of Cu)	Qian et al. 2011
Cu, Cr, Cd, Zn, Pb		Significant inhibition of growth at earlier exposure time. Inhibition of chlorophyll fluorescence by Cu, while Zn promoted fluorescence	Ouyang et al. 2012
Cr		Concentration-dependent increase in antioxidant enzymes (CAT, APX, SOD), carotenoid, and MDA level	Rai et al. 2013
Hg	*Chlorella pyrenoidosa*	Inhibition of cell division	Kamp-Nielsen 1971
Cu^{2+} , Chlortetracycline	*Chlorella pyrenoidosa* *Microsystis aeruginosa*	Increase in SOD activity under initial exposure. Soluble protein contents was decreased under initial exposure and the effect was more severe under re-exposure.	Lu et al. 2015
Cu	*Anabaena doliolum*	Inhibition of chlorophyll-a accumulation, despite significant activation of SOD	Mallick and Rai 1999
Zn, Pb, Cu	*Spirulina platensis*	Increase in MDA and SOD in response to different concentration gradients of Zn, Pb, and Cu	Choudhary et al. 2007
$CuCl_2$, PCB	*Prorocentrum minimum*	Significant up-regulation of *CYP*at lower concentration of Cu, along with induction of ROS	Ponmani et al. 2015
Cd, 4-n-nonylphenol	*Chlorella sorokiniana*	Growth inhibition with significant increase in SOD activity at earlier exposure time	Wang et al. 2018

(Mochida et al. 2006; Su et al. 2012; Qu et al. 2013) particularly on single and/or synergistic effects of metals in the microalga *C. vulgaris* have been reported (Rai et al. 1981a; Franklin et al. 2002; Qian et al. 2009, Qian et al.2011). For example, the combined nitrogen limitation and cadmium stresses have led to significant inhibition of growth and cell density of *C. vulgaris* (Chia et al. 2015). However, a detailed recent study illustrates the additive and synergistic effects on *C. vulgaris* in response to six metals such as Ni, Fe, Zn, lead (Pb), cadmium (Cd), and chromium (Cr) (Mo et al. 2019). Exposure of the microalga *C. pyrenoidosa* to copper and cadmium, individually and in combination, resulted in growth inhibition (Nugroho et al. 2017). Also, single, combined, and second exposure effect of Cu^{2+} and chlortetracycline (CTC) on the microalgae *C. pyrenoidosa* and *Microcystis aeruginosa* demonstrated variation in toxicity due to differences in recovery potential among the two species (Lu et al. 2015). Moreover, the action of binary mixtures of cetyltrimethyl ammonium chloride (CTAC) and aromatic hydrocarbon showed synergetic and antagonistic effects on *C. vulgaris* (Ge et al. 2010). Similarly, single and combined effects of cadmium and 4-n-nonylphenol (4-n-NP) on growth inhibition and oxidative stress in the microalga *C. sorokiniana* were have been reported (Wang et al. 2018) (Table 2).

Based on the previous study on metal composition in treated electroplating industrial effluent (Ajitha et al. 2019), we selected Zn (upper limit 167.25 mg/L) and Hg (upper limit 104.2 mg/L) as the testing metal elements for this study. Single and combined effects of Zn and Hg were analyzed based on both acute and chronic exposure periods, 48 h and 7 days, respectively. This study aims to better understand how Zn, Hg, and Zn+Hg combination affects the biological process in *C. vulgaris*. To corroborate, we investigated the effects of Zn and Hg and their combination on the accumulation of oxidative radicals, the impairments on physiological parameters (pigments and protein), and the counter-response of antioxidant defense mechanisms (CAT and SOD) in the oxidative stress-induced microalga *C. vulgaris*. Besides, the morpho-variability and aberrations in *C. vulgais* during chronic exposure to metals at different concentrations were observed. Overall, even though without any affirmed mechanism of action of synergism or the combinatorial effects of Zn and Hg on *C. vulgaris*, this study provides insight into the response of *C. vulgaris* in response to Zn, Hg, and Zn+Hg and helps to predict the biological effects, which paves way for the directions for identifying the molecular effects of metal synergism in *C. vulgaris*.

Materials and methods

The microalga *C. vulgaris* was obtained from Central Marine Fisheries Research Institute, Kochi, Kerala in India and maintained axenically at the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT), Kerala, India, until it was used for this study (Ajitha et al. 2019). *C. vulgaris* was maintained in aerated Bold's basal medium (BBM) (Bischoff and Bold 1963) under 16:8 h light and dark cycle at 25±2°C, 45 mmol m^{-2} s^{-1} photon flux intensity. All investigations were carried out following the guidelines provided by the Institutional Biosafety Committee (IBSC) at NCAAH, CUSAT, Kerala in India.

To obtain the elemental Zn and Hg, $ZnCl_2$ and $HgCl_2$ with 99.9% purity were selected. Stock solutions of $ZnCl_2$ (2.4 mM contain 167.25mg/L Zn) and $HgCl_2$ (0.076 mM contain 104.2 mg/L Hg) were prepared with Milli-Q water and considered as 100%. The upper limits of Zn (167.25 mg/L) and Hg (104.2 mg/L) were selected based on the previous findings (Ajitha et al. 2019) and considered as 100%. Various concentrations of Zn and Hg used for the study are given in Tables 4 and 5. To assess single and combined effects of Zn and Hg during acute and chronic toxicity tests, *C. vulgaris* cells were exposed to metal solutions prepared in BBM media in different concentrations (2.5 to 80%). Control was maintained by using the same BBM medium without metals. Experimental cell cultures were initiated at 0.6×10^6 cells/mL. Cell number was determined using a hemocytometer (Improved Neubauer, Rohem, India), as described in Ajitha et al. (2019). Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Field emission scanning electron microscopic (FESEM) analysis was performed using *C. vulgaris* samples with distinct concentrations of Zn and Hg (2.5, 20, and 80%). Microalgal cultures were harvested and washed in 1x phosphate-buffered saline (PBS) for 2–3 times followed by centrifugation at 12,400×*g*. To obtain the cell pellets, 1 mL 2.5% glutaraldehyde was added and kept for overnight at 4°C. After 12 h, cells were harvested and washed in 1x PBS for 2–3 times. One milliliter of 2% osmium tetroxide was added and incubated for 4 h at 4°C. Cells were harvested and washed with 1x PBS followed by the dehydration with acetone and air dry (Grantt 2008). Nova NanoSEM 450UoK scanning electron microscope (Nova NanoSEM, Los Angeles, USA) was used to observe the microalgal cells.

C. vulgaris samples treated with various metal concentrations (2.5 to 80% for both Zn and Hg) were prepared for the assessment of photosynthetic pigments. Two-milliliter microalgal sample was centrifuged at 2200×*g* for 5 min and the pellets were suspended in 2 mL methanol, incubated for 30 min at 45°C. The supernatant was discarded and absorbance was taken at 665.2, 652.4, and 470 nm (Lichtenthaler 1987).

For the evaluation of protein content, six dilutions of metal samples along with the control were tested in *C. vulgaris* cultures. Followed by the standard protocol (Barbarino and Lourenço 2005), protein was extracted. To quantify the total

Table 2 Synergistic effect of heavy metals and their endpoint assays on various algae

Synergistic exposure	Species	Endpoint assays	References
Zn, Hg, CH_3Hg^+	*Chlorella vulgaris*	Total chlorophyll, carotenoid content, growth rate, pH, phosphate,calcium, magnesium	Rai et al. 1981b
Cu, Cd, and Zn	*Chlorella vulgaris*	Cell division rate	Franklin et al. 2002
Cu, Cd	*Chlorella vulgaris*	Reduction in cell growth, chlorophyll content, and increase in ROS content	Qian et al. 2009
Cetyltrimethylammonium chloride (CTAC), benzene, toluene, phenol, nitrobenzene, phenanthrene, fluoranthene	*Chlorella vulgaris*	Biomass, zeta potential	Ge et al. 2010
Cu, Cd	*Chlorella vulgaris*	SOD, peroxidase, Malondialdehyde	Qian et al. 2011
Brassinosteroids, auxins	*Chlorella vulgaris*	Cell number, chlorophyll content, protein, monosaccharides	Bajguz and Piotrowska-Niczyporuk 2013
Cd, N	*Chlorella vulgaris*	Growth rate, biomass, and biochemical composition	Chia et al. 2015
Cu^{2+}, chlortetracycline (CTC)	*Chlorella pyrenoidosa*, *Microcystis aeruginosa*	Chlorophyll fluorescence, MDA, SOD, protein	Lu et al. 2015
Cu, Cd	*Chlorella pyrenoidosa*	Growth inhibition	Nugroho et al. 2017
Cd, 4-n-nonylphenol (4-n-NP)	*Chlorella sorokiniana*	SOD, CAT, GSH, and growth inhibition	Wang et al. 2018
Ni, Fe, Zn, Pb, Cd, Cr	*Chlorella pyrenoidosa*	Toxicity inhibition	Mo et al. 2019

protein contents, Bradford assay (Bradford 1976) was carried out with the precipitated proteins via bovine serum albumin (BSA) as the standard.

Estimation of ROS was done using dihydroxyrhodamine123 (DHR123) dye. Various metal concentrations (2.5, 20, and 80%) were prepared along with the control at 48 h and 7 days. The cell pellets were stained with DHR123 at a final concentration of 5μg/L for 1h. Cells were centrifuged and washed twice in fresh BBM medium. The cultures were resuspended in fresh BBM medium and observed under a confocal microscope (Nikon A1R, Tokyo, Japan) to check the ROS generation in single cells (Sathasivam et al. 2016).

Antioxidant enzyme assays were performed by following our previous paper (Ajitha et al. 2019). Briefly, *C. vulgaris* cultures in response to various concentrations of metals (i.e., zinc and mercury) ranging from 2.5 to 80% were kept for 48h and 7 days. *C. vulgaris* cells (100 mg) were homogenized in 0.5 M PBS (pH7.5), 1 mM ethylene-diamine-tetraacetic-acid (EDTA), and a pinch of polyvinyl polypyrrolidone. The homogenate was centrifuged at 12,400×*g* at 4 °C for 30 min. Enzyme extraction was carried out at 0–4°C and the supernatants were stored as aliquot for enzyme estimation. Catalase activity was examined following the standard protocol (Chance and Maehly 1955). Reaction mixture was prepared with 2.5 mL 10 mM PBS, 0.5 mL H_2O_2, and 0.2 mL enzyme extract. Reduction in absorbance at 230 nm was analyzed in a spectrophotometer (Hitachi U-3900, Tokyo, Japan) and the specific activity was expressed in terms of changes in absorbance/min/extinction coefficient/mg protein. Superoxide dismutase activity was determined by standard protocol (Das et al. 2000). Reaction mixture of 1.5 mL aliquot comprised of 0.3 mL each of 50 mM PBS (pH7.4), 20 mM methionine, 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride, and 50 μM EDTA. To this aliquot, 200 μL, the supernatant was added followed by the pre-incubation at 37°C for 5 min. Eighty microliters of 50 μM riboflavin was added to the tubes and the mixture was placed below a light source for 10 min. One milliliter of Griess reagent was added to each tube and the absorbance of the color formed was measured at 543nm against buffer taken as blank. Each test was performed in triplicates.

One-way analysis of variance (ANOVA) was done to confirm the validity of the data using SPSS® software (version21; SPSS Inc., Chicago, IL, USA) followed by Tukey's post hoc test (Tukey's, $P<0.05$ and $P<0.01$), which shows statistically significant differences in all treatments.

Results and discussion

In the present study, single and combined effects of Zn and Hg were assessed through morphological changes, photosynthetic pigment content, total protein content, ROS generation, and antioxidant enzyme (CAT and SOD) activities in *C. vulgaris*. SEM provided direct observation of microalgal cells in which high magnification and resolving power facilitates the improved examination of morphology and surface attachment. Under the chronic exposure of different concentrations of Zn, *C. vulgaris* cells featured various physical transformations in cell size and structure compared to the control. *C. vulgaris*

cells of the control showed typical size for the species (Fig. 1A and Suppl. Fig. 1A). Morphological variations and aberrations in *C. vulgaris* cells were visible from 2.5% concentration onwards compared to the control. Cell wall showed signs of shrinkage and structural damages illustrated in Fig. 1 Suppl. Fig. 1, and the deformations were severe under the higher concentration (80%). Under Hg exposure, microalgal cells in response to 2.5, 20, and 80% concentrations of Hg also showed structural alterations compared to the control (Fig. 1B), nearly similar to Zn exposed cells. However, in the combined toxicity test, the adverse effects were marginally higher compared to the single metal toxicity, especially at the highest concentration (Fig. 1C). Indeed, ruptured and shrank cells in the test groups demonstrated the severity of metal-induced toxicity on *C. vulgaris* and further suggests that the combined effects of the two metals are likely to exert higher toxicity

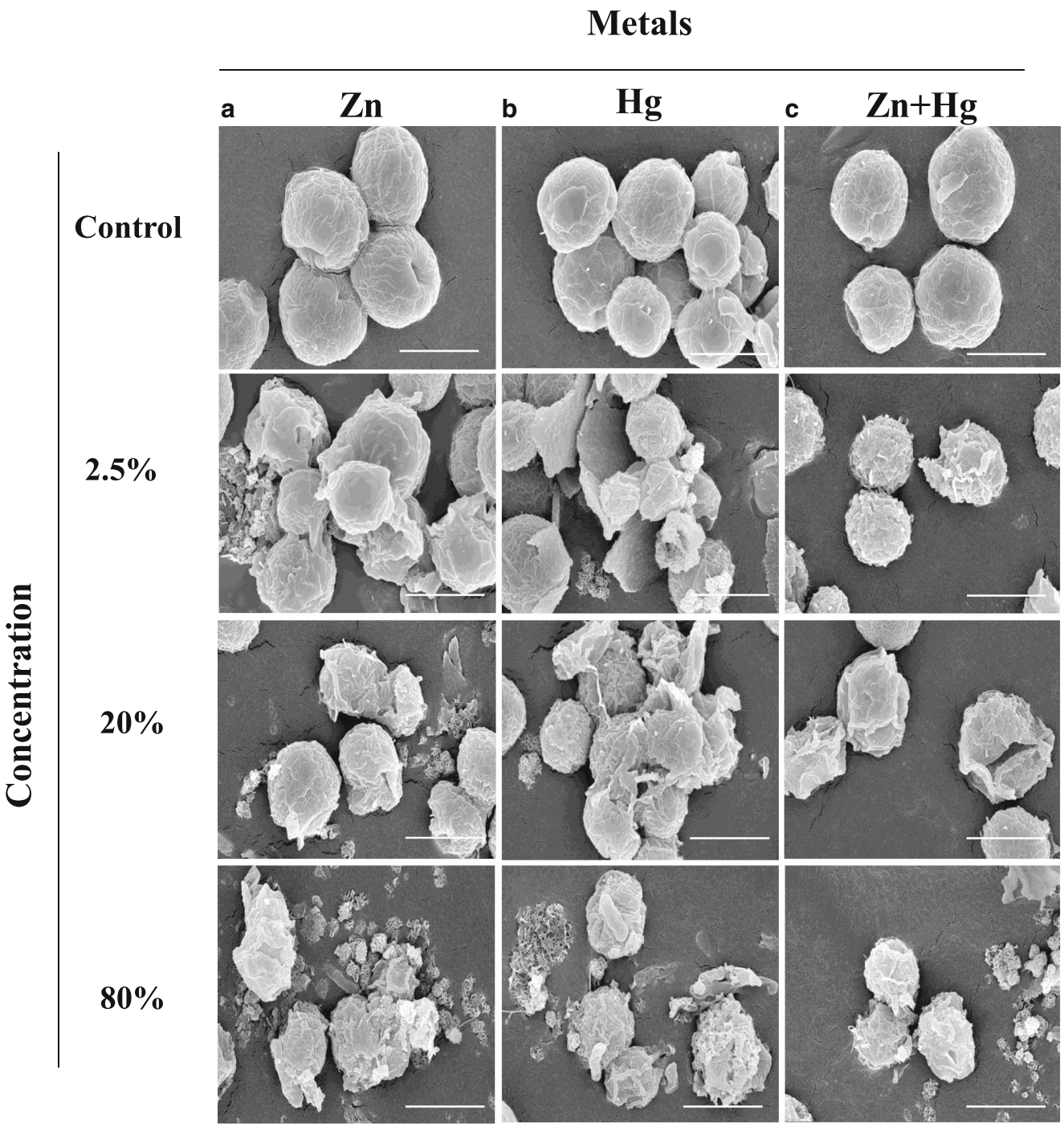


Fig. 1 Scanning electron micrograph of *Chlorella vulgaris* treated with Zn, Hg, and Zn+Hg shows morpho-variation and aberrations. Cell wall damages are observed at 2.5, 20, and 80% heavy metals present in concentrations tested (Scale bar = 3 μm)

within the cells, ultimately leading to cell structural deformities at a higher extent.

In fact, Hg and Zn compounds induced morphological transformations and oxidative stress in microalgal cells including *Chlorella* sp. (Nuzzi 1972; Gold et al. 2003; Li et al. 2006; Morin and Coste 2006; Tripathi and Gaur 2006). Similarly, in the diatoms *Thalassiosira pseudonana* (Sunda 1975) and *Skeletonema costatum* (Morel et al. 1978), morphological aberrations were reported in response to Cu^{2+}. Furthermore, a photosynthetic protist *Euglena gracilis* exposed to chromium showed rigorous morphological and biochemical alterations (Rocchetta et al. 2006), while morphological changes due to metal intoxication were widely reported in Chlorophyceae (Rosko and Rachlin 1977), Chrysophyceae (Davies 1974), Bacillariophyceae (Morel et al. 1978; Nuzzi 1972; Sunda 1975), and ciliates (Tingle et al. 1973). In contrast, few studies in the past have shown ameliorating effects of iron (Fe) against the toxicities of other heavy metals in various algae, including *Micrasterias* (Volland et al. 2011; Volland et al. 2012; Andosch et al. 2012) which showed significant improvements of cell morphogenesis, photosynthesis, cell division rates, and the structures of chloroplasts (Volland et al. 2014). Also, it has been shown that Fe and Zn assist in ameliorating the toxicity of heavy metal such as chromium (Cr) (Mallick et al. 2010; Branzini et al. 2012) possibly induced by the presence of competition for carrier up-take into the cell (di Toppi and Gabbrielli 1999; Shanker et al. 2005). However, due to possible species-specific differences in metal toxicities among algae, further molecular analyses on the metal-uptake potential are required to fully understand the differences in metal toxicities. Aside from species-specific differences in metal-uptake and subsequent induction of toxicity, other factor such as biosorption potential of *C. vulgaris* may account for the physiological malformation due to Zn and Hg-induced toxicity. Indeed, biosorption in aquatic plants cells is crucial against toxicity as they are involved in removal of toxic elements (Mehta et al. 2002; Michalak and Chojnacka 2010) and threshold concentration for different metals has been reported (Wan Maznah et al. 2012), suggesting species-and metal-specific differences in biosorption potential, contributing to concentration-dependent increase in cell deformities in *C. vulgaris* under the two metal exposures. Taken together, it is evident that the observed morphological variations in *C. vulgaris* samples were likely due to metal toxicity (Table 3).

The main photosynthetic pigments are comprised of chlorophyll-α, -β, and carotenoids (Chl-α, Chl-β, and Car) (Yang et al. 2020). Chlorophyll is essential in photosynthesis which enables microalgae and cyanobacteria to generate energy from light absorption, Chl-α, specifically (Takamura et al. 1990; Van Baalen and O'Donnell 1978). The results presented in Fig. 2A and Tables 4 and 5 clearly demonstrate a dose-dependent toxic effect of Hg and Zn on pigment contents of *C. vulgaris* and combined effect of Zn and Hg induced more stress on pigments compared to the single-dose experiment and correspondingly the pigments were decreased. This pattern was observed in both acute (48 h) and chronic (7 days) experiments. However, a random reduction of pigment was observed in chronic toxicity test (Fig. 2A). *C. vulgaris* cultures exposed to different concentrations of Zn during 48 h and 7 days showed a reduction in Chl-α, Chl-β, and Car contents respectively (Fig. 2A, Suppl. Fig. 2). Above 2.5% concentration, diminution of pigments was noticed compared to the control in both acute and chronic studies. Interestingly, both single and combined toxicity of metals induced higher contents of Chl-β compared to that of Chl-α in all concentration treatment. Single effects of Zn on *C. vulgaris* cultures from lower to higher concentration showed a concentration-dependent significant reduction ($P<0.05$) in pigment content and the percentage reductions were 47.64 to 90.93% and 67.09 to 94.17% at lower (2.5) and higher (80) concentrations during acute and chronic exposure (Tables 6 and 7).

Previously, photosynthetic pigments were found to be diminished under excess concentrations of Zn in microalgal cultures (De Filippis and Pallaghy 1976; Rai et al. 1981b). Similarly, Chl-α concentration was reduced in the green microalga *Pseudokirchneriella subcapitata* in response to Zn exposure (Soto et al. 2011). Also, high concentrations of Zn reduced total chlorophyll content, ATPase activity and carotenoid/chlorophyll ratio, and cell division and mobility in the green microalgae *Scenedesmus obliquus* and *S. quadricauda* (Omar 2002).

Hg-exposed cells showed decreased photosynthetic pigments in both acute and chronic tests (from 2.5 to 80%), compared to the control. Under both acute and chronic exposure, the reduction of pigment contents was in a concentration-dependent manner, compared to the control (i.e., gradual reduction of pigment contents). In a single metal exposed experiment, marginal reduction in pigment contents was noticed in Hg-treated cells than Zn which varied at approximately 3–4% variation on toxic endpoints (Tables 6 and 7).

Hg toxicity has been reported to cause perturbation in various biological functions. For example, growth reduction by Hg in *Chlorella* was extensively acknowledged (Gipps and Biro 1978; Hutchinson and Stokes 1975), and the photosynthetic capacity of *Chlorella* was affected at a concentration of 2.5×10^{-5} M $HgCl_2$ (Greenfield 1942).0.1 mg/L Hg, which is highly toxic than Cu or Pb, completely inhibited cell division in *Chlorella* (Hannan and Patouillet 1972). In previous findings, Hg showed a detrimental effect on various microalgae, showing the decreased photosynthetic pigments as the characteristic of Hg-exposed microalgae (Rai et al. 1981b). Based on previous findings and the results obtained in this study (Tables 4, 5, 6, and 7), it

Table 3 Review on morpho-variations and aberrations in the metal exposed microalgae

Species	Metal	Effects	References
Thalassiosira pseudonana	Cu^{2+}	Morphological aberration	Sunda 1975
Chlorella vulgaris	Cd, Cu, Hg, Zn, Pb	Alterations in cell division	Rosko and Rachlin 1977
Skeletonema costatum	Cu^{2+}	Morphological aberration	Morel et al. 1978
Asterionella japonica	Cu, Zn	Reduction in cell division rate, increase in cell size (swollen appearance)	Fisher et al. 1981
Fragilaria capucina var. gracilis	Cd, Zn	Perturbations, frustule formation	Gold et al. 2003
Euglena gracilis	Cr	High number of vacuoles and thylakoid alteration	Rocchetta et al. 2006
Micrasterias denticulata	Al, Zn, Cu, Cd	Distinct cell shape aberration, vacuole formation, malformations in cell shape	Volland et al. 2011
Chara vulgaris, Pithophora oedogonia	$AgNO_3$	Alterations in cell wall, cell surface disruption, shrinkage and extensive surface irregularity reflective of wall rupture, and degradation	Dash et al. 2012
Micrasterias denticulata	Cr III, Cr VI	Increased vacuolization, condensed cytoplasm, and dark precipitations in the cell wall	Volland et al. 2012
Micrasterias denticulata	Cd	Unidirectional disintegration of dictyosomes, autophagy	Andosch et al. 2012
Chlorella vulgaris, Chlamydomonas sp.	Cu, Zn	Irregular and folded appearance of cells, cell shrinkage, and rupturing	Wan Maznah et al. 2012

is suggestive that Hg has higher potential to cause significant toxicity in *C. vulgaris* compared to Zn.

Under chronic exposure, combined toxicity tests with Zn and Hg illustrated the inhibitory effects that were similar to those of the single-exposure experiments with Zn and Hg (Fig. 2A; right panel). In diverse concentrations of combinations of Zn and Hg, gradual decrement of pigment content was shown in *C. vulgaris* cells during 48 h and 7 days, indicating that this is likely due to dose-dependent inhibitory effect of Zn and Hg. Combination of Zn and Hg provides a significantly higher impact ($P<0.05$) on microalgal cells than that of single-exposed effect. Due to the metal-induced effects of Zn and Hg in the combined test, the pigment contents were reduced to >90%, compared to control and the marginal difference between the single and combined one was ~4–23% ($P<0.05$), suggestive of combined metal effect toxicities (Tables 4, 5, 6, and 7).

The main indication of metal toxicity has been prevalently found with the reduction of chlorophyll contents, which is likely associated with oxidative stress. Indeed previous findings from *Euglena* (De Filippis et al. 1981), metal-exposed higher plants (Clijsters et al. 1999), and the lichen *Xanthoria parietina* in response to environmentally relevant concentrations of hexavalent chromium (di Toppi et al. 2004), all showed similar outcomes on the species due to metal-induced oxidative stress. Similar to the present study, except the metal concentration used, a concentration-dependent reduction of photosynthetic pigments in response to Zn and Hg was reported earlier in *C. vulgaris* (Rai et al. 1991). In the pearl millet *Pennisetum typhoideum*, chlorophyll synthesis was suppressed in response to Hg and Pb (Prasad and Prasad 1987). Diminution in chlorophyll pigments was reported in the microalgae *C. kessleri* and *Coelastrum sphaericum* and the delphacid planthopper *Stenocranus acutus* in response to high concentrations of copper (Schiariti et al. 2004). The green microalga *Chlamydomonas reinhardtii* showed chlorophyll pigment reduction in response to Cd and Cu (Prasad et al. 1998). The microalga *C. protothecoides* in response to various concentrations (30–300 μM) of the herbicide SANDOZ 9785(4-chloro-5-[dimethylamino]-2-phenyl-3[2H]pyridazinone) caused a reduction in the Chl-α/Chl-β ratio (Samuel and Bose 1987). Also, Cd and Pb reduced the Chl-α/Chl-β ratio in wheat seedlings (Öncel et al. 2000). Pigment reduction was reported in *C. vulgaris* in response to Cr (Rai et al. 2013). Overall, combined toxicities of metals indeed induced significant loss of chlorophyll contents, possibly by uncontrolled accumulation of metal ions within the cell (Shakya et al. 2007). In addition to the accumulation of heavy metals within a cell, reduction of chlorophyll contents could possibly be due to the inhibition of chlorophyll biosynthesis from heavy metal interference in magnesium in the porphyrin ring of the chlorophyll molecule (Kupper et al. 1998; Kupper et al. 2002). Moreover, loss of chlorophyll pigments is one of the bio-indication for heavy-metal induced injury in plant cells (Muradoglu et al. 2015), suggesting that single and combined toxicity of metals (Zn and Hg) could cause detrimental effect in *C. vulgaris*. In addition, acute exposure (i.e., 96 h) of Zn and Cu decreased pigment content (Kebeish et al. 2014; Kumar et al. 2016; Zeraatkar et al. 2016) and photosynthetic rates in *C. vulgaris* (Saavedra et al. 2018).

C. vulgaris cultures in response to different concentrations of Zn demonstrated a concentration-dependent reduction in protein content. At 2.5% concentration, protein content was

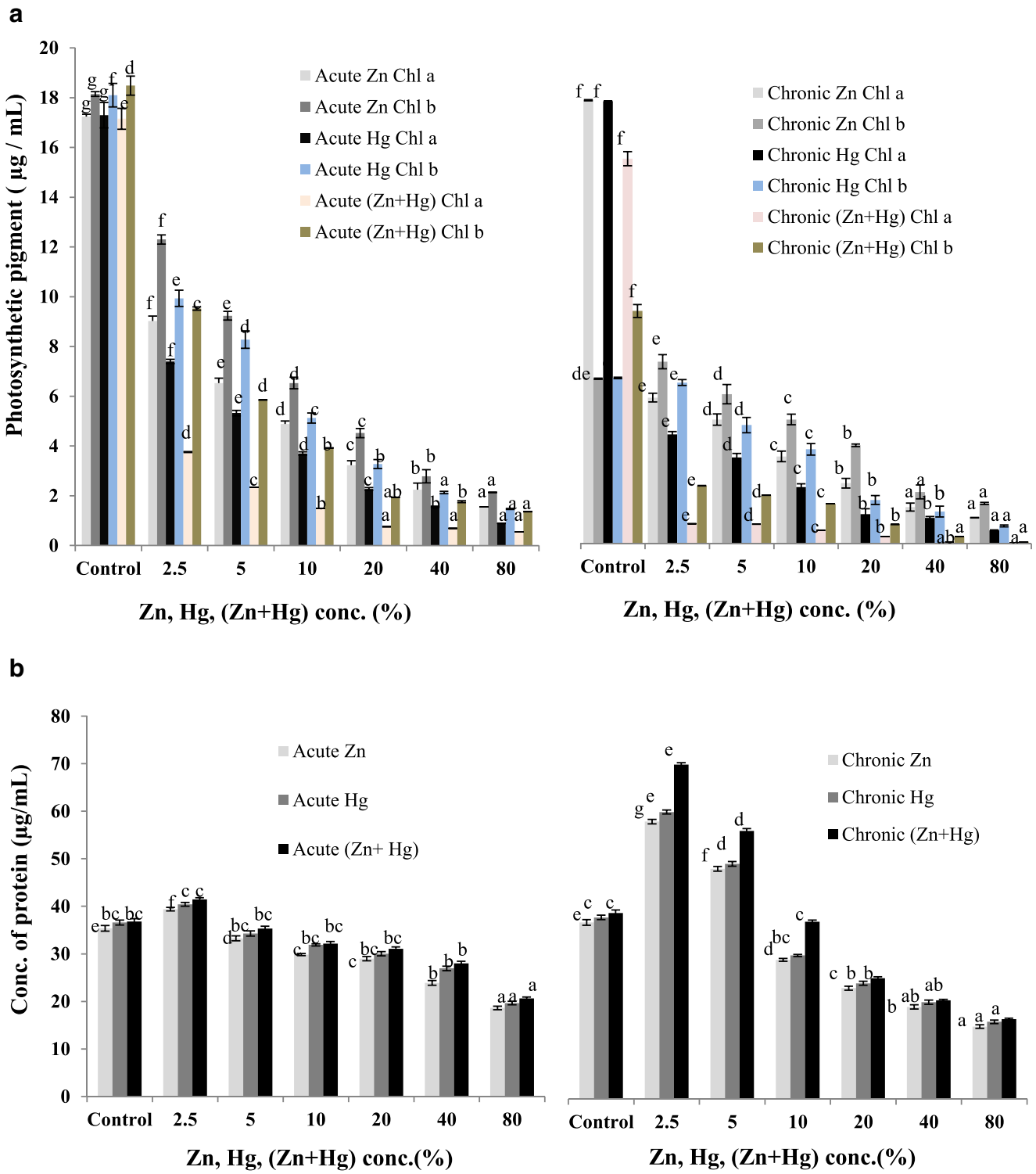


Fig. 2 Effect of Zn, Hg, and Zn+Hg from 48-h and 7-day experiments on photosynthetic pigment production (A) and protein content (B) in *Chlorella vulgaris* cells. All the values are mean of triplicates ± SD. Different letters represent significant differences ($P < 0.05$) in response to different concentrations after Tukey's post hoc analysis

high, supporting with similar findings (Mishra et al. 2006), where the rising of protein content at lower concentration is likely due to the increase of stress proteins including antioxidant enzymes to maximize the defense against toxicity or could be the indication of the maximum defense threshold. Total amino acid content was increased at less concentration of Zn but was decreased at higher Zn concentration (Omar 2002). The reduction

Table 4 Effect of Zn, Hg, and Zn+Hg on *Chlorella vulgaris* after 48h post-exposure

Metals	Concentration		Toxicity endpoints						
	(%)	(mg/L)	Chl-a (µg/mL)	Chl-b (µg/mL)	Carotenoid (µg/mL)	Protein (µg/mL)	ROS (AU)	CAT (U/mg protein)	SOD (U/mg protein)
Zn	0	0	17.2±0.09	18.1±0.07	0.305±0.17	36.3±0.58	531.15±3.5	0.0314±0.00	2.45±0.01
	2.5	4.2	9.02 ±0.19	12.3±0.18	0	39±0.37	3203±4.7	0.084±0.00	5.6±0.02
	5	8.4	6.53±0.19	9.23±0.17	0	33±0.51		0.09±0.00	4.2±0.01
	10	16.7	4.88±0.12	6.52±0.23	0	29±0.22		0.103±0.01	3.4±0.01
	20	33.6	3.22±0.18	4.52±0.18	0	28±0.43	4693±3.9	0.15±0.00	2.04±0.01
	40	66.9	2.24±0.25	2.78±0.26	0	23±0.47		0.22±0.00	1.28±0.02
	80	133.8	1.56±0.005	2.13±0.01	0	18±0.37	6003±2.6	0.32±0.01	0.57±0.007
Hg	0	0	17.29±0.51	18.12±0.47	0.46±0.04	36.5±0.51	532.49±4.9	0.031±0.001	2.62±0.01
	2.5	2.6	7.4±0.08	9.9±0.32	0	40±0.37	3745±4.2	0.093±0.003	6.21±0.01
	5	5.2	5.32±0.1	8.27±0.35	0	34±0.52		0.104±0.002	5.3±0.01
	10	10.4	3.7±0.06	5.12±0.20	0	31±0.22		0.11±0.007	4.5±0.01
	20	20.8	2.27±0.05	3.27±0.18	0	30±0.44	5004±4.15	0.16±0.004	3.05±0.02
	40	41.7	1.56±0.02	2.13±0.04	0	26±0.47		0.24±0.007	2±0.01
	80	83.4	0.887±0.04	1.47±0.01	0	19±0.36	6445±4.2	0.36±0.008	0.8±0.001
Zn+ Hg	0	0	17.15±0.42	18.48±0.38	0.49±0.21	36.8±0.58	545.48 ±3.02	0.0313±0.00	2.83±0.01
	2.5	6.8	3.76±0.01	9.52±0.06	0	41±0.37	4419±1.01	0.1203±0.003	7.08±0.01
	5	13.6	2.35±0.002	5.85±0.003	0	35±0.52		0.124±0.002	6.02±0.01
	10	27.1	1.48±0.009	3.92±0.005	0	32±0.22		0.13±0.008	5.2±0.01
	20	54.4	0.75±0.002	1.93±0.005	0	31±0.39	5649±1.4	0.19±0.005	4±0.005
	40	108.6	0.688±0.01	1.76±0.03	0	28±0.45		0.26±0.009	3±0.01
	80	217.2	0.542±0.02	1.36±0.006	0	20±0.36	7112±1.5	0.39±0.009	1±0.00

of protein content was due to increased proteolytic activity.

Significant dose-dependent reduction ($P<0.05$) in the protein content was observed in both acute and chronic studies. Interestingly, in all concentration, the protein content in the combined metal exposed cells was higher than the single-dose Zn-exposed cells, suggesting an elevated level of stress protein accumulation in the algal cells. This pattern was observed in both acute and chronic toxicity studies (Fig. 2B and Tables 4, 5, 6, and 7).

C. vulgaris cells in response to Hg for 48 h and 7 days showed a similar trend as shown in Zn-exposed microalgal cells. Significant enhancement ($P<0.05$) of protein content at a lower concentration of Hg indicates the generation of stress proteins through which they eradicate their stress but requires further studies to confirm it.

In combined toxicity tests, *C. vulgaris* cultures in response to different concentrations of Zn and Hg showed similar changes, but at a higher level, to those of single-exposed cultures with Zn and Hg single treatment. Due to the combined effect of Zn and Hg, the metal-induced effects on *C. vulgaris* cells were higher than the individual exposed effect.

A percentage reduction of protein content in Zn during acute exposure at 20% concentration compared to control was found to be 20.2 ± 2.6, and in Hg, it was around 17 ± 1.1 while in the combined test (Zn+Hg), the value was significantly reduced to 15 ± 1.1 (Table 6) which is highly significant $P<0.01$ and $P<0.05$, respectively, compared to that of the individual tests. In chronic exposure, a significantly higher percentage reduction in protein content was observed for both individual and combined tests in a concentration-dependent manner (Table 7).

Induction of protein content at 2.5% concentration was further supported by earlier findings such as an increase of protein content at lower doses (Osman et al. 2004) and it would be one of the mechanisms either eliminating toxic effects or increasing the cellular respiration leading to utilization of carbohydrate in favor of protein accumulation. In microalgae, the toxicity of metals leads the binding to sulfhydryl groups in proteins or the disruption of an essential element and/or interruption of protein structure (Tripathi and Gaur 2006). Elevation in oxidative stress-induced protein degradation demonstrates a correlation between protein reduction and proteolytic activity in response to oxidative stress (Romero-Puertas et al. 2002). Indeed, in agreement to other

Table 5 Effect of Zn, Hg, and Zn+Hg on *Chlorella vulgaris* after 7th day post-exposure

Metals	Concentration		Toxicity endpoints						
	(%)	(mg/L)	Chl-a (μg/mL)	Chl-b (μg/mL)	Carotenoid (μg/mL)	Protein (μg/mL)	ROS (AU)	CAT (U/mg protein)	SOD (U/ mg protein)
Zn	0	0	15.1±0.00	5.6±0.01	2.83±0.105	38.02±0.59	532.14±2.99	0.02±0.001	2.46±0.008
	2.5	4.2	5.008±0.14	5.24±0.24	0	59±0.49	4004±4.4	0.04±0.007	3.8±0.02
	5	8.4	4.25±0.19	5.12±0.33	0	49±0.52		0.06±0.001	1.6±0.01
	10	16.7	2.98±0.18	4.25±0.18	0	29±0.31		0.08±0.003	1.3±0.02
	20	33.6	2.07±0.16	3.37±0.04	0	23±0.43	5124±3.2	0.2±0.004	0.8±0.01
	40	66.9	1.25±0.13	1.7±0.24	0	19±0.45		0.32±0.006	0.53±0.003
	80	133.8	0.88±0.005	1.37±0.03	0	15±0.37	7135±1.5	0.34±0.006	0.31±0.004
Hg	0	0	15.2±0.01	5.68±0.02	3.98±0.01	39.1±0.49	533.18±5.9	0.021±0.002	2.54±0.008
	2.5	2.6	3.75±0.09	5.52±0.1	0	61±0.43	4294±4.5	0.047±0.003	4.1±0.02
	5	5.2	2.95±0.13	4.06±0.26	0	51±0.49		0.07±0.002	2.8±0.02
	10	10.4	1.93±0.1	3.23±0.19	0	30±0.23		0.092±0.005	1.8±0.031
	20	20.8	1.01±0.17	1.49±0.15	0	24±0.42	5424±3.9	0.22±0.006	1.3±0.02
	40	41.7	0.87±0.05	1.09±0.18	0	20±0.45		0.34±0.008	0.75±0.002
	80	83.4	0.44±0.01	0.603±0.03	0	16±0.38	7533±3.2	0.36±0.008	0.54±0.002
Zn+ Hg	0	0	13.2±0.24	7.98±0.22	3.99±0.01	40.06±0.62	541.5±4.7	0.022±0.00	2.58±0.00
	2.5	6.8	0.67±0.007	1.98±0.005	0	72±0.38	4898±2.6	0.056±0.001	5±0.01
	5	13.6	0.66±0.004	1.65±0.005	0	58±0.51		0.092±0.004	3.2±0.008
	10	27.1	0.45±0.001	1.36±0.001	0	38±0.37		0.1±0.008	2.2±0.01
	20	54.4	0.23±0.001	0.65±0.003	0	26±0.31	5964±4.04	0.25±0.009	1.7±0.02
	40	108.6	0.04±0.004	0.23±0.001	0	21±0.24		0.37±0.009	1.07±0.01
	80	217.2	0.021±0.00	0.059±0.00	0	17±0.2	8125±4.5	0.38±0.009	0.67±0.002

previously reported studies, the results obtained in *C. vulgaris* in response to Zn and Hg, under both single and combined as well as acute and chronic exposure, have demonstrated significant increase at the lowest concentration tested, most likely attributing to a wide array of metabolic processes including expression of stress-related proteins for defense against environmental stressors (Xu et al.2008). Taken together, increase in the protein content under a low concentration of both single and combined effect of metals (i.e., Zn and Hg), possibly suggests that *C. vulgaris* can cope with the metal-induced stress only up to certain concentrations; however, the threshold concentration would most likely be in a species-specific manner.

To analyze whether metals induce oxidative stress, single and combined toxicity tests in *C. vulgaris* cultures in response to 2.5, 20, and 80% concentrations of Zn and Hg at 48 h and 7

Table 6 Percentage increase (+)/decrease(−) from control of Zn, Hg, and Zn+Hg on *Chlorella vulgaris* after 48h post-exposure

Metals	Concentration		Percentage increase /decrease compared to control					
	(%)	(mg/L)	Chl-a (μg/mL)	Chl-b (μg/mL)	Protein (μg/mL)	ROS (AU)	CAT (U/mg protein)	SOD (U/mg protein)
Zn	2.5	4.2	−47.64 ± 1.1	−32.22 ± 1.1	+8.36 ± 3.4	+503.2 ± 4.8	+108.65 ± 0.43	+129.2 ± 0.7
	20	33.6	−81.27 ± 1.1	−75.09 ± 1.1	−20.29 ± 2.6	+783.71 ± 6.2	+386.5 ± 1.2	−16.71 ± 0.4
	80	133.8	−90.93 ± 0.09	−88.25 ± 0.003	−48.75 ± 0.24	+1030.2 ± 7.5	+940.95 ± 2.9	−76.58 ± 0.7
Hg	2.5	2.6	−57.20 ± 1.5	−45.12 ± 2.9	+10.48 ± 0.5	+603.45 ± 6.4	+207.5 ± 1.4	+136.8 ± 0.6
	20	20.8	−86.84 ± 0.3	−81.9 ± 1.07	−17.95 ± 1.1	+839.87 ± 7.1	+414.9 ± 1.2	−6.5 ± 0.14
	80	83.4	−94.87 ± 0.3	−91.86 ± 0.19	−46.29 ± 0.06	+1110.5 ± 6.3	+1056 ± 2	−69.13 ± 2
Zn+ Hg	2.5	6.8	−78.06 ± 0.59	−48.47 ± 1.3	+12.53 ± 1.3	+710.4 ± 5.1	+300.6 ± 1.4	+149.8 ± 0.9
	20	54.4	−95.6 ± 0.11	−89.52 ± 0.2	−15.6 ± 1.8	+935.68 ± 5.2	+534.43 ± 2	−3.7 ± 0.15
	80	217.2	−96.83 ± 0.12	−92.62 ± 0.2	−43.9 ± 0.2	+1203.9 ± 7.1	+1212.18 ± 1.4	−64.59 ± 0.6

Table 7 Percentage increase (+)/decrease (−) from control of Zn, Hg, and Zn+Hg on *Chlorella vulgaris* after 7th day post-exposure

Metals	Concentration		Percentage increase/decrease compared to control					
	(%)	(mg/L)	Chl-a (μg/mL)	Chl-b (μg/mL)	Protein (μg/mL)	ROS (AU)	CAT (U/mg protein)	SOD (U/mg protein)
Zn	2.5	4.2	−67.09 ± 1	−6.69 ± 4.7	+57.15 ± 3.6	+652.59 ± 3.8	+83.15 ± 0.28	+58.19 ± 0.6
	20	33.6	−86.39 ± 1	−40.37 ± 0.8	−37.22 ± 0.9	+863.01 ± 4.9	+826.95 ± 1.1	−63.89 ± 0.6
	80	133.8	−94.17 ± 0.03	−75.67 ± 0.5	−59.01 ± 0.97	+1240.9 ± 7.1	+1446.4 ± 2.7	−87.21 ± 0.2
Hg	2.5	2.6	−75.33 ± 0.6	−2.77 ± 1.8	+58.07 ± 2	+705.51 ± 6.2	+114.7 ± 1.4	+63.97 ± 0.6
	20	20.8	−93.34 ± 1.1	−73.69 ± 2.7	−36.31 ± 1.68	+917.5 ± 6.3	+910.51 ± 1.4	−48.84 ± 0.28
	80	83.4	−97.04 ± 0.1	−89.37 ± 0.5	−57.53 ± 0.58	+1312.9 ± 6.4	+1553.8 ± 1.5	−78.73 ± 0.21
Zn+Hg	2.5	6.8	−94.89 ± 0.04	−75.15 ± 0.64	+79.84 ± 2.8	+804.54 ± 5.1	+151.3 ± 0.9	+79.28 ± 0.5
	20	54.4	−98.18 ± 0.02	−91.76 ± 0.2	−35.09 ± 1.7	+1001.5 ± 5.2	+1016.8 ± 1.4	−35.92 ± 0.8
	80	217.2	−99.83 ± 0.004	−99.25 ± 0.02	−56.12 ± 0.1	+1400.5 ± 6.4	+1605.2 ± 1.7	−73.83 ± 0.9

days were performed. In this investigation, ROS generation was found to be increasing significantly ($P<0.05$) in both metal exposures, in both single and combined effects (Figs. 3 and 4; Tables 4, 5, 6, and 7) in concentration-dependent manner. In the individual toxicity test with Zn, during acute exposure at 2.5% concentration, 5-fold increase in ROS production was observed compared to the control, and in Hg, ~ 6-fold increase was observed; whereas in the combined test (Zn+Hg), 7-fold difference was observed (Table 6). All the values were statistically significant at $P<0.01$ and $P<0.05$. While compared to the acute test significantly higher percentage increase in ROS production was noticed in the chronic test (Table 7). ROS generation by the various concentrations of metal compounds suggests induction of oxidative stress in *C. vulgaris*, which further verifies the toxic nature of the metals in *Chlorella.*

Previously, ROS generation was observed in various algae in response to metals and xenobiotics. For example, exposure of CuO on the green microalga *C. reinhardtii* induced oxidative stress (Melegari et al. 2013) and exposure to polychlorinated biphenyl (PCB) in the dinoflagellate *Lingulodinium polyedrum* (da Leitao et al. 2003) led to significant induction of oxidative stress. Also, ROS generation was noticed in the dinoflagellate *Prorocentrum minimum* in response to $CuCl_2$ and PCB (Ponmani et al. 2015), while the accumulation of the intracellular ROS was reported in the microalgae *C. vulgaris* and *P. subcapitata*in response to Cu (Knauert and Knauer 2008). In agreement with our results, the similar tendency in ROS generation has been reported in the microalgal cells of *Anabaena* sp. in response to Zn^{2+}, reaching the maximum peak at a concentration higher than 0.7 mg/L Zn^{2+}. In fact, heavy metals promote oxidative damage in two ways, by increasing the cellular concentrations of ROS (Winterbourn 1982) and/or by reducing the cellular antioxidant potential (Sies 1999). The adverse effects of ROS accumulation on cellular levels are highly associated with protein oxidation, lipids, and nucleic acids, which ultimately lead to alterations in cell structure and mutagenesis (Halliwell and Gutteridge 1999; Pinto et al. 2003). Furthermore, negative effects of metal and/or xenobiotic-induced ROS generation and consequent oxidative stress pose a higher threat in photosynthetic organisms compared to animals, as the common biological source of oxygen is acquired through intense electron flux within the microenvironment, which is already filled with elevated oxygen levels and metal ion concentrations, making photosynthetic organisms highly susceptible to oxidative stress (Pinto et al. 2003). Taken together, the single and combined effect of metal (Zn and Hg) induced a significant increase in ROS levels in *C. vulgaris*, which may be closely associated with concentration-dependent cell morphological deformity; however, further studies on morphological alteration in association to intracellular ROS levels are required to fully elucidate this phenomenon due to the presence of ambiguity in mechanisms of heavy metal toxicity.

Microalgae have diverse antioxidant enzymes to mitigate the increased generation of ROS caused by metals. For ROS scavenging, antioxidant enzymes perform a vital role (Kang et al. 1999; Sharma et al. 2012). Antioxidant enzymes are well-known biomarkers of protection in response to oxidative stress and generation of antioxidant enzymes is regarded to be one of the ways to avoid or overcome the metal-induced cell destruction (Wu and Lee 2008). CAT, SOD, glutathione reductase (GR), and glutathione peroxidase (GPx) are meant to safeguard cells and tissues from oxidative damages and to counteract the toxicity of ROS (Ensibi et al. 2013). CAT is the key enzyme for the conversion of H_2O_2 to H_2O and O_2. CAT involved in the mechanism to shield the cells against the damage caused by ROS to cellular components including nucleic acids, lipids, and proteins (Imlay 2002).

The initial key enzyme for ROS scavenging is considered to be SOD in plants and other organisms, playing an important role in active O_2 metabolism and altering superoxide radicals (O_2^-) to H_2O_2 at a rapid rate. SOD, among other antioxidant enzymes, detoxifies superoxide anions (Beyer et al. 1991;

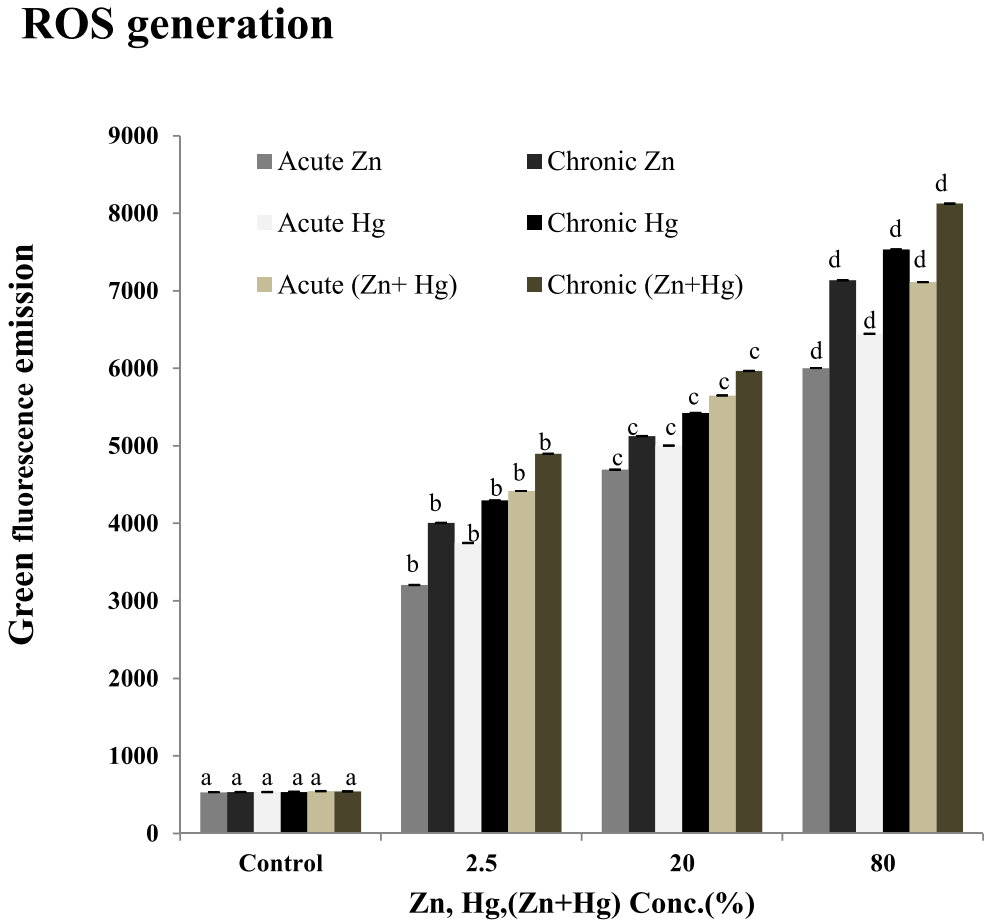
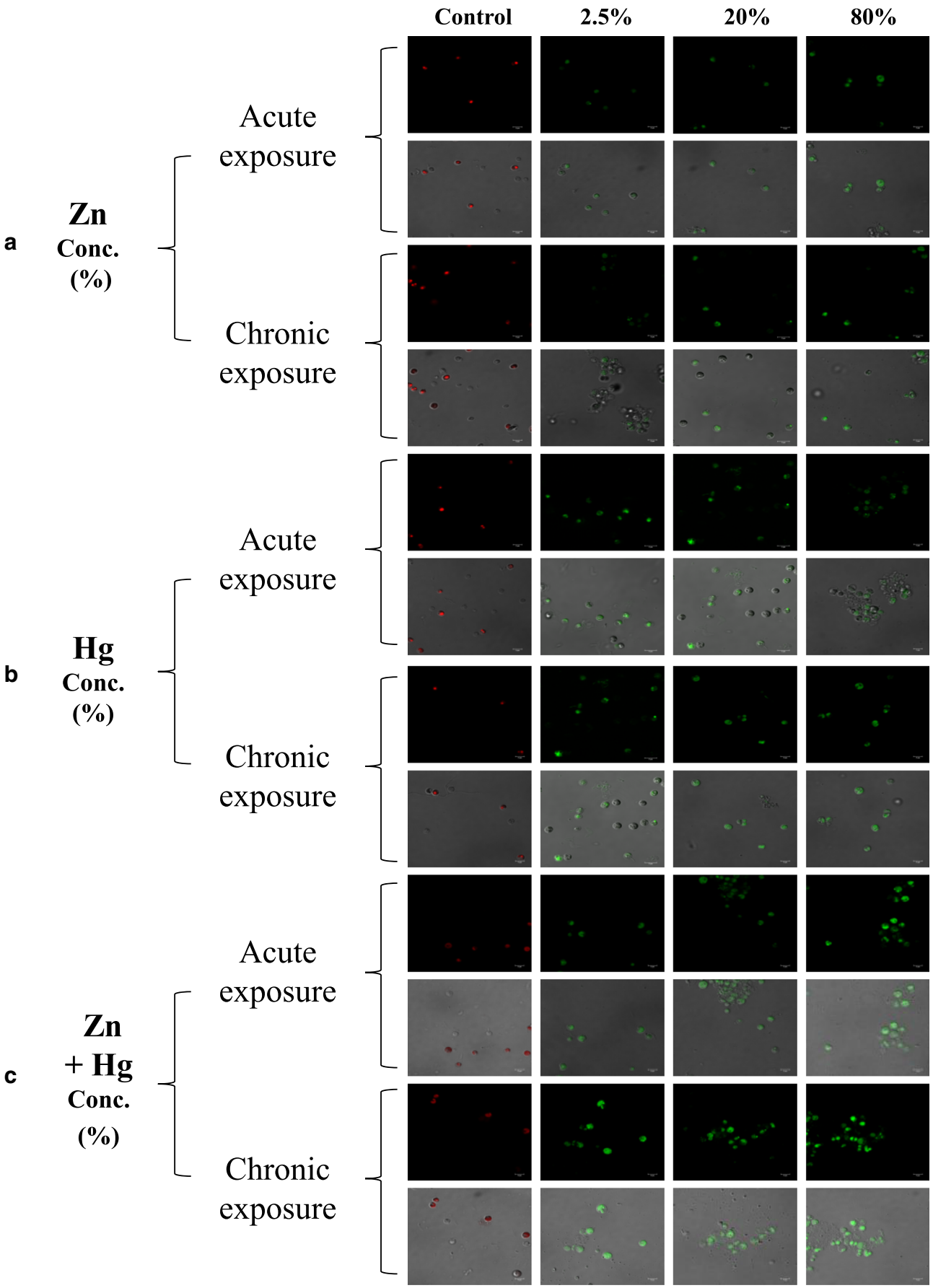


Fig. 3 Generation of reactive oxygen species in response to Zn, Hg, and combination (Zn+Hg) during acute and chronic exposure. Different letters represent significant differences ($P < 0.05$) in response to different concentrations after Tukey's post hoc analysis. Data are the mean ± SD of triplicates

Mellado et al. 2012) and considered to be a renowned biomarker of defense in response to oxidative stress (Assche and Clijsters 1990; Chongpraditnun et al. 1992). In this study, both single and combined exposure to Zn and Hg in *C. vulgaris* resulted in significant elevation at initial concentration, followed by gradual reduction under acute and chronic exposures (Fig. 5A and Tables 4, 5, 6, and 7). A bell-shaped concentration-response pattern was noticed in the SOD activity. In the individual toxicity tests of Zn and Hg, the percentage reduction in SOD activity during acute exposure at 20% concentration was found to be 16.7± 0.4 and 6.5 ± 0.1, while in the combined test, the value was significantly reduced to 3.7 ± 0.1 due to the combined effect of Zn and Hg (Table 6). All the values were statistically significant at $P<0.01$ and $P<0.05$. A concentration-dependent significant percentage reduction was noticed in both acute and chronic tests with Zn, Hg, and Zn+ Hg (Tables 6 and 7).

In plant cells, SOD activity increased as a result of different kind of chemical compounds and physical stresses (Mittler 2002). Induction of superoxide anion content was also shown in metals-exposed macroalgae (Çelekli et al. 2016; Wu et al. 2014). Increased SOD activity was observed in the cyanobacterium *Spirulina platensis* in response to Zn, Pb, Cu over a concentration gradient of 0.05–0.20 mg/L (Choudhary et al. 2007). Also, the combined effects of Cu and Cd increased the SOD activity in *C. vulgaris* (Qian et al. 2011) and the second exposure effect of Cu^{2+} and CTC showed increased SOD activity on the microalgae *C. pyrenoidosa* and *M. aeruginosa* (Lu et al. 2015). Furthermore, single and combined effects of Cd and 4-n-NP on the microalga *C. sorokiniana* for 48 h, 72 h, and 96 h showed induction of SOD activity and reduced during the exposure time increased (Wang et al. 2018). Overall, initial induction of SOD activity under acute and chronic exposures to single and combined treatment of Zn and Hg in *C. vulgaris* may suggest that both mitochondrial and chloroplast electron transport systems may be affected by heavy metal-induced oxidative stress (Pinto et al. 2003). Also, an initial increase in SOD activity relative

Fig. 4 Fluorescent confocal microscopy images demonstrate Zn-, Hg-, and Zn+Hg-induced reactive oxygen species (ROS) generation in *Chlorella vulgaris*. (A) Non-treated cells (control), cells treated with 2.5, 20, and 80% concentrations of Zn for 48 h and 7 days; (B) Non-treated cells (control), cells exposed to different concentrations of Hg (2.5, 20, and 80%) incubated for 48 h and 7 days; Non-treated cells (control), cells exposed to different concentrations of Zn+Hg (2.5, 20, and 80%) incubated for 48 h and 7 days. Red fluorescence is the auto-fluorescence of *C. vulgaris* cells, green fluorescence originated during the reaction of ROS with DHR123. (Scale bar = 5 μm) ▶


Control
2.5%
20%
80%
a
Zn
Conc.
(%)
Acute
exposure
Chronic
exposure
b
Hg
Conc.
(%)
Acute
exposure
Chronic
exposure
c
Zn
+ Hg
Conc.
(%)
Acute
exposure
Chronic
exposure

to a reduction over time may suggest disruption of oxidative balance which possibly depends on the severity of the stress and metal properties.

When *C. vulgaris* cultures were exposed to various concentrations of Zn and Hg under acute and chronic periods (48 h and 7 days, respectively), a significant increase ($P<0.05$) in CAT activity was observed as shown in Fig. 5B and Tables 4, 5, 6, and 7. In the single toxicity tests, during acute exposure, the percentage increase in CAT activity compared to control at 80% concentration in Zn and Hg were 941 ± 2 (9-fold) and 1056 ± 2 (10-fold), respectively; whereas in the combined test (Zn+Hg), increase in CAT activity was significantly higher ($P<0.01$ and $P<0.05$) (1212 ± 1.4 [12-fold]) compared to the single exposure tests (Table 6). While compared to the acute exposure, percentage increase in CAT activity during chronic exposure was high in both individual and combined test (Table 7).

In the marine microalga *Pavlova viridis*, the antioxidant enzymatic activities were increased at the highest concentrations in response to Zn and Cu (Li et al. 2006). Similar to that of the findings in the toxicity test of *C. vulgaris* cells with Zn, significant increasing trend ($P<0.05$) in CAT activity was

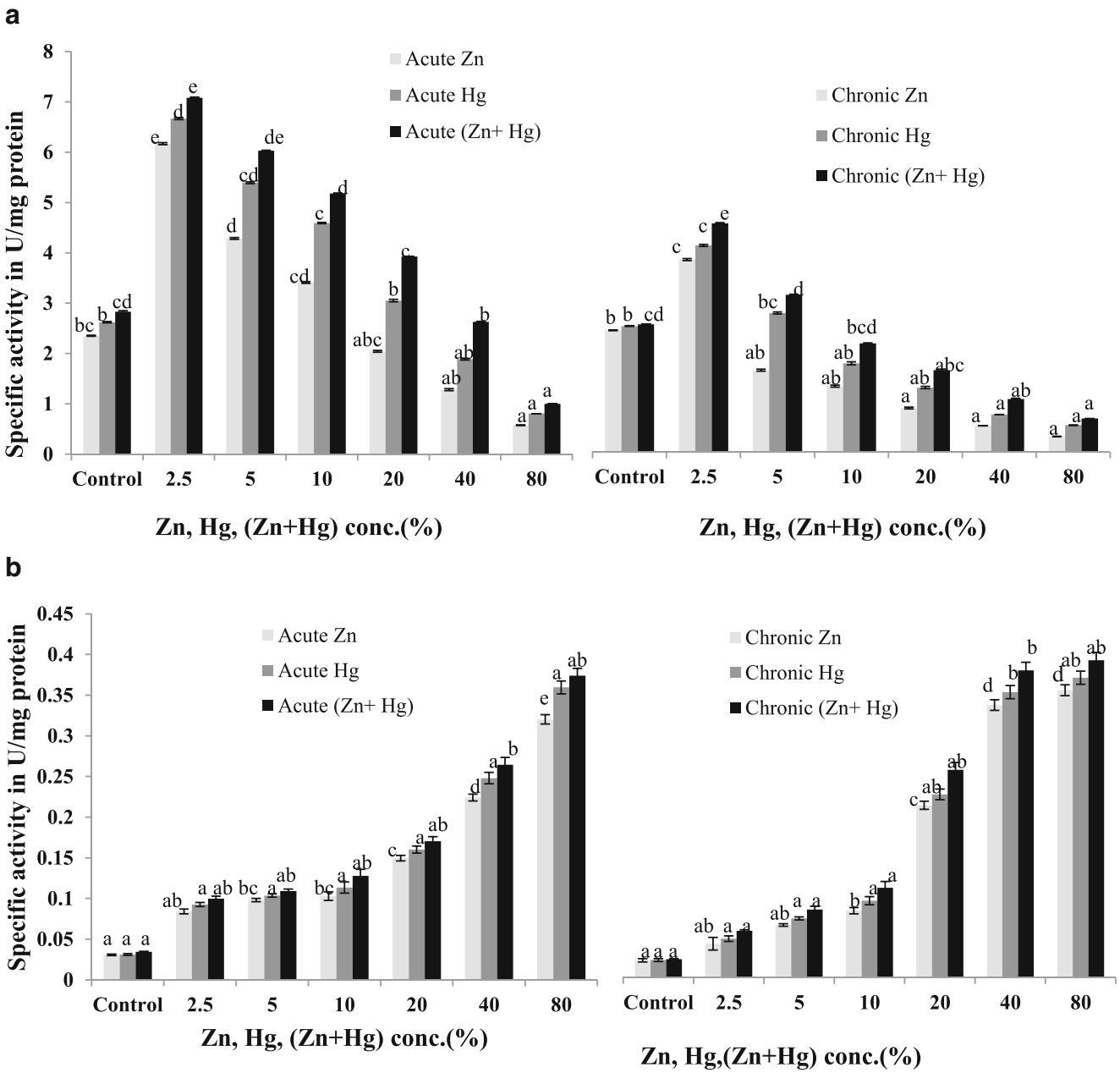


Fig. 5 Effects of diverse concentrations of Zn, Hg, and Zn+Hg for 48-h and 7-day exposure on superoxide dismutase activity (A) and catalase activity (B) in *Chlorella vulgaris* cells. Different letters represent significant differences ($P < 0.05$) in response to different concentrations after Tukey's post hoc analysis. Data are the mean ± SD of triplicates

observed for both individual toxicity and combined toxicity (Fig. 5B). A dose-dependent increase in the antioxidant activity was reported in *C. vulgaris* in response to Cu, Pb, and Cd (Bajguz 2010; Cheng et al. 2016). Antioxidant enzymes interact together to prevail over metal impacts in the marine microalgae *Acanthophora spicifera* and *Chaetomorpha antennina* and the seaweed *Ulva reticulate* (Babu et al. 2014). CAT demonstrates an essential function in the microalga *P. subcapitata* at greater toxicant concentrations (Soto et al. 2011). Activation of CAT activity occurred in the freshwater cyanobacterium *Anabaena doliolum* in response to Cu (Mallick and Rai 1999). Production of antioxidant enzymes such as CAT, GR, and GPx in *C. vulgaris* in response to Cd exposure illustrates that these antioxidant enzymes perform together to reduce the toxic effects of metals (Cheng et al. 2016). Indeed, increase in CAT activity is referred to be an adaptation method developed by plants (Reddy et al. 2005). Single toxicity tests of Cu and Cd on *C. vulgaris* showed a slight increase in CAT activity (Qian et al. 2011). Besides, single and combined effects of Cd and 4-n-NP on the microalga *C. sorokiniana* demonstrated initial stimulation in CAT activity which then decreased over time (Wang et al. 2018). In this study, however, metal-induced (Zn and Hg) elevation in ROS was not successfully scavenged by the antioxidant CAT, despite significant increase in CAT activity in *C. vulgaris*, which may suggest incapability of CAT in the clearance of Zn and Hg-induced ROS, possibly due to concentration-specific modulations of SOD activities, which may not have fully catalyzed superoxide into oxygen in response to the two metals

In summary, Zn and Hg established a significant impact on the *C. vulgaris* cells. The observation from this study clearly documents a higher level of dose-dependent toxic effect of Hg and Zn in combination than in single, which suggests synergistic effects. These effects were observed in both acute and chronic toxicity studies through significant flection in photosynthetic pigment content, total protein content, ROS production, antioxidant enzymes (SOD and CAT) along with morphological aberrations. However, elaborative study with large data sets including the genomics and proteomics data are required to find out the synergistic effects of metals at the molecular level in *C. vulgaris*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-12950-6.

Author contribution Vayampully Ajitha and Chandrasekharan Parvathi Sreevidya carried out the experiments and statistical analysis. Vayampully Ajitha, Manomi Sarasan, and Jun Chul Park prepared the main manuscript including all figures and tables. Ambat Mohandas, Isaac Sarojini Bright Singh, Jayesh Puthumana, and Jae-Seong Lee supervised all the experiments and finally approved the manuscript.

Funding This research was supported by the University Grants Commission (UGC), Government of India under Basic Scientific Research (BSR) Programme (SES/53A/2014-15/04) and CUSAT-SMNRI Programme for New Research Initiatives (UGC 1/SPG/SMNRI/2018-19). The first author thanks UGC for fellowships.

Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval All investigations were carried out following the guidelines of the Institutional Biosafety Committee (IBSC) at NCAAH, CUSAT, Kerala in India.

Consent to participate Not applicable.

Consent to publish All authors approved the manuscript to be published and all the work is appropriately investigated.

Competing interests The authors declare no competing interests.

References

Ajitha V, Sreevidya CP, Kim JH, Bright Singh IS, Mohandas A, Lee J-S, Puthumana J (2019) Effect of metals of treated electroplating industrial effluents on antioxidant defense system in the microalga *Chlorella vulgaris*. Aquat Toxicol 217:105317

Andosch A, Affenzeller MJ, Lütz C, Lütz-Meindl U (2012) A freshwater green alga under chromium stress: ameliorating calcium effects on ultrastructure and photosynthesis in the unicellular model *Micrasterias*. J Plant Physiol 169:1489–1500

Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:195–206

Azevedo-Pereira HMVS, Soares AMVM (2010) Effects of mercury on growth, emergence, and behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). Arch Environ Contam Toxicol 59:216–224

Babu MY, Palanikumar L, Nagarani N, Devi VJ, Kumar SR, Ramakritinan CM, Kumaraguru AK (2014) Cadmium and copper toxicity in three marine macroalgae: evaluation of the biochemical responses and DNA damage. Environ Sci Pollut Res 21:9604–9616

Bajguz A (2010) An enhancing effect of exogenous brassinolide on the growth and antioxidant activity in *Chlorella vulgaris* cultures under heavy metals stress. Environ Exp Bot 68:175–179

Bajguz A (2000) Blockade of heavy metals accumulation in *Chlorella vulgaris* cells by 24-epibrassinolide. Plant Physiol Biochem 38:797–801

Bajguz A, Piotrowska-Niczyporuk A (2013) Synergistic effect of auxins and brassinosteroids on the growth and regulation of metabolite content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Physiol Biochem 71:290–297

Barbarino E, Lourenço SO (2005) An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J Appl Phycol 17:447–460

Beyer W, Imlay J, Fridovich I (1991) Superoxide dismutases. pp. 221–253.

Bidar G, Garçon G, Pruvot C, Dewaele D, Cazier F, Douay F, Shirali P (2007) Behavior of *Trifolium repens* and *Lolium perenne* growing in a heavy metal contaminated field: Plant metal concentration and phytotoxicity. Environ Pollut 147:546–553

Bischoff HW, Bold HC (1963) Phycological studies IV : some soil algae from enchanted rock and related algal species. University of Texas, Austin

Boening DW (2000) Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40:1335–1351

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Branzini A, Gonzalez RS, Zubillaga M (2012) Absorption and translocation of copper, zinc and chromium by *Sesbania virgata*. J Environ Manag 102:50–54

Cargnelutti D, Tabaldi LA, Spanevello RM, de Oliveira JG, Battisti V, Redin M, Linares CEB, Dressler VL, de Moraes Flores ÉM, Nicoloso FT, Morsch VM, Schetinger MRC (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65:999–1006

Çelekli A, Gültekin E, Bozkurt H (2016) Morphological and biochemical responses of *Spirogyra setiformis* exposed to cadmium. CLEAN 44: 256–262

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. Methods Enzymol 2:764–775

Chen X, Zhu X, Li R, Yao H, Lu Z, Yang X (2012) Photosynthetic toxicity and oxidative damage induced by nano-Fe3O4 on *Chlorella vulgaris* in aquatic environment. Open J Ecol 2:21–28

Cheng J, Qiu H, Chang Z, Jiang Z, Yin W (2016) The effect of cadmium on the growth and antioxidant response for freshwater algae *Chlorella vulgaris*. Springerplus 5:1290

Chia MA, Lombardi AT, da Graça Gama Melão M, Parrish CC (2015) Combined nitrogen limitation and cadmium stress stimulate total carbohydrates, lipids, protein and amino acid accumulation in *Chlorella vulgaris* (Trebouxiophyceae). Aquat Toxicol 160:87–95

Chongpraditnun P, Mori S, Chino M (1992) Excess copper induces a cytosolic Cu, Zn-superoxide dismutase in soybean root. Plant Cell Physiol 33:239–244

Choudhary M, Jetley UK, Abash Khan M, Zutshi S, Fatma T (2007) Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. Ecotoxicol Environ Saf 66:204–209

Chouteau C, Dzyadevych S, Chovelon J-M, Durrieu C (2004) Development of novel conductometric biosensors based on immobilised whole cell *Chlorella vulgaris* microalgae. Biosens Bioelectron 19:1089–1096

Clijsters H, Cuypers A, Vangronsveld J (1999) Physiological responses to heavy metals in higher plants: defence against oxidative stress. Z Naturforsch C 54:730–734

Das K, Samanta L, Chainy GBN (2000) A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. Indian J Biochem Biophys 37:201–204

Dash A, Singh AP, Chaudhary BR, Singh SK, Dash D (2012) Effect of silver nanoparticles on growth of eukaryotic green algae. Nano-Micro Lett 4:158–165

Davies AG (1974) The growth kinetics of *Isochrysis galbana* in cultures containing sublethal concentrations of mercuric chloride. J Mar Biol Assoc UK 54:157–169

De Filippis LF, Hampp R, Ziegler H (1981) The effects of sublethal concentrations of zinc, cadmium and mercury on *Euglena*. Growth and pigments. Z Pflanzenphysiol 101:37–47

De Filippis LF, Pallaghy CK (1976) The effect of sub-lethal concentrations of mercury and zinc on *Chlorella*. Z Pflanzenphysiol 79:323–335

Dinesh Kumar S, Santhanam P, Ananth S, Shenbaga Devi A, Nandakumar R, Balaji Prasath B, Jeyanthi S, Jayalakshmi T, Ananthi P (2014) Effect of different dosages of zinc on the growth and biomass in five marine microalgae. Int J Fish Aquacult 6:1–8

Dirilgen N (2011) Mercury and lead: assessing the toxic effects on growth and metal accumulation by *Lemna minor*. Ecotoxicol Environ Saf 74:48–54

di Toppi LS, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41:105–130

Durrieu C, Guedri H, Fremion F, Volatier L (2011) Unicellular algae used as biosensors for chemical detection in Mediterranean lagoon and coastal waters. Res Microbiol 162:908–914

Ensibi C, Pérez-López M, Soler Rodríguez F, Míguez-Santiyán MP, Yahya MND, Hernández-Moreno D (2013) Effects of deltamethrin on biometric parameters and liver biomarkers in common carp (*Cyprinus carpio* L.). Environ Toxicol Pharmacol 36:384–391

Fisher NS, Jones GJ, Nelson DM (1981) Effects of copper and zinc on growth, morphology, and metabolism of Asterionella japonica (Cleve). J Exp Mar Biol Ecol 51:37–56

Foster PL (1982) Metal resistances of chlorophyta from rivers polluted by heavy metals. Freshw Biol 12:41–61

Franklin NM, Stauber JL, Lim RP, Petocz P (2002) Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environ Toxicol Chem 21:2412–2422

Ge F, Xu Y, Zhu R, Yu F, Zhu M, Wong M (2010) Joint action of binary mixtures of cetyltrimethyl ammonium chloride and aromatic hydrocarbons on *Chlorella vulgaris*. Ecotoxicol Environ Saf 73:1689–1695

Gipps JF, Biro P (1978) The use of *Chlorella vulgaris* in a simple demonstration of heavy metal toxicity. J Biol Educ 12:207–214

Godbold DL (1991) Mercury-induced root damage in spruce seedlings. Water Air Soil Pollut 56:823–831

Gold C, Feurtet-Mazel A, Coste M, Boudou A (2003) Impacts of Cd and Zn on the development of periphytic diatom communities in artificial streams located along a river pollution gradient. Arch Environ Contam Toxicol 44:189–197

Grantt E (2008) Handbook of phycological methods: developmental and cytological methods. Cambridge University Press.

Greenfield SS (1942) Inhibitory effects of inorganic compounds on photosynthesis in *Chlorella*. Am J Bot 29:121–131

Gupta M, Chandra P (1994) Lead accumulation and toxicity in *Vallisneria spiralis* (L.) and *Hvdrilla vertieillata* (l.f.). Royal J Environ Sci Health A 29:503–516

Halliwell B, Gutteridge JBC (1999) Free radicals in biology and medicine, 3rd edn. Oxford University Press, New York, p 936

Hannan PJ, Patouillet C (1972) Effect of mercury on algal growth rates. Biotechnol Bioeng 14:93–101

Harding JPC, Whitton BA (1976) Resistance to zinc of *Stigeoclonium tenue* in the field and the laboratory. Br Phycol J 11:417–426

Hutchinson T, Stokes P (1975) Heavy metal toxicity and algal bioassays, in: Water quality parameters, 19428-2959 edn. ASTM International, West Conshohocken, pp 320–324

Imlay JA (2002) How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. AdvMicrob Physiol 46:111–153

Israr M, Sahi S, Datta R, Sarkar D (2006) Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. Chemosphere 65:591–598

Kamp-Nielsen L (1971) The effect of deleterious concentrations of mercury on the photosynthesis and growth of *Chlorella pyrenoidosa*. Physiol Plant 24:556–561

Kang K-S, Lim C-J, Han T-J, Kim J-C, Jin C-D (1999) Changes in the isozyme composition of antioxidant enzymes in response to aminotriazole in leaves of *Arabidopsis thaliana*. J Plant Biol 42: 187–193

Kebeish R, El-Ayouty Y, Hussein A (2014) Effect of copper on growth, bioactive metabolites, antioxidant enzymes and photosynthesis-related gene transcription in *Chlorella vulgaris*. World J Biol Biol Sci 2:34–43

Knauert S, Knauer K (2008) The role of reactive oxygen species in copper toxicity to two fresh green algae. J Phycol 44:311–319

Kumar D, Pandey LK, Gaur JP (2016) Metal sorption by algal biomass: from batch to continuous system. Algal Res 18:95–109

Kumar KS, Dahms H-U, Won E-J, Lee J-S, Shin K-H (2015) Microalgae - a promising tool for heavy metal remediation. Ecotoxicol Environ Saf 113:329–352

Kupper H, Kupper F, Spiller M (1998) *In situ* detection of heavy metal substituted chlorophylls in water plants. Photosynth Res 58:123–133

Kupper H, Setlik I, Spiller M, Kupper FC, Prasil O (2002) Heavy metal-induced inhibition of photosynthesis: targets of in vivo heavy metal chlorophyll formation. J Phycol 38:429–441

da Leitao MAS, Cardozo KHM, Pinto E, Colepicolo P (2003) PCB-induced oxidative stress in the unicellular marine dinoflagellate *Lingulodinium polyedrum*. Arch Environ Contam Toxicol 45:59–65

Li M, Hu C, Zhu Q, Chen L, Kong Z, Liu Z (2006) Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). Chemosphere 62:565–572

Lichtenthaler HK (1987) [34] Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. pp. 350–382.

Liu H, Li L, Yin C, Shan B (2008) Fraction distribution and risk assessment of heavy metals in sediments of Moshui Lake. J Environ Sci 20:390–397

Lu L, Wu Y, Ding H, Zhang W (2015) The combined and second exposure effect of copper (II) and chlortetracycline on freshwater algae, *Chlorella pyrenoidosa* and *Microcystis aeruginosa*. Environ Toxicol Pharmacol 40:140–148

Mallick N (2004) Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: response of the antioxidant system. J Plant Physiol 161:591–597

Mallick N, Rai LC (1999) Response of the antioxidant systems of the nitrogen fixing cyanobacterium *Anabaena doliolum* to copper. J Plant Physiol 155:146–149

Mallick S, Sinam G, Kumar Mishra R, Sinha S (2010) Interactive effects of Cr and Fe treatments on plants growth, nutrition and oxidative status in *Zea mays* L. Ecotoxicol Environ Saf 73:987–995

Mehta SK, Singh A, Gaur JP (2002) Kinetics of adsorption and uptake of Cu^{2+} by *Chlorella vulgaris*: influence of pH, temperature, culture age, and cations. J Environ Sci Health A 37:399–414

Melegari SP, Perreault F, Costa RHR, Popovic R, Matias WG (2013) Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. Aquat Toxicol 142(143):431–440

Mellado M, Contreras RA, González A, Dennett G, Moenne A (2012) Copper-induced synthesis of ascorbate, glutathione and phytochelatins in the marine alga *Ulva compressa* (Chlorophyta). Plant Physiol Biochem 51:102–108

Michalak I, Chojnacka K (2010) Interactions of metal cations with anionic groups on the cell wall of the macroalga *Vaucheria* sp. Eng Life Sci 10:209–217

Mishra S, Srivastava S, Tripathi RD, Kumar R, Seth CS, Gupta DK (2006) Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation. Chemosphere 65:1027–1039

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mo L-Y, Zhao D-N, Qin M, Qin L-T, Zeng H-H, Liang Y-P (2019) Joint toxicity of six common heavy metals to *Chlorella pyrenoidosa*. Environ Sci Pollut Res 26:30554–30560

Mochida K, Ito K, Harino H, Kakuno A, Fujii K (2006) Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (*Pagrus major*) and toy shrimp (*Heptacarpus futilirostris*). Environ Toxicol Chem 25:3058–3064

Morel NML, Rueter JC, Morel FMM (1978) Copper toxicity to *Skeletonema costatum* (Bacillariophyceae). J Phycol 14:43–48

Morin S, Coste M (2006) Metal-induced shifts in the morphology of diatoms from the Riou Mort and Riou Viou streams (South West France). In: Acs E, Kiss KT, Padisak J, Szabo K (eds) Use of algae for monitoring rivers VI. Hungarian Algological Society, Göd, Hungary, Balatonfüred, pp 91–106

Muradoglu F, Gundogdu M, Ercisli S, Encu T, Balta F, Jaafar HZE, Zia-Ul-Haq M (2015) Cadmium toxicity affects chlorophyll a and b content, antixodiant enzyme activities and mineral nutrient accumulation in strawberry. Biol Res 48:11

Nugroho AP, Handayani NSN, Pramudita IGA (2017) Combined effects of copper and cadmium on *Chlorella pyrenoidosa* H.Chick: subcellular accumulation, distribution, and growth inhibition. Toxicol Environ Chem 99:1368–1377

Nuzzi R (1972) Toxicity of mercury to phytoplankton. Nature 237:38–40

Omar H (2002) Bioremoval of zinc ions by *Scenedesmus obliquus* and *Scenedesmus quadricauda* and its effect on growth and metabolism. Int Biodeterior Biodegrad 50:95–100

Öncel I, Keleş Y, Üstün A (2000) Interactive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. Environ Pollut 107:315–320

Osman ME, El-Naggar AH, El-Sheekh MM, El-Mazally EE (2004) Differential effects of Co^{2+} and Ni^{2+} on protein metabolism in *Scenedesmus obliquus* and *Nitzschia perminuta*. Environ Toxicol Pharmacol 16:169–178

Ouyang H, Kong X, He W, Qin N, He Q, Wang Y, Wang R, Xu F (2012) Effects of five heavy metals at sub-lethal concentrations on the growth and photosynthesis of *Chlorella vulgaris*. Chin Sci Bull 57:3363–3370

Pinto E, Sigaud-kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P (2003) Heavy metal-induced oxidative stress in algae. J Phycol 39:1008–1018

Pokora W, Tukaj Z (2010) The combined effect of anthracene and cadmium on photosynthetic activity of three *Desmodesmus* (Chlorophyta) species. Ecotoxicol Environ Saf 73:1207–1213

Ponmani T, Guo R, Ki J-S (2015) A novel cyclophilin gene from the dinoflagellate *Prorocentrum minimum* and its possible role in the environmental stress response. Chemosphere 139:260–267

Prasad DDK, Prasad ARK (1987) Altered δ-aminolevulinic acid metabolism by lead and mercury in germinating seedlings of Bajra (*Pennisetum typhoideum*). J Plant Physiol 127:241–249

Prasad MNV, Drej K, Skawinska A, Stratkaka K (1998) Toxicity of cadmium and copper in *Chlamydomonas reinhardtii* wild-type (WT 2137) and cell wall deficient mutant strain (CW 15). Bull Environ Contam Toxicol 60:306–311

Qian H, Li J, Pan X, Sun L, Lu T, Ran H, Fu Z (2011) Combined effect of copper and cadmium on heavy metal ion bioaccumulation and antioxidant enzymes induction in *Chlorella vulgaris*. Bull Environ Contam Toxicol 87:512–516

Qian H, Li J, Sun L, Chen W, Sheng GD, Liu W, FuZ (2009) Combined effect of copper and cadmium on *Chlorella vulgaris* growth and photosynthesis-related gene transcription. Aquat Toxicol 94:56–61

Qu R, Wang X, Liu Z, Yan Z, Wang Z (2013) Development of a model to predict the effect of water chemistry on the acute toxicity of cadmium to *Photobacterium phosphoreum*. J Hazard Mater 262:288–296

Rai LC, Gaur JP, Kumar HD (1981a) Protective effects of certain environmental factors on the toxicity of zinc, mercury, and methylmercury to *Chlorella vulgaris*. Environ Res 25:250–259

Rai LC, Gaur JP, Kumar HD (1981b) Phycology and heavy-metal pollution. Biol Rev 56:99–151

Rai LC, Singh AK, Mallick N (1991) Studies on photosynthesis, the associated electron transport system and some physiological variables of *Chlorella vulgaris* under heavy metal stress. J Plant Physiol 137:419–424

Rai UN, Singh NK, Upadhyay AK, Verma S (2013) Chromate tolerance and accumulation in *Chlorella vulgaris* L.: role of antioxidant enzymes and biochemical changes in detoxification of metals. Bioresour Technol 136:604–609

Reddy MK, Alexander-Lindo RL, Nair MG (2005) Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell

proliferation by natural food colors. J Agric Food Chem 53:9268–9273

Rocchetta I, Mazzuca M, Conforti V, Ruiz L, Balzaretti V, de Molina del Carmen M (2006) Effect of chromium on the fatty acid composition of two strains of *Euglena gracilis*. Environ Pollut 141:353–358

Romero-Puertas MC, Palma JM, Gómez M, Del Río LA, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 25:677–686

Rosko JJ, Rachlin JW (1977) The effect of cadium, copper, mercury, zinc and lead on cell division, growth, and chlorophyll a content of the chlorophyte *Chlorella vulgaris*. Bull Torrey Bot Club 104:226

Saavedra R, Muñoz R, Taboada ME, Vega M, Bolado S (2018) Comparative uptake study of arsenic, boron, copper, manganese and zinc from water by different green microalgae. Bioresour Technol 263:49–57

Samuel K, Bose S (1987) Bleaching of photosynthetic pigments in *Chlorella protothecoides* grown in the presence of SANDOZ 9785 (4-chloro-5-dimethylamino-2-phenyl-3 (2H) pyridazinone). J Biosci 12:399–404

Sathasivam R, Ebenezer V, Guo R, Ki J-S (2016) Physiological and biochemical responses of the freshwater green algae *Closterium ehrenbergii* to the common disinfectant chlorine. Ecotoxicol Environ Saf 133:501–508

Schiariti A, Juárez ÁB, Rodríguez MC (2004) Effects of sublethal concentrations of copper on three strains of green microalgae under autotrophic and mixotrophic culture conditions. Arch Hydrobiol Suppl 114:143–157

Shakya K, Chettri MK, Sawidis T (2007) Impact of heavy metals (copper, zinc, and lead) on the chlorophyll content of some mosses. Arch Environ Contam Toxicol 54:412–421

Shanker AK, Cervantes C, Loza-Tavera H, Avudainayagam S (2005) Chromium toxicity in plants. Environ Int 31:739–753

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Aust J Bot 2012:1–26

Shehata FHA, Whitton BA (1982) Zinc tolerance in strains of the blue-green alga *Anacystis nidulans*. Br Phycol J 17:5–12

Sies H (1999) Glutathione and its role in cellular functions. Free Radic Biol Med 27:916–921

Soto P, Gaete H, Hidalgo ME (2011) Assessment of catalase activity, lipid peroxidation, chlorophyll a, and growth rate in the freshwater green algae *Pseudokirchneriella subcapitata* exposed to copper and zinc. Lat Am J Aquat Res 39:280–285

Su L, Zhang X, Yuan X, Zhao Y, Zhang D, Qin W (2012) Evaluation of joint toxicity of nitroaromatic compounds and copper to Photobacterium phosphoreum and QSAR analysis. J Hazard Mater 241/242:450–455

Sunda W (1975) The relationship between cupric ion activity and the toxicity of copper to phytoplankton. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution, Woods Hole

Takamura N, Kasai F, Watanabe MM (1990) Unique response of cyanophyceae to copper. J Appl Phycol 2:293–296

Takamura N, Kasai F, Watanabe MM (1989) Effects of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. J Appl Phycol 1:39–52

Tingle LE, Pavlat WA, Cameron IL (1973) Sublethal cytotoxic effects of mercuric chloride on the ciliate *Tetrahymena pyriformis*. J Protozool 20:301–304

di Toppi LS, Musetti R, Marabottini R, Corradi MG, Vattuone Z, Favali MA, Badiani M (2004) Responses of *Xanthoria parietina* thalli to environmentally relevant concentrations of hexavalent chromium. Funct Plant Biol 31:329–338

Tripathi BN, Gaur JP (2006) Physiological behavior of *Scenedesmus* sp. during exposure to elevated levels of Cu and Zn and after with drawal of metal stress. Protoplasma 229:1–9

Tukaj S, Tukaj Z (2010) Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae *Desmodesmus subspicatus*: heat pretreatment increases cadmium resistance. J Therm Biol 35:239–244

Van Baalen C, O'Donnell R (1978) Isolation of a nickel-dependent blue-green alga. J Gen Microbiol 105:351–353

Volland S, Andosch A, Milla M, Stöger B, Lütz C, Lütz-Meindl U (2011) Intracellular metal compartmentalization in the green algal model system *Micrasterias denticulata* (Streptophyta) measured by transmission electron microscopy-coupled electron energy loss spectroscopy. J Physiol 47:565–579

Volland S, Bayer E, Baumgartner V, Andosch A, Lütz C, Sima E, Lütz-Meindl U (2014) Rescue of heavy metal effects on cell physiology of the algal model system *Micrasterias* by divalent ions. J Plant Physiol 171:154–163

Volland S, Lütz C, Michalke B, Lütz-Meindl U (2012) Intracellular chromium localization and cell physiology response in the unicellular alga *Micrasterias*. Aquat Toxicol 109:59–69

Wan Maznah WO, Al-Fawwaz AT, Surif M (2012) Biosorption of copper and zinc by immobilised and free algal biomass, and the effects of metal biosoprtion on the growth and cellular structure of *Chlorella* sp. and *Chlamydomonas* sp. isolated from rivers in Penang, Malaysia. J Environ Sci 24:1386–1393

Wang C, Wang X, Su R, Liang S, Yang S (2011) No detected toxic concentrations in *in situ* algal growth inhibition tests-a convenient approach to aquatic ecotoxicology. Ecotoxicol Environ Saf 74:225–229

Wang L, Kang Y, Liang S, Chen D, Zhang Q, Zeng L, Luo J, Jiang F (2018) Synergistic effect of co-exposure to cadmium (II) and 4-n-nonylphenol on growth inhibition and oxidative stress of *Chlorella sorokiniana*. Ecotoxicol Environ Saf 154:145–153

Winterbourn CC (1982) Superoxide dependent formation of hydroxyl radicals in the presence of iron salts is a feasible source of hydroxyl radicals in vivo. Biochem J Lett 205:461–463

Wu S, Zhang H, Yu X, Qiu L (2014) Toxicological responses of *Chlorella vulgaris* to dichloromethane and dichloroethane. Environ Eng Sci 31:9–17

Wu T-M, Lee T-M (2008) Regulation of activity and gene expression of antioxidant enzymes in *Ulva fasciata Delile* (Ulvales, Chlorophyta) in response to excess copper. Phycologia 47:346–360

Xu Y, Feng L, Jeffrey PD, Shi YG, Morel FMM (2008) Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. Nature 452:56–61

Yang Y, Zhang L, Huang X, Zhou Y, Quan Q, Li Y, Zhu X (2020) Response of photosynthesis to different concentrations of heavy metals in *Davidia involucrata*. PLoS One 15:e0228563

Zeb B, Ping Z, Mahmood Q, Lin Q, Pervez A, Irshad M, Bilal M, Bhatti ZA, Shaheen S (2017) Assessment of combined toxicity of heavy metals from industrial wastewaters on *Photobacterium phosphoreum* T3S. Appl Water Sci 7:2043–2050

Zeraatkar AK, Ahmadzadeh H, Talebi AF, Moheimani NR, McHenry MP (2016) Potential use of algae for heavy metal bioremediation, a critical review. J Environ Manag 181:817–831

Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9