

Lead (Pb²⁺) causes chlorophyll related changes and oxidative damage in *Chlorella ellipsoides* (Chlorophyceae)

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Abstract. The increasing production of anthropological wastes containing heavy metals has resulted to their discharge and contamination into freshwater ecosystems. Hence, the effects of heavy metals are of health concern for aquatic biodiversity. This study investigated the short term effects of Pb²⁺ (0.0, 10, 20, 40, 60, 80 and 100 µg.L⁻¹) on the biomass (cell density, chlorophyll *a*, *b*) and antioxidant (catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPx), glutathione reductase (GRx), and malondialdehyde (MDA)). *Chlorella ellipsoides* (Chlorophyceae) was sensitive to Pb²⁺, a significant decrease ($p < 0.05$) of chlorophyll *a* and *b* was observed with increasing concentrations of Pb²⁺. Antioxidant Catalase, SOD, GPx and GRx relatively decreased significantly ($p < 0.05$) after exposure of microalga to Pb²⁺. However, MDA increased significantly ($p < 0.05$) after microalgae was exposed to Pb²⁺. The finding of this study indicates that exogenous concentrations are harmful for the welfare of *C. ellipsoides*. This study is important as it demonstrates the potential impact of Pb²⁺ on microalgae. Field studies in African freshwater biodiversity and monitoring of aquatic ecosystems are recommended to assess the level and impact of Pb²⁺ in aquatic ecosystems.

Keywords: *Chlorella ellipsoides*; Stress; Toxicant; Antioxidative.

Introduction

Metals are part of the main constituents necessary in the constitution of several animal or plant organisms. However, the development of anthropogenic and industrial activity is nowadays responsible for the increase of heavy metals above limit in many compartments of animals and plants (Tchounwou et al., 2012). A manifold of reports have demonstrated that some activities are important source of

pollutants over time; some of such activities include mining and smelting, fuel production, pesticide and fertilizer industry, leather industry, photography, electric appliance, iron and steel etc (Tchounwou et al., 2012). Therefore, everything leads to the fact that heavy metals remain a major problem. The entry of heavy metals into the aquatic environment through anthropological efflux poses a serious health risk to aquatic webs.

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These metals are revealed to disrupt the metabolism by inactivating the photosynthetic systems of plant, enzymes pathways, metabolic nutrient and transport availability in cells (Mallick and Rai, 1992). However; inhibition of growth, limited photosynthesis, respiration, inhibited biosynthesis of chlorophyll and carotenoid and reduce phosphorylation are reported symptoms of heavy metal toxicity (Poskuta et al., 1996). In aquatic ecosystems, microalgae are first affected by heavy metal effluents because they are directly in contact with the media which are separated by the cytoplasmic membrane and the cell wall (Visiviki and Rachlin, 1994; Shehata et al., 1999), with harmful effect on the structure of organelles (Wong et al., 1994).

Toxicity levels and sensitivity to heavy metal are considered to vary among different microalgae (Soldo and Behra, 2000) with a number of studies establishing the effect of toxicity of heavy metal on microalgae with respect to species (Saçan et al., 2007). The concentration of lead in stagnant surface water ranges from 6 to 1,410 μgL^{-1} and the toxicological effect on growth, chlorophyll and antioxidant responses on microalgal cells have been widely reported by Patel et al. (2006) on various species of microalga.

Relatively, little is known on the toxic effects of lead (Pb) on microalgae isolated from African freshwater. Also, very little attention has been given to *Chlorella* species from the African freshwater bodies. In this study, *Chlorella* species is a microalgae collected from Kainji Lake. Kainji Lake like many other lakes in Nigeria is exposed to human activities in and around the lake such as the hydroelectric plant and agricultural activities; it is likely that the microalgae *C. ellipsoidea* inhabiting the lake suffers the effects of pollutants such as lead (Pb^{2+}) discharged into the Lake. Hence, it is necessary to understand the impact of such a stressful compound on

C. ellipsoidea. Thus, this study determines the toxic effect of Pb^{2+} on growth, chlorophyll and anti-oxidative responses in *C. ellipsoidea* isolated from Kainji Lake.

Materials and Methods

Algal culture

All toxicity tests were conducted using freshwater *C. ellipsoidea* provided by the National Institute for Freshwater and Fisheries Research (NIFFR), Kainji, New Bussa, Nigeria.

The microalgae was cultured in 250 mL flask containing 150 mL of sterilized modified B11 medium which was made of the following chemicals: 31.43 mgL^{-1} organic Nitrate (NO_3), 40.80 mgL^{-1} Phosphorus (PO_4), 170.08 mgL^{-1} Potassium (K_2O), 42.0 mgL^{-1} Magnesium (MgO), 4.66 mgL^{-1} Sulfur (SO_4), 0.42 mgL^{-1} , Calcium (Ca) 2.53 mgL^{-1} , Iron (Fe), 39.27 mgL^{-1} Sodium (Na) 20.62 mgL^{-1} , Chlorine (Cl) and Zinc (Zn) 0.29 mgL^{-1} . An alga in this study was cultured at $25\text{ }^\circ\text{C} \pm 2\text{ }^\circ\text{C}$ on a 14: 10-h: light: dark cycle with a light intensity of 100 $\mu\text{Em}^{-2}\cdot\text{s}^{-1}$. *C. ellipsoidea* cells at exponential growth phase were used for all experiments, and the initial cell density counted under Neubauer haemocytometer (Optik labor, Qiujiang, QJ1102) was 2×10^4 cells. mL^{-1} .

Chemicals

Lead (II) nitrate 99% solid form CAS: 10099-74-8, Pcode: 101620642 was obtained from Sigma-Aldrich (St. Louis, MO 63103, USA). Chemical was of analytical grade. Appropriate chemical was added to prepare aqueous solutions. Stock solution of chemical was prepared in distilled water at a concentration of 100 μgL^{-1} . Stocks were, then diluted with distilled water to obtain the expected concentrations for chemicals tests.

Experimental set up

C. ellipsoidea cells obtained from Kainji Lake were taken to the laboratory of Biology Baze University for culturing

and sub-culturing in culture medium free from Pb²⁺. Experiments investigated the toxicity of different concentrations of lead (lead nitrate). Lead was selected because lead in nature is usually found in many wastes. Microalgal cells in exponential phase of growth were transferred in 1 L beakers containing B11 medium and exposed to concentrations (10, 20, 40, 60, 80 and 100 µg.L⁻¹) of PbNO₃ according to the OECD (1984).

Toxicity study

Toxicity of Pb²⁺ on *C. ellipsoides* was measured as toxicity of the pollutant as estimated by determining the percentage of reduction in cell viability with respect to control on haemocytometer under Nikon binocular optical microscope. Toxicity endpoints measured in this study include growth inhibition, chlorophyll *a* and *b* content and antioxidant enzymes activities. All experiments were carried out in triplicates.

Growth determination

Growth of algal cells was monitored by direct count of viable cells under microscope using a Neubauer haemocytometer. Percentage inhibition of growth was calculated as (Adeleye et al., 2016):

$$GI = \frac{N_c - N_t}{N_c} \times 100$$

Where GI is the percent inhibition in average cell density; N_c is the average cell density in the control group, and N_t is the average cell density for the treatment group.

Chlorophyll *a* and *b* determination

The extraction and analysis of chlorophyll *a* were carried out according to the procedure described by Amaral (2012). Chlorophyll *a* and *b* extracted using absolute methanol. The chlorophyll *a* (Chl*a*) concentration was calculated using the equation:

$$\text{Chl}a \text{ (mgL}^{-1}\text{)} = (11.47 * \text{OD}_{664}) - (0.4 * \text{OD}_{630}) x/y$$

Where *x* is the total volume of extraction solvent used and *y* represents the volume of culture filtered.

Chlorophyll *b* (Chl*b*) was calculated according to the formula (Lichtenthaler and Wellburn, 1985):

$$\text{(Chl}b\text{) (mgL}^{-1}\text{)} = 27.05 \text{OD}_{653} - 11.21 \text{OD}_{666}$$

Where OD₆₃₀, OD₆₅₃, OD₆₆₄, and OD₆₆₆, is optical density at a wavelength of 630, 653, 664 nm and 666 nm, respectively. OD was determined using a UV-2600 spectrophotometer (Shimadzu Scientific Instrument).

Antioxidant assay

Microalgal cells (50 mL) of each culture were centrifuged at 12,000 g for 10 min. Centrifuged algal cells were ground in 1 mL of 20 mM phosphate

buffer (pH 7.4), 0.1g of white quartz sand in a chilled tissue grinder was added to the mixture. The mixture was centrifuged at 12,000 g for 10 min at 4 °C to obtain the supernatant for further

analysis. The supernatant was stored as aliquot for antioxidant estimations.

Catalase

Catalase activity was determined using UV absorption method (Gao, 2005). Sample was divided into two tubes added with live enzymes in one and another with dead enzymes, then Tris-HCl buffer (pH 7.0), distilled water were added in each tube and the mixture in each tube was preheated with 200 mmol.L⁻¹ H₂O₂ for 3 min using a water bath at 25 °C. The absorbance was measured immediately at 240 nm. The result was expressed in U.mg⁻¹.FW.min⁻¹.

Superoxide dismutase

Superoxide dismutase (SOD) activity followed the method described by Gao (2005) which consisted of the reduction of tetrazolium. One unit is the amount required to inhibit 50% of NBT photoreduction. The control tube, the light tube and the measuring tube were divided for each sample. Each tube contained 550 mmol L⁻¹ potassium phosphate buffer (pH 7.8), 130 mmol L⁻¹ methionine solution, 750 µmol L⁻¹ NBT solution, 20 µmol L⁻¹ riboflavin solution, 100 µmol L⁻¹ EDTA-Na₂, distilled water, and the enzyme solution was added to the measuring tube, the same amount of distilled water was added to the other tubes. The experiment was conducted under 1000 Lx Fluorescent color reaction for 15 min, the dark control tube was used as a blank. The absorbance was read at 560nm and the result expressed as U.mg⁻¹FW.h⁻¹.

Malondialdehyde (MDA)

Malondialdehyde (MDA) measurements, used to estimate the level of lipid peroxidation in algal cells, were carried out according to Heath and Packer (1968). 2 mL of 10% trichloroacetic acid (TCA), containing 0.5% thiobarbituric acid (TBA) was added to 1 mL of the microalgal suspension. The mixture was then heated in a water bath for 15 min and allowed to

cool in an icebath then, centrifuged at 3,000 rpm for 5 min and the absorbance of the supernatant was read at 532 nm and 600 nm. The concentration of MDA was computed using an extinction coefficient of 155 mM⁻¹ cm⁻¹ and the unit expressed in µmol MDA/g⁻¹ FW.

Guaiacol peroxidase

Peroxidase activity was measured using guaiacol and H₂O as the donor as substrate. The substrate mixture contained 10 mL 1% guaiacol, 10 mL 0.3% hydrogen peroxide and 100 mL 0.05M sodium phosphate buffer (pH 6.5). The mixture in the cuvette was made up of 2.87 mL substrate, 0.1 mL of crude extract, and 0.03 mL antioxidant solution in a total volume of 3 mL (Hemeda and Klein, 1990). The control contained 0.03mL of ethanol. Peroxidase activity was determined spectrophotometrically at 25 °C and 470nm. The result was expressed as µg.mg⁻¹ FW min⁻¹.

Glutathione reductase

Glutathione reductase (GRx, EC 1.6. 4.2) activity was determined according to Schaedle and Bassham (1977).

GR catalyzed following reaction: GSSH + NADPH → GSH + NADP+. GR activity was evaluated by measuring the change of NADPH. 1 mL reaction mixture containing 50 mmol.L⁻¹ potassium phosphate buffer (pH 7.8), 20 mmol.L⁻¹ EDTA, 1.5 mM NADPH, 5 mM GSSG, 200 µL enzyme solution, and measured the change of absorbance at 340 in 1 min under 20 °C immediately (extinction coefficient is 6.2 mmol.L⁻¹.cm⁻¹). The result was expressed in U.mg⁻¹ protein.

Data analysis

For algal growth inhibition tests, the EC₅₀ values (metal concentration required to cause a 50% reduction in growth) were computed using curve Weibull model analysis on Regression toxicology software for Excel. The data obtained from the study was subjected to

Levene's test for homogeneity of variance and ANOVA was used to determine the differences in means parameters (inhibition (%), biomass and enzymes activities) using GraphPad Prism7 and OriginLab 8.1 (Northampton, MA, USA). Tests where significant differences were observed, separation of means was done using Tukey's HSD post hoc test. Values were considered significantly different when the probability was less than 0.05 or 0.01.

Results

Biomass production of *C. ellipsoides*

The microalgae exposed to treatments showed that there was growth inhibition of cells. The increase of growth inhibition percentage seems to be concentration dependant, with the highest concentration having the highest percentage of inhibition (67.69%). However, when the treatments were compared to the control after exposure, one way ANOVA revealed that there was no significant ($p > 0.05$) increase in inhibition of microalgal growth of *C. ellipsoides* (Figure 1).

Chlorophyll a and b decreased significantly ($p < 0.01$) with increasing

concentrations of Pb²⁺ compared to the control (Figure 2). However, the highest reduction of chlorophyll a and b compared to the control was observed in cells exposed to 100 mg/L of Pb²⁺. Chlorophyll b content was lower than chlorophyll a.

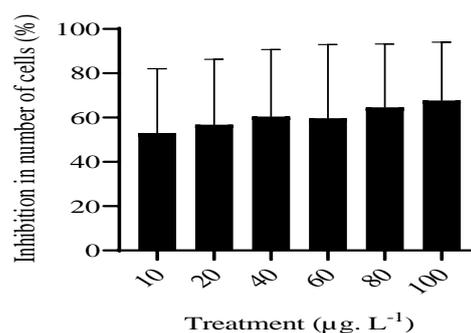


Figure 1. Number of cells percent inhibition of *Chlorella ellipsoides* exposed to Pb concentrations after 72 h. Data are expressed as mean \pm SD of three replicate samples. $P > 0.01$ indicates no significant differences between exposure groups.

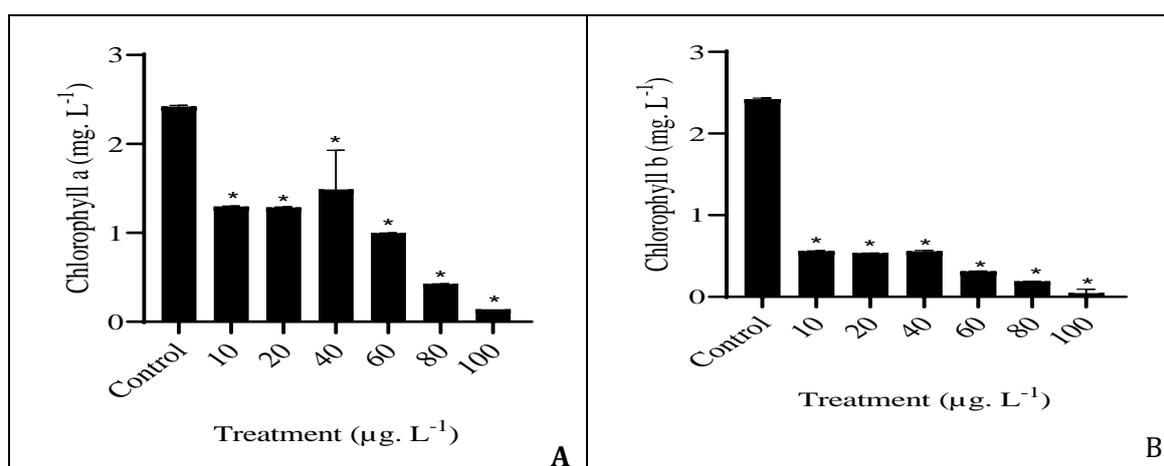


Figure 2. Chlorophyll a and b content from *Chlorella ellipsoides* exposed to lead (Pb) concentrations (0, 10, 20, 40, 60, 80 and 100 µg/L) after 72 h. Data are expressed as mean \pm SD of three replicate samples. * $p < 0.01$ indicate significant differences between exposure group and the corresponding control group (ANOVA followed by Tukey's test).

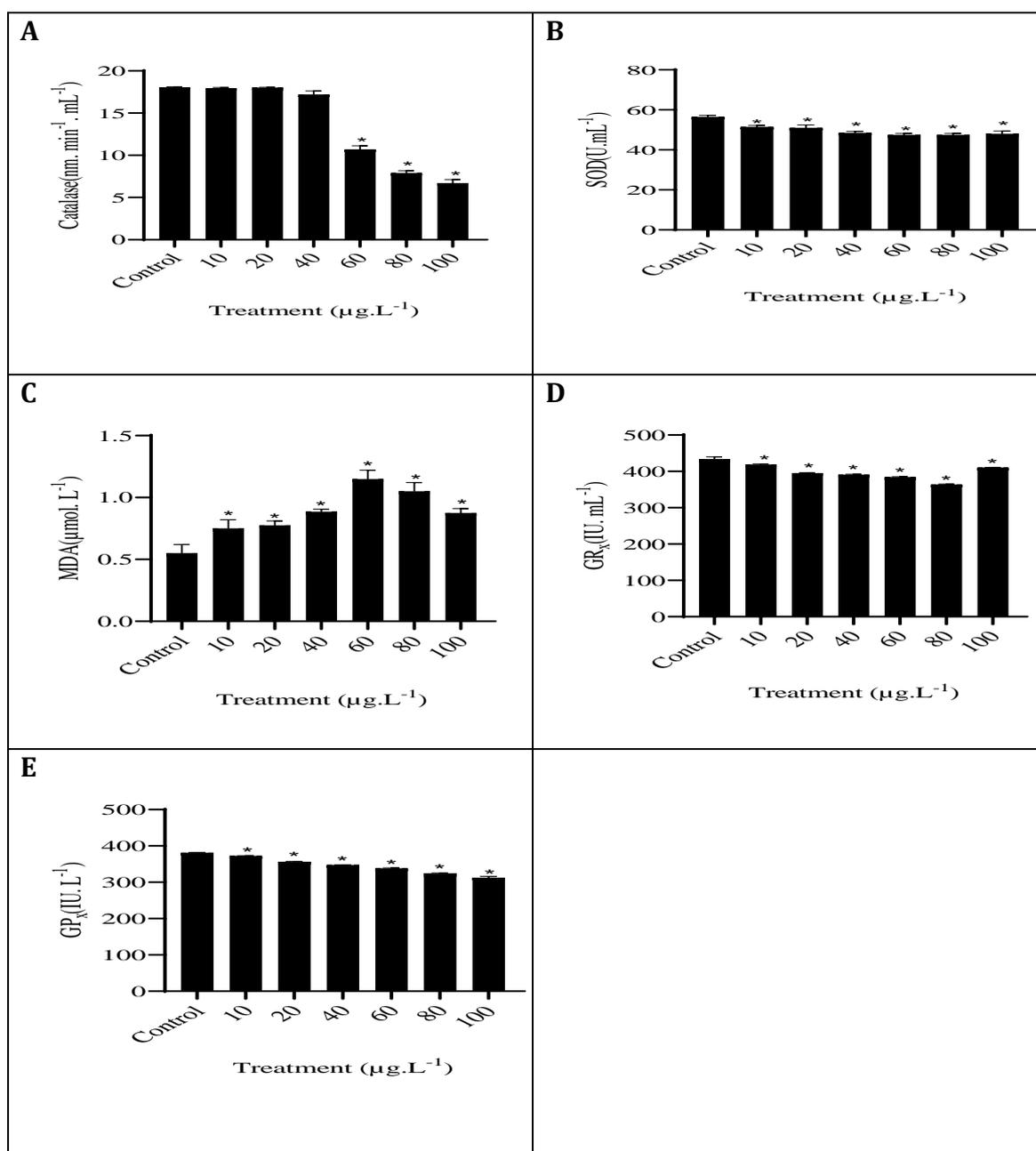


Figure 3. Antioxidant activities of *Chlorella ellipsoides* exposed to Pb concentrations (0, 10, 20, 40, 60, 80 and 100 $\mu\text{g/L}$) after 72h. Data are expressed as mean \pm SD of three replicate samples. * $p < 0.01$ indicate significant differences between exposure group and the corresponding control group (ANOVA followed by Tukey's test).

Antioxidant activities on the algal cells exposed to Pb^{2+} (Figure 3) significantly decreased ($p < 0.01$) for CAT, SOD, GPx and GRx. Amongst the antioxidant previously mention, catalase production was mostly reduced with the

most elevated treatment of 100 $\mu\text{g.L}^{-1}$. However, the highest level of MDA was recorded in cells exposed to 60 $\mu\text{g.L}^{-1}$. MDA level showed a significant increase ($p < 0.01$). The significant antioxidant changes observed in microalgal cells

throughout this study revealed the toxic impact of Pb²⁺.

Discussion

Biomass *C. ellipsoides*

Growth inhibition occurring in this study could be attributed to the reduction of photosynthetic activity of microalgae. This corroborates with the reduction of growth of *Chlorella sorikoniana* exposed to lead (Pb²⁺) (Carfagna et al., 2013).

Inhibition of chlorophyll a and b in algal cells was concentration dependent; in this regard this study indicates that chlorophyll production was adversely related to the process of photosynthesis. Heavy metal can disrupt photosynthesis by substituting the central Mg²⁺ in the central chlorophyll molecule but also impairs photosynthetic electron transport (Krupper et al., 1996). The decrease of Chl a and b may also be an indication of the disruption of the photosynthetic apparatus in these cells. The ultrastructural alteration in the shape and organization of thylakoids and chloroplast could lead to the decrease of chlorophyll content (Carfagna et al., 2013). Probably, Pb²⁺ effect on photosynthesis could have inhibited the photosystem II as a result of damage of thylakoid membranes and reaction centers (Rai et al., 2013) which agreed with the present study.

Anti-oxidative changes in *C. ellipsoides*

Chloroplast alteration due to exposure (oxidative stress) of *Chlorella sorikoniana* to Cd and Pb revealed an increase of production of reactive oxygen species (ROS). Reactive oxygen is dominated by the increase production of superoxide (O²⁻), hydroxide (OH⁻), hydroxyl (.OH), hydroxide peroxide (H₂O₂) (Dai, 2012). ROS is known to damage proteins, nucleic acid, amino acid, membrane lipid (Zhou et al., 2001). Heavy metal in this study produced toxic effect due to exposure of *Chlorella*

ellipsoides to Pb²⁺ which caused stress and led to reduction of CAT, SOD, GPx and GRx. SOD has the function to dismutate O²⁻ radical to H₂O₂, Px, CAT and AOx act as H₂O₂ scavengers. The production of antioxidant enzymes play an important role as it can protect cells against injury (Zhou et al., 2001). In the present study, reduction of CAT, SOD, GPx and GRx is an indication of saturation and accumulation of ROS in cells and caused inhibition of enzyme production. These findings are in disagreement with report demonstrating the up-regulation of antioxidant enzymes following treatment with heavy metals in microalgae (Pinto et al., 2003). This inhibition of antioxidant enzymes may be due to the fact that, data were collected 72 h after exposure and could be inferred that the presence of lead (Pb²⁺) after that period of time must have overwhelmed the microalgal cells thereby causing an enhancement of ROS species that could have inhibited the antioxidant production.

However, the up-regulation of MDA observed in the present study, indicates that the presence of Pb²⁺ in the medium contributes to the production of hydroxyl (HO⁻) from superoxide (O₂⁻) ions. Heavy metal treatment is reported to increase the amount of H₂O₂. The risen of MDA content suggests that metal ions enhanced free radicals (Chouldary et al., 2007). Several studies supported the correlation between the increase production of MDA and the concentration of Pb²⁺ with consequences on the cell wall (Malar et al., 2014; Khan et al., 2013).

Conclusions

The present study provides the novel evidence regarding the effects of heavy metal (Pb²⁺) exposed to *Chlorella ellipsoides* in five different concentrations. In particular, growth inhibition, chlorophyll content and antioxidant responses were investigated. It was observed an increase of inhibition

of growth of cells depending on the concentration increment; increase of concentration of Pb^{2+} led to the occurrence of a substantiated decrease of chlorophyll a, b and antioxidant responses (SOD, CAT, GRx, GPx and MDA). This present study highlighted the necessity to further understand the discrepancy in toxicological responses of microalgae exposed to heavy metal contaminants, but also provided additional information on *Chlorella* sp.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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