Responses of the alga *Pseudokirchneriella subcapitata* to long-term exposure to metal stress

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HIGHLIGHTS

- Heavy metals provoke a perturbation of the physiological status of *Pseudokirchneriella subcapitata*.
- Cd(II), Cr(VI) and Cu(II), at high concentrations, cause the loss of membrane integrity.
- Cd(II), Cr(VI), Cu(II) and Zn(II) inhibit esterase activity in a dose dependent manner.
- Heavy metals affect mitochondrial function and photosynthetic activity.
- Fluorescent probes are a useful tool in the identification of toxicity targets of the heavy metals.

GRAPHICAL ABSTRACT

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ABSTRACT

The green alga *Pseudokirchneriella subcapitata* has been widely used in ecological risk assessment, usually based on the impact of the toxicants in the alga growth. However, the physiological causes that lead algal growth inhibition are not completely understood. This work aimed to evaluate the biochemical and structural modifications in *P. subcapitata* after exposure, for 72 h, to three nominal concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II), corresponding approximately to 72 h-EC₁₀ and 72 h-EC₅₀ values and a high concentration (above 72 h-EC₉₀ values). The incubation of algal cells with the highest concentration of Cd(II), Cr(VI) or Cu(II) resulted in a loss of membrane integrity of 16, 38 and 55%, respectively. For all metals tested, an inhibition of esterase activity, in a dose-dependent manner, was observed. Reduction of chlorophyll *a* content, decrease of maximum quantum yield of photosystem II and modification of mitochondrial membrane potential was also verified. In conclusion, the exposure of *P. subcapitata* to metals resulted in a perturbation of the cell physiological status. Principal component analysis revealed that the impairment of esterase activity combined with the reduction of chlorophyll *a* content were related with the inhibition of growth caused by a prolonged exposure to the heavy metals.

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1. Introduction

Although heavy metals occur naturally, their presence in the environment is due, mostly, to anthropogenic activities. Since the industrial revolution, there has been an increasing use of heavy metals with the subsequent release into the environment and contamination of surface and groundwater. Metals are a cause of concern since they can provoke a detrimental effect on ecosystems and public health due to their toxicity and persistence in the environment. Several metals, such as copper and zinc, can have a dual role: at low concentration are essential to metabolism; at high concentration are toxic. Other metals, like Pb or Hg, do not have any known cellular function [1].

High levels of cadmium ($\geq 9 \mu\text{mol L}^{-1}$ Cd) can be found in polluted areas, such as in the vicinity of Cd-bearing deposits [2]. Cd is generally considered a non-essential element. Its toxicity seems to be associated to the high affinity to sulfhydryl groups, the displacement or substitution of essential metal ions (such as Zn) and the modification of the conformation of the bio molecules [3]. Toxic effects of cadmium on algae include the inhibition of growth [4,5] and photosynthesis [6]. Cd affects the ultra-structure and photosynthesis of the unicellular freshwater green alga *Micrasterias denticulata* as a consequence of the disturbance of calcium homeostasis probably by displacement of Ca by Cd [7]. Cadmium induced the production of reactive oxygen species (ROS) [8] and activated the transcription of a large number of genes involved in the oxidative stress defence mechanisms in the alga *Chlamydomonas reinhardtii* [9].

In polluted rivers, chromium can be found at $17\text{--}140 \mu\text{mol L}^{-1}$ [10]. Chromium (III) and chromium (VI) are the two common oxidation states of chromium in the environment. Trivalent chromium is considered a micronutrient, essential for the proper function of living organisms, while hexavalent chromium can display toxic effects on biological systems. Cr(VI) crosses cellular membrane and exhibits strong oxidative potential [11]. In the alga *Pseudokirchneriella subcapitata*, Cr(VI) inhibited growth and induced genetic mutations [12]. Cr(VI) also induced the intracellular ROS accumulation in the alga *C. reinhardtii* [8]. Studies using transmission electron microscopy showed ultra-structural modifications (such as increased vacuolization, condensed cytoplasm and dark precipitation in the cell wall) in cells of the alga *M. denticulata* exposed to Cr(VI) during three weeks [13]. Proteomic analysis revealed that the treatment of *P. subcapitata* alga cells with Cr(VI) altered the expression of proteins associated with the metabolism of amino acids glutamine, arginine and methionine [14].

Copper concentrations as high as $1 \times 10^3 \mu\text{mol L}^{-1}$ can be found in waters receiving drainage from mining operations and abandoned copper mines [15]. Cu, at low concentration, is an essential micronutrient for the metabolism of different organisms including cyanobacteria and algae. At high concentration, Cu induced toxic effects such as inhibition of growth, respiration and nitrate uptake and loss of membrane integrity [16,17]. Copper is a strong inhibitor of photosynthesis [17] and reduces total thiol content in *Scenedesmus* sp. [18]. Copper induced the intracellular ROS accumulation in *P. subcapitata*, *Chlorella vulgaris* and *C. reinhardtii* [8,19],

Fig. 1. Impact of the heavy metals on the esterase activity of the alga *P. subcapitata*. Algal cells were exposed to heavy metals for 72 h. Esterase activity was evaluated using fluorescein diacetate. Each bar represents the mean of five independent experiments performed in quintuplicate ($n = 25$). The means with different letters are significantly different ($P < 0.05$).

as well as a strong up regulation of transcripts encoding enzymes associated with defence against oxidative stress [20].

Zinc is also a micronutrient required for many biological functions, which plays a structural and catalytic role in several enzymes [21]. In a polluted river, a zinc concentration of 172–667 $\mu\text{mol L}^{-1}$ was found [10]. High Zn concentration can promote severe toxic effects, which include inhibition of photosynthetic activity in the cyanobacterium *Synechocystis aquatilis* [22] and in the green alga *Scenedesmus* sp. [18]. Inhibition of growth, respiration and nitrate uptake [17], as well as the reduction of thiol content in *Scenedesmus* sp. [18] was also described.

The worldwide industrialization occurred in 19th and 20th century. As a consequence, ecological problems increased and triggered the development of acute and chronic laboratory toxicity tests [23]. The green alga *P. subcapitata* has been widely used to assess the toxicity of substances and wastes due to its availability, ease of culture, ecological relevance and sensitivity to toxicants [24]. The standard procedures are based on the potential effects of the toxicant on the algal population growing exponentially during 72–96 h [25,26]. The criterion most frequently used to evaluate the toxic effect is algal growth inhibition based on the change of biomass, determined by cell counts, dry weight or chlorophyll *a* measurements [24]. Despite the use of *P. subcapitata* in the toxicity assessment, namely in heavy metals toxicity studies, the physiological causes that result in the growth inhibition of algal population are not completely known.

In the present study, our objective was to contribute for the elucidation of the main targets of heavy metals toxicity in the alga *P. subcapitata*. For this purpose, the impact of Cd(II), Cr(VI), Cu(II) and Zn(II) on membrane integrity, esterase activity, mitochondrial function, photosynthetic activity and chlorophyll *a* content of the alga, exposed for 72 h to different concentrations of the toxicants, was evaluated. Principal component analysis (PCA) was carried out to determine the relationship among metabolic parameters and the growth inhibition of *P. subcapitata* cells exposed to different metals concentrations.

2. Material and methods

2.1. Strain, media and culture conditions

In this work, the freshwater green alga *Pseudokirchneriella subcapitata* (strain 278/4) was used. The original strain was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK.

The starter-cultures were prepared weekly by inoculating a loop of algal cells (from an agar slant) in 20 mL OECD algal test medium [26], in 100 mL Erlenmeyer flasks. Cultures with an initial cell concentration of 5×10^4 cells mL^{-1} were inoculated from the pre-cultures (in mid-exponential phase of growth) and incubated for 2 days, at 25 °C, on an orbital shaker at 100 rpm, under continuous “cool white” fluorescent light, with an intensity of 55 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the surface of the flask, as previously described [27].

Cell concentration was determined as previously described [28].

2.2. Metals exposure

Based on previous growth inhibition assays [27], algal cultures with 2 days of growth were exposed to three nominal concentrations of each metal: a low concentration [0.12 $\mu\text{mol L}^{-1}$ Cd(II), 2.7 $\mu\text{mol L}^{-1}$ Cr(VI), 0.080 $\mu\text{mol L}^{-1}$ Cu(II) and 0.15 $\mu\text{mol L}^{-1}$ Zn(II)]; an intermediate concentration [0.50 $\mu\text{mol L}^{-1}$ Cd(II), 11 $\mu\text{mol L}^{-1}$ Cr(VI), 0.32 $\mu\text{mol L}^{-1}$ Cu(II) and 0.60 $\mu\text{mol L}^{-1}$ Zn(II)] and a high concentration [1.9 $\mu\text{mol L}^{-1}$ Cd(II), 41 $\mu\text{mol L}^{-1}$ Cr(VI), 1.3 $\mu\text{mol L}^{-1}$ Cu(II) and 2.5 $\mu\text{mol L}^{-1}$ Zn(II)]. For this purpose,

Fig. 2. Visualization of mitochondria of the alga *P. subcapitata*. Microphotographs illustrative of rhodamine 123 (Rh123) stained cells.

appropriate volumes of CdCl_2 , $\text{K}_2\text{Cr}_2\text{O}_7$, $\text{Cu}(\text{NO}_3)_2$, and ZnCl_2 solutions were added from standard solutions (Merck) or from a primary standard of $\text{K}_2\text{Cr}_2\text{O}_7$. Cells were incubated for 72 h, in OECD medium, at 25 °C, on an orbital shaker at 100 rpm, under continuous light. In addition, control cultures were prepared without the addition of heavy metals.

Free metal concentrations (Table 1) were calculated using the MINEQL+ software (version 4.5) [29]. Computational studies were performed at pH 7.5 considering the total heavy metals and ligands concentrations, as well as all the affinity constants between heavy metals and the different ligands and solubility product constants [30].

Table 1

Loss of cell membrane integrity (SYTOX Green positive cells) of the alga *P. subcapitata*, exposed to heavy metals for 72 h, in function of total and free metal ion concentration.

Metal	Total [Me] ($\mu\text{mol L}^{-1}$)	Free [Me] ($\mu\text{mol L}^{-1}$)	SYTOX Green positive cells (%)
Control	0		0.5 ± 0.2
Cd(II)	0.12	2.4×10^{-4}	ND
	0.50	0.22	0.9 ± 0.9
	1.9	1.2	15.5 ± 2.4
Cr(VI)	2.7	2.4	0.2 ± 0.2
	11	9.9	2.0 ± 0.8
	41	37	38.1 ± 7.2
Cu(II)	8.0×10^{-2}	6.2×10^{-7}	0.4 ± 0.4
	0.32	7.6×10^{-3}	0.3 ± 0.3
	1.3	7.8×10^{-2}	55 ± 2
Zn(II)	0.15	7.2×10^{-4}	ND
	0.60	0.32	ND
	2.5	2.0	1.3 ± 0.5

ND – not determined.

SYTOX Green positive cells mean values were obtained from at least three experiments performed in duplicate ($n \geq 6$).

Fig. 3. Effect of the heavy metals on the mitochondria membrane potential of the alga *P. subcapitata*. Algal cells were exposed to the action of heavy metals, for 72 h, and subsequently stained with rhodamine 123. Each bar represents the mean of three independent experiments performed in quintuplicate ($n = 15$). The means with different letters are significantly different ($P < 0.05$).

2.3. Staining protocols

After contact with metal, cells were harvested by centrifugation ($2500 \times g$, 5 min) and suspended in OECD medium (membrane integrity and esterase activity) or 100 mmol L^{-1} PBS buffer (pH 7.0) (mitochondrial activity).

Membrane integrity of algal cells was monitored with the dye SYTOX Green (SG) (Molecular Probes, Invitrogen). SG is excluded in *P. subcapitata* algal cells with intact membrane. Cells with a damaged plasma membrane are penetrated by the fluorescent probe, which displayed a marked green fluorescent [31]. Cells ($1 \times 10^6 \text{ mL}^{-1}$) were incubated with $0.5 \mu\text{mol L}^{-1}$ SG, for 40 min, at 25°C , in the dark. The percentage of SG positive cells was determined by microscopy using a Leica DLMB epifluorescence microscope, equipped with a HBO-100 mercury lamp and a filter set GFP from Leica. In each experiment and for every metal concentration, four samples of at least 100 cells (total > 400 cells) were scored in randomly selected microscope fields. As a positive control (cells with permeabilized membrane), algal cells were heated at 65°C , for 1 h.

Esterase activity was measured using fluorescein diacetate (FDA) (Sigma–Aldrich). Cells ($5 \times 10^5 \text{ mL}^{-1}$) were stained with $20 \mu\text{mol L}^{-1}$ FDA, in the dark, at 25°C , for 40 min, as previously described [32]. Cell suspensions were dispensed in quintuplicate ($200 \mu\text{L}$ per well) in a 96-well flat microplate (Orange Scientific). Fluorescence intensity was measured in a microplate reader at a fluorescence excitation wavelength of 485/14 nm and an emission wavelength of 535/25 nm. Fluorescence was corrected (subtracting cell, culture medium and dye autofluorescence) and normalized considering the cell concentration. As negative control, algal cells were metabolically inactivated (65°C , for 1 h) and stained in the

same conditions of live cells. The percentage of esterase inhibition was calculated as previously described [32].

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated using rhodamine 123 (Rh123) (Sigma–Aldrich) as previously described [28]. Rh123 is a cell-permeant, cationic, probe that is concentrated by active mitochondria [33]. Cells ($1 \times 10^6 \text{ mL}^{-1}$) were stained with $5 \mu\text{mol L}^{-1}$ Rh123 solution, for 30 min, at 25°C , in the dark. After staining, cells were washed (two times) and suspended in PBS buffer. For each metal concentration, cells were dispensed in quintuplicate in a microplate and the fluorescence intensity was measured, corrected and normalized as described above (evaluation of esterase activity). Heavy metal toxicity was expressed as the ratio between the fluorescence in the assay and the fluorescence in the control (non-treated cells). As negative control, cells were treated with sodium azide (NaN_3 ; 20 mmol L^{-1}) (Sigma–Aldrich), for 10 min; subsequently, cells were stained with Rh123 as described above. The visualization of algal mitochondria was carried out using an epifluorescence microscope, equipped with a filter set GFP from Leica. Images were acquired with a Leica DC 300 F camera and processed using Leica IM 50-Image Manager software.

2.4. Determination of photosynthetic efficiency and chlorophyll *a* content

After metals exposure for 72 h, cells were centrifuged ($2500 \times g$, 5 min) and resuspended in OECD medium at $1 \times 10^7 \text{ cells mL}^{-1}$.

Photosynthetic activity was assessed using a pulse amplitude modulated (PAM) fluorescence assay. Algal cells were dark adapted for 30 min and chlorophyll *a* fluorescence was measured using a Walz JUNIOR-PAM chlorophyll fluorometer. PAM fluorometer

Fig. 4. Influence of the heavy metals on the photosynthetic performance of the alga *P. subcapitata*. Algal cells were exposed to the action of the heavy metals, for 72 h, and subsequently dark adapted for 30 min. Maximum quantum yield of PSII (ϕ_m) was evaluated using a PAM fluorometer. Each bar represents the mean of three independent experiments performed in sevenfold ($n = 21$). The means with different letters are significantly different ($P < 0.05$).

measured the minimal fluorescence (F_0) of dark-adapted cells and the maximum fluorescence (F_m) obtained with a saturating light pulse of $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The maximum quantum yield of PSII (ϕ_m) was automatically calculated, using the WinControl software (version 3.2.2), as the ratio F_v/F_m (F_v is variable fluorescence) from fluorescence values F_0 and F_m [34–36], according to the equation:

$$(\Phi_m) = \frac{F_v}{F_m} = \frac{(F_m - F_0)}{F_m}$$

The maximum PSII quantum yield is a measure of intrinsic (maximum) efficiency of PSII, i.e., the quantum efficiency if all PSII centres were open. This F_v/F_m ratio has been used as a sensitive indicator of the photosynthetic performance [35]. The impact of heavy metals on photosynthetic activity was expressed as ratio between the ϕ_m in the assay and the ϕ_m in the control.

The measurement of chlorophyll *a* (chl *a*) content was carried out using a spectrophotometric method as previously described [37]. The values of absorbance were corrected, for turbidity, by subtracting the value of the absorbance at 750 nm. Chl *a* content was calculated as previously described [38]. The values of chl *a* content were normalized considering the cell concentration. The impact of heavy metals on chl *a* content was expressed as the ratio between chl *a* content in the assay and chl *a* content in the control (non-treated cells).

2.5. Recovery studies

After treatment for 72 h with metal, in OECD culture medium, cells were harvested by centrifugation ($2500 \times g$, 5 min), washed (two times) and suspended in fresh OECD culture medium, at a density of 5×10^4 cells mL^{-1} . The growth was monitored daily as described above [28].

2.6. Reproducibility of the results and statistical analysis

Data presented are mean values $\pm$ SD (presented with 95% confidence value) of three-six independent experiments carried out under identical conditions. Statistical differences between non-treated and treated algal cells with heavy metals were tested by one-way ANOVA followed by Tukey–Kramer multiple comparison method.

Principal component analysis (PCA) [39,40] was carried out to determine the relationship among metabolic parameters (esterase activity, cell membrane integrity, maximum quantum yield of PSII, chlorophyll *a* content and mitochondrial membrane potential) and the growth of *P. subcapitata* cells exposed to different metals concentrations (see above, Section 2.2). The PCA was performed with the software CANOCO (Microcomputer Power), version 4.5.

3. Results

The algal cells of *P. subcapitata* were exposed to three nominal concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II), based on previous

Fig. 5. Influence of the heavy metals on the chlorophyll *a* content of the alga *P. subcapitata*. Algal cells were exposed to the action of the heavy metals, for 72 h, and subsequently chlorophyll *a* was determined spectrophotometrically. Each bar represents the mean of three independent experiments performed in triplicate ($n=9$). The means with different letters are significantly different ($P<0.05$).

growth inhibition assays [27]. Total and free metal ion concentrations are summarized in Table 1.

3.1. Impact of heavy metals on cell membrane integrity

Cell membrane integrity can be a critical target of the action of the pollutants. The exposure of *P. subcapitata* algal cells, in exponential phase of growth, to a low metal concentration [$2.7 \mu\text{mol L}^{-1}$ Cr(VI) and $8.0 \times 10^{-2} \mu\text{mol L}^{-1}$ Cu(II)] did not induce a loss of cell membrane integrity (Table 1). Similarly, $\leq 2\%$ of cell population loss membrane integrity (Table 1) when algal cells were exposed to heavy metals concentrations close to 72 h-EC₅₀ values [$0.50 \mu\text{mol L}^{-1}$ Cd(II), $11 \mu\text{mol L}^{-1}$ Cr(VI) and $0.32 \mu\text{mol L}^{-1}$ Cu(II)]. The exposure of algal cells to high concentrations [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI) and $1.3 \mu\text{mol L}^{-1}$ Cu(II)] resulted in a loss of cell membrane integrity. Zn(II) did not induce a loss of membrane integrity up to $2.5 \mu\text{mol L}^{-1}$ (Table 1).

These results show that Zn(II) inhibited growth in the absence of damage of cell membrane integrity. Cd(II), Cr(VI) and Cu(II) induced a reduction of algal growth (10–50%) in the absence of a loss of cell membrane integrity. Only the exposure to high concentration of these metals, where the growth was arrested, induced a loss of membrane integrity (Table 1). Algal cells exposed to $1.3 \mu\text{mol L}^{-1}$ Cu(II) for a short time period (30 min) retained membrane integrity. This result indicated that the loss of membrane integrity occurred in cells exposed to high Cu(II) concentration [$1.3 \mu\text{mol L}^{-1}$ Cu(II)] was not a rapid event. Together, these results prompted us to investigate the impact of heavy metals on algal metabolic activity.

3.2. Impact of heavy metals on esterase activity and mitochondrial function

The presence of Cd(II), Cr(VI), Cu(II) and Zn(II) reduced significantly ($P<0.05$) the esterase activity in a dose-dependent manner (Fig. 1). Even for the lowest concentrations tested [$0.12 \mu\text{mol L}^{-1}$ Cd(II), $2.7 \mu\text{mol L}^{-1}$ Cr(VI), $8.0 \times 10^{-2} \mu\text{mol L}^{-1}$ Cu(II) and $0.15 \mu\text{mol L}^{-1}$ Zn(II)], esterase activity was significantly ($P<0.05$) reduced (Fig. 1), which shows the high sensitivity of the esterases to the presence of metals.

In *P. subcapitata* algal cells, the mitochondria display a tubular arrangement along the inner face of the chloroplast as revealed by electron microscopy [41]. Mitochondrial morphology analysis by fluorescence microscopy, using the mitochondria-specific fluorescent dye rhodamine 123 (Rh123), confirmed this type of mitochondria morphology (Fig. 2).

Rh123 enters freely the cell by simple diffusion through the plasma membrane and is electrophoretically distributed in the mitochondrial matrix in response to mitochondrial membrane potential ($\Delta\Psi_m$) [42,43]. It was shown that Rh123 is a reliable method for mitochondrial function studies with algal cells [44]. In this context, Rh123 was used to investigate the changes in the mitochondrial bioenergetics in response to the presence of heavy metals. The exposure of algal cells to all Cu(II) concentrations or $0.15 \mu\text{mol L}^{-1}$ Zn(II) induced a significant ($P<0.05$) increase in mitochondrial membrane potential ($\Delta\Psi_m$) (hyperpolarisation) (Fig. 3). The exposure to $1.9 \mu\text{mol L}^{-1}$ Cd(II), 11 and $41 \mu\text{mol L}^{-1}$ Cr(VI) or 0.60 and $2.5 \mu\text{mol L}^{-1}$ Zn(II) induced a significant ($P<0.05$)

Fig. 6. PCA biplots representing the variation in the metabolic parameters (arrows) and the growth of algal cells (circles) not exposed (control) or exposed to heavy metals at different 72 h-EC values. Metabolic parameters: esterase activity (EA), cell membrane integrity (MI), maximum quantum yield of PSII (ϕ_m), chlorophyll *a* content (chl *a*) and mitochondrial membrane potential ($\Delta\Psi_m$).

reduction of the $\Delta\Psi_m$ (Fig. 3). As negative control, algal cells were treated with NaN₃ (inhibitor of mitochondrial electron transport chain). Cells treated with NaN₃ displayed a very significant ($P < 0.01$) reduction of the $\Delta\Psi_m$ (data not shown), comparatively to non-treated cells, and did not display fluorescent mitochondria upon staining with Rh123 (Fig. 2).

3.3. Impact of heavy metals on photosynthetic activity and chlorophyll *a* content

The effect of heavy metals on photosynthetic activity of algal cells was assessed using a pulse amplitude modulated (PAM) fluorescence assay. Non treated algal cells (control) displayed a maximum quantum yield of PSII (ϕ_m) of 0.63 ± 0.03 ($n = 33$), which is in agreement with the values described in the literature for *P. subcapitata* (0.62–0.64) [45,46]. The exposure of algal cells to the lowest [$0.12 \mu\text{mol L}^{-1}$ Cd(II), $2.7 \mu\text{mol L}^{-1}$ Cr(VI), $8.0 \times 10^{-2} \mu\text{mol L}^{-1}$ Cu(II) and $0.15 \mu\text{mol L}^{-1}$ Zn(II)] or intermediary concentrations [$0.50 \mu\text{mol L}^{-1}$ Cd(II), $11 \mu\text{mol L}^{-1}$ Cr(VI), $0.32 \mu\text{mol L}^{-1}$ Cu(II) and $0.60 \mu\text{mol L}^{-1}$ Zn(II)] of the different heavy metals did not provoke a significant ($P < 0.05$) modification of ϕ_m (Fig. 4). Photosynthetic performance was significantly ($P < 0.05$) decreased when algal cells were incubated with the highest metal concentrations [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI), $1.3 \times 10^{-2} \mu\text{mol L}^{-1}$ Cu(II) and $2.5 \mu\text{mol L}^{-1}$ Zn(II)]. This effect was markedly observed for Cr(VI) (Fig. 4).

The exposure of algal cells to the lowest concentrations of all metals did not modify significantly ($P < 0.05$) the chl *a* content (Fig. 5). The incubation of algal cells with intermediary concentrations of Cr(VI) or Zn(II), provoked a significant reduction ($P < 0.05$) of the pigment (Fig. 5). The exposure of algal cells to the highest

metals concentrations induced a significant reduction ($P < 0.05$) of the chl *a* (Fig. 5).

Taken together, the results presented above suggest that photosynthesis was not affected for the lowest metals concentration, where a 10% growth reduction was observed. For the highest concentrations tested, which arrested algal growth, the photosynthesis was affected as revealed by the decrease of maximum quantum yield of PSII and the reduction of chl *a* content. For intermediary values [$0.50 \mu\text{mol L}^{-1}$ Cd(II), $11 \mu\text{mol L}^{-1}$ Cr(VI), $0.32 \times 10^{-2} \mu\text{mol L}^{-1}$ Cu(II) and $0.60 \mu\text{mol L}^{-1}$ Zn(II)], the maximum quantum yield of PSII was not modified (Fig. 4); however, a reduction of chl *a* content was observed for Cr(VI) and Zn (II) (Fig. 5).

3.4. Variation among metabolic parameters and growth of algal cells exposed to metals

In order to evaluate the weight of each metabolic parameter studied on the growth inhibition of algal cells exposed to heavy metals, a PCA was carried out. In the PCA biplots presented in Fig 6, each metabolic parameter is represented by an arrow. The arrow direction indicates the greatest change for each measured parameter, whereas the length of the arrow is related to the variation of each parameter among the analysed samples. Samples are represented by circles.

The variance of the metabolic parameters on the algal cells growth was plotted along axis 1 which depicted 92.3%, 86.8%, 88.9% and 84.9% of the total extracted variance for Cd(II), Cr(VI), Cu(II) and Zn(II), respectively (Fig. 6). For Cr(VI), the parameters most related to this variation were esterase activity (EA) and chlorophyll *a* (chl *a*), which contributed to the separation of the control from cells exposed to different EC values, being 72 h-EC₉₀ the most

Fig. 7. Growth-recovery of *P. subcapitata* algal cells exposed to heavy metals. Algal cells were exposed to the heavy metals, for 72 h. Then, cells were washed and re-inoculated at 5×10^4 cells mL⁻¹ in OECD medium. Each point represents the mean of two independent experiments performed in duplicate.

different (Fig. 6). The weight of these two metabolic parameters was corroborated when the PCA was done including only EA and chl *a*, describing 98.9% of total extracted variance of the samples; in this situation, a gradient according to the increase of metal concentration was observed (data not shown). Cd(II) biplot did not present a gradient that allow the separation of the samples according to metal concentration. Nevertheless, it was possible to observe that 72 h-EC₉₀ was the most different with the lowest values for all parameters (Fig. 6). The same distribution of samples was obtained when EA and chl *a* were used in PCA (data not shown). In the case of Cu(II), it was not possible to separate all the samples. Only 72 h-EC₉₀ was distinct from the others (Fig. 6). The separation of the control and 72 h-EC₁₀ from the 72 h-EC₅₀ and 72 h-EC₉₀ was possible when the PCA was done only with the FDA and chl *a*, demonstrating the sensitivity of these two metabolic parameters to metal concentration (data not shown). Zn(II) biplot showed that EA, chl *a* and mitochondrial membrane potential ($\Delta\Psi_m$) had a higher contribute to the variation among the analyzed samples (higher arrows) (Fig. 6). Nevertheless, these metabolic parameters did not create a gradient of sensitivity with the increase of metal concentration. Two groups are depicted in the biplot: one group which included control and 72 h-EC₁₀; and the other group composed by 72 h-EC₅₀ and 72 h-EC₉₀, with higher and lower values of EA, chl *a* and $\Delta\Psi_m$, respectively (Fig. 6). When only EA and chl *a* were included in the PCA, the percentage of variance depicted along axis 1 increased to 96.8%; control and 72 h-EC₁₀ were more separated along this axis (data not shown).

3.5. Recovery assays

After exposure, for 72 h, to the action of different heavy metals, algal cells were washed and suspended in fresh culture medium at 5×10^4 cells mL⁻¹. The exposure to heavy metals provoked a loss of esterase activity (Fig. 1), in all conditions tested. However, cells were able to resume the growth in a similar way to the control (cells not exposed to heavy metal) (Fig. 7). These results are consistent with those obtained with SYTOX Green, where no loss of membrane integrity was observed when the cells were exposed to 72 h-EC₁₀ and 72 h-EC₅₀ heavy metals values (Table 1). Cells exposed to the highest concentration of the different heavy metals [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI), $1.3 \mu\text{mol L}^{-1}$ Cu(II) and $2.5 \mu\text{mol L}^{-1}$ Zn(II)] re-started the growth more slowly, comparatively to the control (Fig. 7).

4. Discussion

Algae are in the base of food chain and stand in the front line of the aquatic pollution exposure. The impact of three concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II) on the membrane integrity, metabolic activity and photosynthesis of the alga *P. subcapitata* was investigated.

Metal ions can provoke membrane damage and lead to disruption of cellular homeostasis [47]. In the present study, the exposure to high metal concentration [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI) and $1.3 \mu\text{mol L}^{-1}$ Cu(II)] for 72 h induced the loss of

membrane integrity. This effect is particularly noticeable for Cu(II) and Cr(VI) (Table 1). Cid et al. [16] observed that the marine diatom *Phaeodactylum tricornutum* population presented 41% cells with compromised membrane when treated with $8 \mu\text{mol L}^{-1}$ Cu(II) for 72 h. The results presented here show the high sensitivity of *P. subcapitata* to Cu(II) since a higher percentage of cells (55%) displayed disrupted plasma membrane with a lower ($1.3 \mu\text{mol L}^{-1}$) Cu(II) concentration (Table 1). The loss of cell membrane integrity in algal cells exposed for 72 h at high concentrations of Cr(VI) and Cu(II) can explain, at least partially, the delay of growth recovery (Fig. 7). In fact, in the beginning of the recovery assay, only 62 and 45% of the cells presented intact membrane, after being exposed to Cr(VI) and Cu(II), respectively.

The cellular membrane, should not be the primary/immediate target of the action of the metals studied, by the following reasons: (i) all metal tested induced a growth inhibition of 10–50% without loss of membrane integrity; (ii) the exposure of algal cells to high metals concentrations [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI) and $2.5 \mu\text{mol L}^{-1}$ Zn(II)], for 6 h, did not induce the loss of membrane integrity [28]; a similarly result was obtained when algal cells were exposed to $1.3 \mu\text{mol L}^{-1}$ Cu(II) for 30 min. These results strongly suggest that the disruption of membrane integrity, observed for high metals concentrations, was not a rapid event. Probably, the metals induced the reduction of algal growth and metabolic activity; subsequently, occurred the loss of membrane integrity in a so called route from “live” to “dead” [48].

The activity of intracellular esterases was assessed using FDA as a substrate. FDA is hydrolysed by esterases to fluorescein, which exhibits a green fluorescence. Thus, the fluorescence intensity depends primarily on the esterase activity of the cells and reflects the functional (metabolic) status of the living cell [49,50]. Cd(II), Cr(VI), Cu(II) and Zn(II) inhibited the esterase activity of the algal cells of *P. subcapitata*, in a dose-dependent manner (Fig. 1). These results are consistent with those describing a loss of esterase activity after a short time exposure (1–6 h) of the alga *P. subcapitata* to Cu(II) [28,32,51,52], Cd(II) or Zn(II) [28,52]. These metals may interact with the active centre of esterases or modify the conformation of the enzymes, provoking a decrease of the esterase activity. The results presented here show the high sensitivity of these enzymes to the presence of heavy metals and reinforce the potential usefulness of the measurement of esterase activity of algal cells as an alternative endpoint for the detection of metal toxic effect.

Mitochondria are the only known organelle that displays a strong membrane potential ($\Delta\Psi_m$), with a negative charge inside. The movement of electrons through the electron transport chain in the inner mitochondrial membrane originates a transmembrane electrochemical gradient necessary for ATP production by ATP synthase [53]. Thus, $\Delta\Psi_m$ reflects the mitochondrial activity. In the present work, Rh123 was used to evaluate the mitochondrial bioenergetics of *P. subcapitata* algal cells exposed to different heavy metals. This fluorescent probe have been used in mitochondrial function studies with a variety of cell types, such as animal [54,55], plant [56], yeast [43] and algal cells [57,58]. In recent years, bioenergetic studies in algal cells carried out with Rh123 were validated using a polarographic oxygen electrode [44].

The inhibition of ATP synthase, by oligomycin, resulted in an increase of $\Delta\Psi_m$ and, consequently, an augment of mitochondrial staining with Rh123 [43]. Similarly, the exposure of *P. subcapitata* algal cells to all Cu(II) concentrations or $0.15 \mu\text{mol L}^{-1}$ Zn(II) provoked an increase of mitochondrial fluorescence (Fig. 3), which can be attributed to the damage of mitochondrial function (energy production). An analogous effect (enhancement of Ψ_m) was described in the freshwater alga *Scenedesmus obliquus* exposed to perfluorocarboxylic and perfluorosulfonic acids for 72 h [57].

The treatment of *P. subcapitata* cells with sodium azide (electron transport chain inhibitor) originated the abolition of

mitochondrial respiratory function with the consequent reduction of $\Delta\Psi_m$. Under this condition, Rh123 accumulation was practically abolished (Fig. 2). Cd(II) and Cr(VI) at defined concentrations seems to have a similar effect. *P. subcapitata* cells exposed to $1.9 \mu\text{mol L}^{-1}$ Cd(II), 11 or $41 \mu\text{mol L}^{-1}$ Cr(VI), displayed a reduction of mitochondrial staining with Rh123 (Fig. 3). The impact of Zn(II) on mitochondrial function was concentration dependent. At low concentrations, an increase of $\Delta\Psi_m$ was observed; at intermediate and high concentrations, a reduction of $\Delta\Psi_m$ occurred (Fig. 3). A similar pattern of perturbation of mitochondrial function was described by Brickley et al. [44] in *C. reinhardtii* exposed to an herbicide.

Photosynthetic pigments are organized in clusters called *photosystem I* (PSI) and *photosystem II* (PSII). The maximum quantum yield of PSII (ϕ_m) allows to indicate the potential capacity of photosynthetic cells to convert light energy into chemical energy [59]. At higher concentrations [$1.9 \mu\text{mol L}^{-1}$ Cd(II), $41 \mu\text{mol L}^{-1}$ Cr(VI), $1.3 \mu\text{mol L}^{-1}$ Cu(II) and $2.5 \mu\text{mol L}^{-1}$ Zn(II)], the metals tested provoked a perturbation on algal photosynthesis, as revealed by the reduction of ϕ_m (Fig. 4), which was accompanied to the decrease of chl *a* content (Fig. 5). Similarly, a reduction of 30% of ϕ_m in *P. subcapitata* exposed to $0.16 \mu\text{mol L}^{-1}$ Cu for 96 h was described [59]. Studies with *Chlorella pyrenoidosa* also showed a reduction of maximum quantum yield when algal cells were exposed for 96 h to Cd (II) [60] or Cr(VI) [61]. The exposure of *M. denticulata* to $150 \mu\text{mol L}^{-1}$ Cd for 4 days, inhibited almost completely the function of PSII [7]. The inhibition of the activity of PSII in the cyanobacterium *S. aquatilis*, exposed to Zn(II), was also described [22]. Additionally, the reduction of chl *a* content was also observed in the alga *Scenedesmus* sp. exposed to Cu (2.5 and $10 \mu\text{mol L}^{-1}$) and Zn (5 and $25 \mu\text{mol L}^{-1}$) [17]. Probably, heavy metals inhibit chl *a* biosynthesis [62].

The exposure of algal cells to $11 \mu\text{mol L}^{-1}$ Cr(VI) or $0.60 \mu\text{mol L}^{-1}$ Zn(II) did not affect, significantly ($P < 0.05$) the ϕ_m ; however, a decrease of chl *a* content was observed (Fig. 4 and 5), which suggest a different adaptation response to heavy metals stress. The results here presented are in agreement with those which describe that Cr(VI), at $\sim 7 \mu\text{mol L}^{-1}$, induced a decrease in chl *a* and chl *b* as well as down-regulated photosynthetic proteins in *P. subcapitata* [14].

In conclusion, the exposure of *P. subcapitata* to Cd(II), Cr(VI), Cu(II) and Zn(II), for 72 h, had a negative impact on alga physiology and metabolism. Although a compromising of membrane integrity was observed for higher concentrations of Cd(II), Cr(VI) and Cu(II), cell membrane should not be the primary target of the action of metals. The main toxicity targets of the heavy metals under study have an intracellular localization. The PCA biplots revealed that the inhibition of esterase activity combined with the reduction of chlorophyll *a* content were related to the growth inhibition caused by the prolonged exposure (72 h) to Cr(VI) and Cu (II). In the case of Zn (II), in addition to these metabolic parameters, the damage of mitochondrial function (with the consequent impairment of energy production) was related with the growth inhibition caused by the exposure to this metal. The identification of the targets of the heavy metals studied contributes to the elucidation of the mechanisms of action of these toxicants on the alga *P. subcapitata*.

Conflict of interest

Authors declare no conflict of interest.

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