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Research Article

Short- and Long-Term Exposure to Heavy Metals Induced Oxidative Stress Response in *Pseudokirchneriella subcapitata*

Algal cells can be exposed to toxicants for a short-term due to accidental discharges or, more commonly, for a long-term. The present work aimed to assess the ability of Cd, Cr, Cu, and Zn to induce accumulation of reactive oxygen species (ROS) in the alga *Pseudokirchneriella subcapitata* after a short (6 h) or a long (72 h) exposure time. The relationship between the ROS induction and the content of reduced glutathione (GSH) was also examined. For this purpose, three nominal concentrations of each metal were used corresponding approximately to 72 h-EC_{10} and 72 h-EC_{50} values and a high concentration (>72 h-EC_{90} values). Intracellular ROS accumulation and GSH content were evaluated using a fluorescent-based approach. A long-term (chronic) exposure of algal cells to Cd, Cu, and Zn, at the highest concentrations tested, induced an increase of intracellular ROS and GSH content. The increase of GSH content might be a form of algal cells to redress the imbalance caused by the oxidative stress. However, the increase of GSH was not enough to protect the algal cells against the long-term exposure to oxidative stress. The exposure of algal cells to low or intermediate metals concentrations induced a modification of GSH content; however, no increase of ROS production was detected, which indicates that the toxic symptoms exhibited by algal cells, under these conditions, cannot be attributed to intracellular ROS accumulation.

Keywords: 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA); Monochlorobimane (mBCl); Reactive oxygen species (ROS); Reduced glutathione (GSH); Toxicity

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1 Introduction

Although metals occur naturally in earth's crust, most environmental contamination results of anthropogenic sources, like mining, electroplating, and smelting industries as well as agrochemicals [1]. Some metals, such as Cu, Zn, Mn, and Fe, at trace amounts, are essential to support biological functions. Other metals, such as Cd, Cr, Pb, and Hg have no biological effect and are toxic even at low concentrations [2]. According to the International Agency for Research on Cancer, Cd and Cr(VI) are considered carcinogenic to humans [3].

Aquatic systems are particularly exposed to increasing amounts of metals that provoke a negative impact on organisms. Algae, as primary producers and essential components of the aquatic systems, are the basis of the food chain. Thus, toxic effects on algae will affect all ecosystems. The freshwater alga *Pseudokirchneriella subcapitata* is highly sensitive to heavy metals [4]; therefore, it is frequently used as a standard organism in toxicological tests [5, 6]. Cd, Co, Cr, Cu, Mn, and Zn had a detrimental effect on cell structure and metabolism of *P. subcapitata* [7–13]. Bossuyt and Janssen [13] observed an increase of chlorophyll and carotenoids content, and a decrease of biomass of *P. subcapitata* exposed to 0.007–1.6 μmol L^{-1} Cu. Copper ions caused significant inhibition of the cell division rate, chlorophyll *a* fluorescence and esterase activity [14, 15]. Machado et al. [9] reported that Cd, Cr, Cu, and Zn have different effects on growth, cell division, and cell volume of *P. subcapitata*, suggesting that distinct mechanisms underlie the action of each metal. The 72 h exposure of the alga to 1.9 μmol L^{-1} Cd, 41 μmol L^{-1} Cr, 1.3 μmol L^{-1} Cu, and 2.5 μmol L^{-1} Zn resulted in a severe perturbation of the cell physiological status by loss of membrane integrity, reduction of chlorophyll *a* content, and the maximum quantum yield of photosystem II, inhibition of esterase activity, and alteration of mitochondrial membrane potential [10]. Soto et al. [8] also reported a significant decrease in chlorophyll *a* in algal cells exposed to 1.2 μmol L^{-1} Cu

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Abbreviations: **DCF**, 2′,7′-dichlorofluorescein; **EC_{10}, EC_{50}, EC_{90}**, concentration of the toxicant that caused the inhibition of 10%, 50%, or 90% of algal growth; **GSH**, reduced glutathione; **H_2DCF**, 2′,7′-dichlorodihydrofluorescein; **H_2DCFDA**, 2′,7′-dichlorodihydrofluorescein diacetate; **mBCl**, monochlorobimane; **OECD**, organization for economic co-operation and development; **ROS**, reactive oxygen species; **SD**, standard deviation.

and 1.1 μmol L^{-1} Zn. High concentrations of Mn induced several changes in the cell ultrastructure, namely at cytoplasm, chloroplast, and membrane levels [12].

Heavy metals are also able to induce the production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, singlet oxygen, and the hydroxyl radical [16]. Induction of ROS generation was reported when *P. subcapitata* and *Chlorella vulgaris* were exposed to Cu [17] and when the chlorophyte *Chlamydomonas reinhardtii* was exposed to Pb, Fe, Cd, Ag, Cu, As, Cr, Zn, and Ni [18, 19]. The green alga *Scenedesmus quadricauda* also produced ROS in the presence of Cd, Ni, Cr [20, 21]. The freshwater alga *Micrasterias denticulata* increased the ROS production when exposed to Cr(VI) [22]. Collen et al. [23] observed that Cu and Cd induced oxidative stress in the red macro alga *Gracilaria tenuistipitata*. The green algae *Chlorella pyrenoidosa* and *Scenedesmus* sp. exhibited oxidative stress and activation of antioxidative enzymes when used to treat a tannery effluent containing Cr, Cu, Pb, and Zn [24].

Algal cells respond to ROS production by induction of protective antioxidants mechanisms, such as the induction of several enzymes (*e.g.*, catalase, peroxidase, superoxide dismutase, and ascorbate peroxidase) and/or the synthesis of several low molecular weight compounds (*e.g.*, ascorbic acid, flavonoids, α-tocopherol, carotenoids, and reduced glutathione (GSH) [25]. When ROS levels exceed the antioxidant capacity of the cell, an oxidative stress occurs, which can damage lipids of membranes, proteins, DNA, and affect cell viability [26].

ROS result of normal oxygen metabolism (respiration and photosynthesis processes). High light, UV radiation, and metal exposure, can stimulate an excessive production of ROS [16]. Redox active metals (Cr(VI), Cu(II), and Fe(III)) induce ROS formation via the Fenton or Haber–Weiss reactions. Redox-inactive metals (Cd, Hg, and Pb) induce ROS production by indirect mechanisms: displacement of redox-active metals from cellular binding sites, inhibition of enzymatic antioxidant defences, or GSH pool depletion [25].

Cysteine-containing peptides such as GSH are responsible for metal sequestration in living cells. GSH is simultaneously a general reductant and a substrate for enzymatically catalysed reactions [27]. Cellular antioxidative defences of algal cells, when exposed to environmental pollutants, can be depleted. Some metals, like Cd, can promote indirectly oxidative stress by depleting cellular antioxidants [19]. Antioxidants also have the ability to inhibit heavy metal uptake and contribute for the detoxification or decreasing of the cells damage. Therefore, GSH has multiple functions in the biosynthetic pathways, metal detoxification, antioxidant biochemistry, and redox homeostasis [28, 29].

The toxic impact of heavy metals on organisms can be associated, at least partially, with ROS production [30]. Since oxidative stress can be correlated with GSH synthesis or depletion, the elucidation of the relation between these two parameters is an important issue. Occasionally, algal cells can be exposed to heavy metals for a short-term due to accidental discharges in water bodies. More commonly, algae are exposed to waters polluted with heavy metals for a long period of time. In the present study, an aquatic organism model, the freshwater green alga *P. subcapitata*, was used to investigate the impact of different heavy metals (Cd, Cr, Cu, and Zn) under two scenarios: a short (6 h) and long (72 h) term exposure. In order to contribute for the understanding of the mechanisms associated with the biological responses of algal cells exposed to heavy metals, the intracellular accumulation of ROS and the content of GSH were evaluated.

2 Materials and methods

2.1 Strain and growth conditions

The freshwater green alga *P. subcapitata* (strain 278/4) was used. The strain was purchased from the Culture Collection of Algae and Protozoa (CCAP), UK.

The alga was maintained in the Organization for Economic Co-Operation and Development (OECD) algal test medium [6] with 20 g L^{-1} agar (Merck) in the dark at 4°C. Medium stock solutions were prepared, sterilized, and stored according to the OECD guidelines [6].

The starter-cultures were prepared weekly by inoculating a loop of algal cells (from OECD agar medium slant) in 20 mL OECD medium, in 100 mL Erlenmeyer flasks. The cells were incubated with orbital shaking for 2 days, at 25°C, under continuous "cool white" fluorescent light with an intensity of 4000 lux at the surface of the flask. The cultures were prepared by inoculating 100 or 400 mL of OECD medium in 250 mL or 1 L Erlenmeyer flasks, respectively, with an initial cell concentration of $\sim 5 \times 10^4$ cells mL^{-1} from the pre-culture. Cells were incubated under the conditions previously described for the starter-cultures.

The algal cell concentration was determined by measuring the absorbance at 750 nm [5] or using an automated cell counter (TC10, Bio-Rad).

2.2 Exposure of algal cells to metals

Algal cells in exponential phase of growth (2 days) were centrifuged at 2500×*g* for 5 min and resuspended at 5.5×10^6 cells mL^{-1} in deionized water. Three different concentrations of each metal (Cd(II), Cr(VI), Cu(II), and Zn(II)) were used as previously described [9]: low concentrations (0.12 μmol L^{-1} Cd, 2.7 μmol L^{-1} Cr, 0.080 μmol L^{-1} Cu, 0.15 μmol L^{-1} Zn), medium concentrations (0.50 μmol L^{-1} Cd, 11 μmol L^{-1} Cr, 0.32 μmol L^{-1} Cu, 0.60 μmol L^{-1} Zn), and high concentrations (1.9 μmol L^{-1} Cd, 41 μmol L^{-1} Cr, 1.3 μmol L^{-1} Cu, 2.5 μmol L^{-1} Zn). These concentrations corresponded approximately to 10, 50, and >90% of algal growth inhibition, respectively, after 72 h of exposure when compared to the control (cells not exposed to the metal). The assays were carried out in 1 L Erlenmeyer flasks containing OECD medium and the appropriate volume of metal added from stock solutions (Merck) of $CdCl_2$, $Cu(NO_3)_2$, and $ZnCl_2$ or from a primary standard $K_2Cr_2O_7$ in a final volume of 400 mL. The flasks were inoculated with 5×10^4 cells mL^{-1} of algal cells and incubated for a short or a long exposure time (6 or 72 h, respectively) in the growth conditions described above. As a control, cells were incubated under the same conditions but without metals.

2.3 Detection of ROS production

Intracellular ROS accumulation was monitored with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). The cell-permeant H_2DCFDA diffuses passively into cells and is retained intracellularly after cleavage of the acetate groups by intracellular esterases. Upon oxidation by several types of ROS (including hydrogen peroxide, hydroxyl radical, and peroxynitrite), the non-fluorescent 2′,7′-dichlorodihydrofluorescein (H_2DCF) is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [31].

After 6 or 72 h of metal exposure, cells were harvested by centrifugation (2500×*g*, 5 min, 4°C) and resuspended in 0.1 mol L^{-1}

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phosphate buffered saline (PBS), pH 7, at 1×10^6 cells mL^{-1}. Subsequently, cells were stained with H_2DCFDA (Sigma) (in a final concentration of 10 $\mu mol\,L^{-1}$) and placed in quintuplicate in a 96-well flat microplate (Orange Scientific, Belgium), 200 μL per well. As negative control, cells were exposed to 10 $mmol\,L^{-1}$ of ascorbic acid (an antioxidant that quenches ROS) for 30 min in the dark. After an incubation of 90 min, at 25°C in the dark, the fluorescence intensity was measured as relative fluorescence units, RFUs, in a microplate reader (Victor3, Perkin–Elmer) at a fluorescence excitation wavelength of 485/14 nm and an emission of 535/25 nm. Fluorescence was corrected (subtracting cell, culture medium, and dye fluorescence) and normalized considering the cell concentration. Intracellular ROS accumulation was expressed as the ratio of fluorescence of the assay/fluorescence of the control (cells not exposed to metals).

2.4 Assessment of intracellular-reduced glutathione content

Intracellular GSH content was evaluated using monochlorobimane (mBCl) in an assay that requires minimal algal cells manipulation. The mBCl is a non-fluorescent cell permeant probe; once inside the cell, mBCl reacts with GSH, and forms stable fluorescent bimane-glutathione adducts [32].

After 6 or 72 h of heavy metals exposure, cells were harvested by centrifugation (2500×*g*, 5 min, 4°C), resuspended in OECD medium at 1×10^6 cells mL^{-1}, and incubated with mBCl (in a final concentration of 50 $\mu mol\,L^{-1}$) at 25°C in the dark, during 90 min, in black 96-well microplates (OptiPlate-96 F, Perkin–Elmer) as previously described [32]. As negative control, cells were exposed to 1 $mmol\,L^{-1}$ iodoacetamide (an alkylating agent that depletes glutathione). Fluorescence intensity was measured using a microplate reader at a fluorescence excitation of 355/40 nm and an emission of 460/25 nm. Fluorescence was corrected and normalized as described above. Intracellular GSH content was expressed as the ratio of fluorescence of the assay/fluorescence of the control (cells not exposed to metals).

2.5 Reproducibility of the results

The data are mean values of three to six independent experiments. Results, expressed as the mean ± standard deviation (SD), are presented with 95% confidence limits.

3 Results

3.1 The exposure to heavy metals induced the accumulation of ROS

The alga *P. subcapitata* was exposed to Cd, Cr, Cu, and Zn for a short- (6 h) or a long-term (72 h) period of time. The short-time exposure simulated an acute toxic effect due to accidental discharges of heavy metals in water bodies. This period of time corresponded, approximately, to half of the doubling (generation) time of the alga *P. subcapitata* under the cultural conditions used [33]. The long-term exposure corresponded to a chronic exposure of the algal cells to heavy metals. This time is proposed by OECD guidelines for testing chemicals toxicity [6]. For each metal, three nominal concentrations were used: a low concentration, which corresponded to a metal concentration closer to 72 h-EC_{10}; an intermediary concentration, closer to 72 h-EC_{50} and a concentration >72 h-EC_{90}, where the growth was arrested [9]. Algal cells were exposed to heavy metals in OECD medium under continuous illumination and agitation [6]. The level of intracellular ROS was monitored using the fluorescent probe H_2DCFDA.

Algal cells exposed for 6 h to low or intermediary metals concentrations of Cd, Cu, and Zn did not present levels of ROS higher than the control (Fig. 1). In addition, no increase of intracellular ROS was observed for cells exposed to Cr during 6 h for all concentrations tested (Fig. 1). Algal cells exposed for 6 h to the highest concentration of Cd, Cu, and Zn (1.9 $\mu mol\,L^{-1}$ Cd, 1.3 $\mu mol\,L^{-1}$ Cu, and 2.5 $\mu mol\,L^{-1}$ Zn) presented a higher intracellular ROS level comparatively to the control (Fig. 1).

P. subcapitata cells long-term (72 h) exposed to low or intermediary metals concentrations (0.12 or 0.50 $\mu mol\,L^{-1}$ Cd, 2.7 or 11 $\mu mol\,L^{-1}$ Cr, 0.080 or 0.32 $\mu mol\,L^{-1}$ Cu, and 0.15 or 0.60 $\mu mol\,L^{-1}$ Zn) did not present a higher level of intracellular ROS (Fig. 1). Nevertheless, algal cells exposed to the highest concentrations of the metals for 72 h induced an increase of ROS. This increase was particularly notorious for Cu; cells treated with Cu for 72 h displayed a ROS level ~16 times higher compared to control. The hierarchy of intracellular ROS accumulation was the following: $Cu >> Zn \approx Cd > Cr$ (Fig. 1).

3.2 The exposure of algal cells to metals modified GSH content

The intracellular GSH content of *P. subcapitata* exposed to heavy metals was evaluated in situ with mBCl using a cell-based assay [32]. The exposure of algal cells with Cd and Zn for all concentrations tested, during 6 h, did not affect the GSH content (Fig. 2). However, the exposure of algae cells to intermediate and higher Cr concentrations, during 6 h, reduced the GSH content. Intermediate and higher Cu concentrations increased 1.2 and 1.3 times the GSH content, respectively (Fig. 2).

Algal cells exposed to all Cd concentrations tested, during 72 h, originated an increase of GSH content (Fig. 2). An increase of 1.2, 1.4, and 3 times of GSH was observed for low, intermediate, and high concentrations of Cd, respectively. Cells exposed to the highest concentration of Zn presented an increase of GSH content (~2.6 times), comparatively to the control cells (Fig. 2). Similarly to what happened with the short-term exposure, algal cells incubated for 72 h with intermediate and high concentrations of Cr displayed a reduced GSH content comparatively to the control. Cu induced an enlargement of GSH content for intermediate and high concentrations; for the highest Cu concentration, an enhancement of ~2.5 times of GSH was observed (Fig. 2).

4 Discussion

The mitigation of damages caused by heavy metals in aquatic systems requires the development of new methodologies that either detect earlier these elements but also elucidate their main targets in cells. ROS control various processes in the cell, namely cell cycle, stress response, and antioxidant defence [34]. High production of ROS damages the antioxidant defence of cells and induces oxidative stress. In the present study, the effect of three nominal concentrations of two redox active (Cu and Cr) and two non-redox active metals (Cd and Zn) on the alga *P. subcapitata* was evaluated.

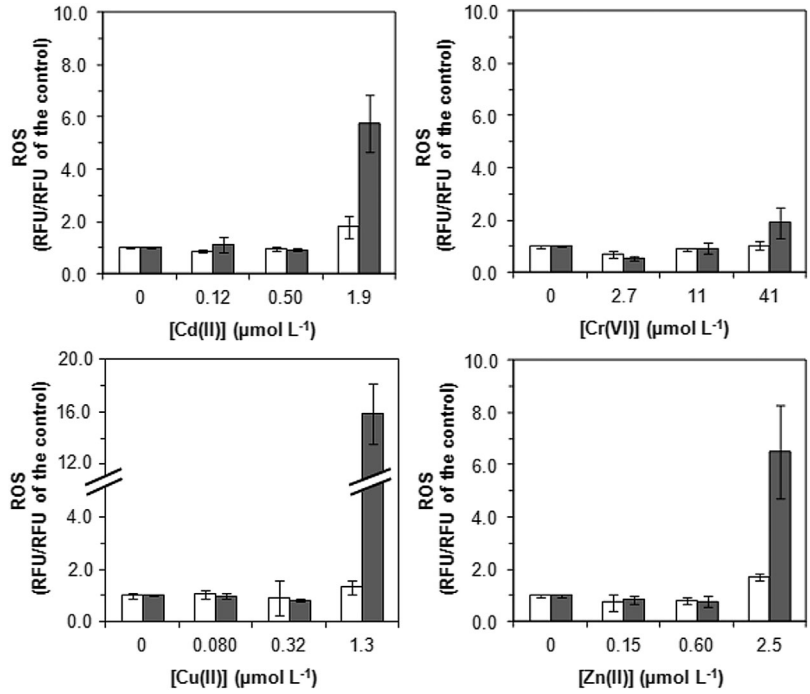

Figure 1. ROS production in *P. subcapitata* exposed to heavy metals. Algal cells were exposed to the action of heavy metals for 6 h (white bar) or 72 h (gray bar). Subsequently, cells were washed and ROS production was evaluated using H_2DCFDA. Each bar represents the mean of three to five independent experiments performed in quintuplicate ($n \geq 15$). The error bars represent the SD calculated with 95% confidence limits.

Algal cells exposed for a long-term to high metals concentration (1.9 μmol L^{-1} Cd, 1.3 μmol L^{-1} Cu, and 2.5 μmol L^{-1} Zn) displayed an increase of ROS production (Fig. 1) and GSH content (Fig. 2). These results suggest that the chronic exposure of algal cells to ROS lead to an increase of the antioxidant defences to redress the imbalance caused by the oxidative stress. Consistent with this possibility, the oxidative stress caused by H_2O_2, Cd, Cu, and Zn promoted the GSH synthesis [35–40]. However, the increase of intracellular GSH content, found in the present work, was not enough to sustain the intracellular accumulation of ROS.

The exposure of *P. subcapitata* for a short- or long-term to Cd, Cr, Cu, and Zn, for concentrations up to 72 h-EC_{50} values did not induce an increase of intracellular ROS (Fig. 1). For those metal concentrations, these results indicated that ROS generation cannot be the main mechanism of metals toxicity since for 72 h-EC_{10} and 72 h-EC_{50} values occurred the inhibition of algal growth [9], and esterase

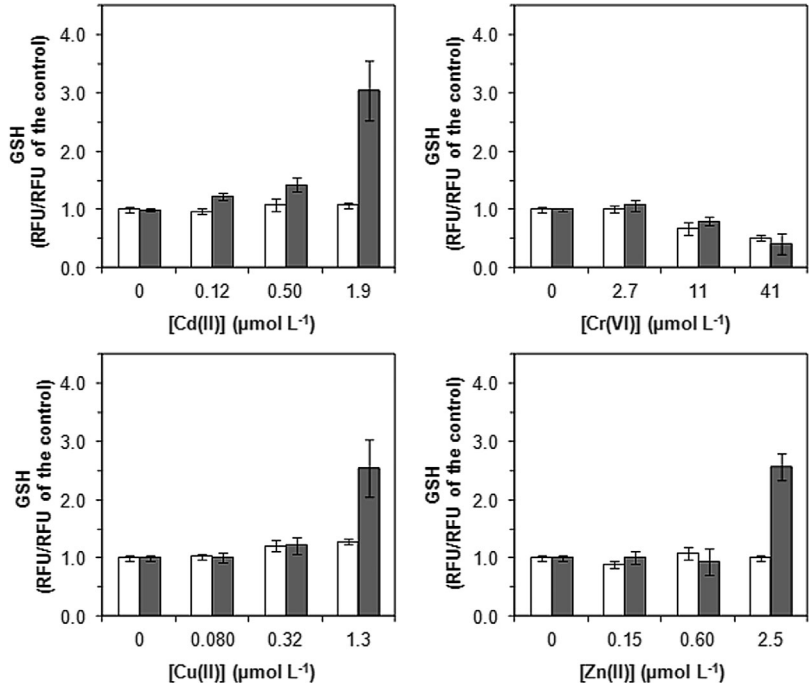

Figure 2. Effect of the heavy metals on GSH content of the alga *P. subcapitata*. Algal cells were exposed to the action of heavy metals for 6 h (white bar) or 72 h (gray bar). Subsequently, cells were washed and stained with mBCl. Each bar represents the mean of three to five independent experiments performed in quintuplicate ($n \geq 15$). The error bars represent the SD calculated with 95% confidence limits.

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activity [10, 11] without ROS production (Fig. 1). However, a small increase of GSH content was observed in algal cells exposed for 6 or 72 h to 0.32 $\mu mol\,L^{-1}$ Cu or for 72 h to 0.12 or 0.50 $\mu mol\,L^{-1}$ Cd (Fig. 2). A reduction of GSH content in algal cells exposed for a short- or long-term to 11 $\mu mol\,L^{-1}$ Cr was also observed (Fig. 2). These results suggest that cells exposed to Cd, Cr, and Cu, at concentrations up to 72 h-EC_{50} values, perturbed the redox state of the algal cells, although a rise in the intracellular ROS was not observed.

Cell membrane injury is one important effect of metal ions. It was described that intracellular ROS can originate extensive peroxidation of membrane lipids, which lead to the loss of membrane integrity and cell death [26]. In fact, lipid peroxidation in *P. subcapitata* exposed to 0.39 $\mu mol\,L^{-1}$ Cu and 1.53 $\mu mol\,L^{-1}$ Zn was described [8]. Cu concentrations of $\geq$40 $\mu mol\,L^{-1}$ induced ROS overproduction in marine diatom *Phaeodactylum tricornutum*, which led to membrane deterioration [41]. In a previous work [10], the exposure of algal cells for 72 h to 1.9 $\mu mol\,L^{-1}$ Cd, 41 $\mu mol\,L^{-1}$ Cr, and 1.3 $\mu mol\,L^{-1}$ Cu, induced a loss of membrane integrity of 16, 38, and 55%, respectively. Under these conditions, an increase of ROS accumulation was observed (Fig. 1). However, the level of ROS production cannot be correlated with the amount of cells that presented cell membrane disruption. These results suggest that other mechanisms, such as direct lipid peroxidation by transition elements [42], should be associated with cell membrane disruption. On the other hand, an increased accumulation of intracellular ROS was observed for 2.5 $\mu mol\,L^{-1}$ Zn (Fig. 1) without the loss of membrane integrity [10]. Together, these results suggest that cell death of *P. subcapitata* induced by heavy metal cannot be directly correlated with the induction of intracellular ROS accumulation.

Algal cells exposed for 6 h to 1.9 $\mu mol\,L^{-1}$ Cd or 2.5 $\mu mol\,L^{-1}$ Zn presented increased values of intracellular ROS (Fig. 1) without disturbance of the photosynthetic activity [11]. Conversely, the exposure to 11 and 41 $\mu mol\,L^{-1}$ Cr or to 0.32 $\mu mol\,L^{-1}$ Cu for 6 h inhibited photosynthesis [11] without detectable increase of intracellular ROS accumulation (Fig. 1). These results support the idea that, for several heavy metals, the induction of ROS is a consequence of a direct interaction of the metal with the donor or acceptor side of photosystem II, which originates an interruption of the photosynthetic electron chain [43]. In this case, the inhibition of photosynthesis precedes the ROS production. The chronic exposure to oxidative stress can induce the damage of protein and lipid components of thylakoid membrane, and lead to photosynthesis inhibition [17]. Consistent with this possibility, a causal link between the intracellular ROS accumulation (Fig. 1) and the decrease of the maximum quantum yield of photosystem II in *P. subcapitata* exposed for 72 h to 1.9 $\mu mol\,L^{-1}$ Cd, 41 $\mu mol\,L^{-1}$ Cr and 1.3 $\mu mol\,L^{-1}$ Cu and 2.5 $\mu mol\,L^{-1}$ Zn was observed [10].

Algal cells exposed for 6 or 72 h to 11 or 41 $\mu mol\,L^{-1}$ Cr presented a reduction of GSH content (Fig. 2). Nevertheless, no appreciable amount of intracellular ROS was detected (Fig. 1). These results suggest the involvement of GSH in the protection of algae against oxidative stress since GSH primarily quenches ROS. Similarly, the reduction of intracellular GSH content in *S. bijugatus* and *C. reinhardtii* exposed to Cu was observed [44, 45]. These results suggest that Cu alters the equilibrium between GSH synthesis and consumption. GSH depletion in *Scenedesmus* sp. exposed to Cu and Zn [46] and in *S. acutus* in the presence of Cr [47] was also described. Exposure of the marine macroalgae *Acanthophora spicifera*, *Chaetomorpha antennina*, and *Ulva reticulate* to Cu and Cd enhanced oxidative stress and decreased GSH content [48]. The modification of the redox status of the algal cells (modification of intracellular GSH content) without the detection of intracellular ROS induction also suggests that GSH content can be an early biomarker of oxidative stress.

For algal cells submitted to the action of toxicants, the use of the fluorescent probes H_2DCFDA and mBCl showed to be a fast and easy tool in the monitoring of intracellular ROS accumulation and evaluation of GSH content, respectively.

5 Concluding remarks

The results presented here evidence that a long-term (chronic) exposure of algal cells to the highest concentration of Cd, Cu, and Zn induced an increase of intracellular ROS accumulation and GSH content. The increase of the antioxidant defences might be a form of algal cells to redress the imbalance caused by the oxidative stress. Nevertheless, the increase of GSH was not enough to provide a complete protection against a long-term exposure to oxidative stress. A short- or a long-term exposure of algal cell to heavy metals up to 72 h-EC_{50} concentrations perturbed the redox state of the algal cell, since a modification of the intracellular GSH content was observed. However, an increase of ROS production was not observed; this fact suggests that, for these metal concentrations, the oxidative stress should not be the main mechanism of metals toxicity.

Acknowledgments

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