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Use of a fluorescence-based approach to assess short-term responses of the alga *Pseudokirchneriella subcapitata* to metal stress

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Abstract This work explores the use of fluorescent probes to evaluate the responses of the green alga *Pseudokirchneriella subcapitata* to the action of three nominal concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II) for a short time (6 h). The toxic effect of the metals on algal cells was monitored using the fluorochromes SYTOX Green (SG, membrane integrity), fluorescein diacetate (FDA, esterase activity) and rhodamine 123 (Rh123, mitochondrial membrane potential). The impact of metals on chlorophyll *a* (Chl *a*) autofluorescence was also evaluated. Esterase activity was the most sensitive parameter. At the concentrations studied, all metals induced the loss of esterase activity. SG could be used to effectively detect the loss of membrane integrity in algal cells exposed to 0.32 or 1.3 μmol L^{-1} Cu(II). Rh123 revealed a decrease in the mitochondrial membrane potential of algal cells exposed to 0.32 and 1.3 μmol L^{-1} Cu(II), indicating that mitochondrial activity was compromised. Chl *a* autofluorescence was also affected by the presence of Cr(VI) and Cu(II), suggesting perturbation of photosynthesis. In conclusion, the fluorescence-based approach was useful for detecting the disturbance of specific cellular characteristics. Fluorescent probes are a useful diagnostic tool for the assessment of the impact of toxicants on specific targets of *P. subcapitata* algal cells.

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Introduction

Living organisms require trace amounts of some metals, including Zn, Fe, Cu and Co. However, excessive levels of these metals may be highly toxic. Heavy metals tend to accumulate along the food chain, representing potential hazards for the environment and for humans (Bleackley and MacGillivray 2011).

As primary producers, microalgae are the basis of the food chain in aquatic systems and serve as the main food source of the other trophic levels. Any significant change in primary producers will have a strong impact on the ecosystem overall. Conventional chemical analyses can be used to quantify heavy metals at very low levels (ppb). Nevertheless, these measurements do not provide any information about their effects on living organisms. For regulatory purposes, standard algal toxicity tests (US-EPA, OECD and ISO) have been proposed. The green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák has been widely used to measure chronic toxicity (i.e. inhibition of growth over 72–96 h) (US-EPA 2002; OECD 2011). *P. subcapitata* is suitable for the evaluation of toxicity because it exhibits a higher level of sensitivity to heavy metals and herbicides when compared with other algae (Rojickova-Padrtova and Marsalek 1999).

Conventional long-term toxicity tests measure slow responses to toxicants (days). Thus, the development of early warning systems that can detect pollutants within few hours using expeditious techniques will be useful in aquatic toxicology. To this end, attempts have been made to reduce both the time of exposure and the volume of toxicant and to perform automatic readings of the samples (Pardos et al. 1998; van der

Heever and Grobbelaar 1998; Eisentraeger et al. 2003; Fai et al. 2007; Machado and Soares 2012). Blaise et al. (1986) reported the use of a 4-h test. The impact of toxicants on algal cells was detected by ATP measurement. Franklin et al. (2005) and van der Heever and Grobbelaar (1998) described 3- and 4-h assays, respectively, based on evaluations of toxic effects on esterase activity as measured by flow cytometry. A short-term assay (3–6 h) based on the inhibition of esterase activity was also developed by Machado and Soares (2013) using the alga *P. subcapitata*. This assay was carried out in small volumes using microplates, and fluorescence readings were automatically carried out in a microplate reader.

Fluorescent probes can be useful in detecting early responses of algae to toxicants. SYTOX Green (SG) has been used for the assessment of membrane integrity in cyanobacteria, diatoms, dinoflagellates and green algae (Sato et al. 2004; Ribalet et al. 2007; Timmermans et al. 2007; Segovia and Berges 2009; Chang et al. 2011; Peperzak and Brussaard 2011). SG is excluded by intact plasma membranes; thus, SG can penetrate cells with a damaged plasma membrane. This probe exhibits bright green fluorescence upon binding with nucleic acids in permeabilised (dead) cells (SG-positive cells). SG was used to evaluate the toxic impact of CeO_2 nanoparticles (Rogers et al. 2010), rice paddy herbicides (Nagai et al. 2011) and 1-pentanol (Machado and Soares 2012) on *P. subcapitata*.

Enzymes, namely, esterases, have been recognised as potentially useful for the detection of early responses to toxicants in a variety of microorganisms (Dorsey et al. 1989; Blaise et al. 2000; Bitton 2005; Peperzak and Brussaard 2011). Esterase activity has been evaluated using fluorescein diacetate (FDA). This probe is a non-fluorescent, lipophilic molecule that is readily taken up by algal cells. Inside viable cells, FDA is cleaved by non-specific esterases to liberate fluorescein, a highly polar compound that displays strong green fluorescence. As the toxic effect increases, the enzyme activity (and consequently the fluorescence intensity) is reduced. Cells that are metabolically inactive do not display green fluorescence. Therefore, the fluorescence intensity of fluorescein has been used as a marker of the metabolic activity of algal cells. This compound has been used to evaluate the toxicity of heavy metals (Arsenault et al. 1993; Snell et al. 1996; Franklin et al. 2001a; Machado and Soares 2013) and organic toxicants (Gilbert et al. 1992; Snell et al. 1996; Debenest et al. 2011); additionally, it has been used in natural samples (Gilbert et al. 1992; Blaise and Menard 1998; Regel et al. 2002).

One of the dominant roles of mitochondria is the production of ATP through respiration. At the mitochondrial inner membrane, the passage of electrons through the electron transport chain generates an electrochemical proton gradient across the membrane. Therefore, the impact of pollutants in algal cells can also be determined by the measurement of mitochondrial membrane potential ($\Delta\Psi m$). Rhodamine 123 (Rh123) is a cell-permeant cationic fluorophore that is readily accumulated by cells in direct proportion to the $\Delta\Psi m$ (Johnson et al. 1980). The measurement of Rh123 fluorescence has been used to determine the toxic impact of environmental contaminants on the alga *Scenedesmus vacuolatus* (Adler et al. 2007). However, the usefulness of this fluorescent probe in the evaluation of toxicity using *P. subcapitata* algal cells has not been examined.

Chlorophyll *a* (Chl *a*) is a characteristic pigment that exhibits endogenous red (emission at 680 nm) fluorescence (autofluorescence). The quantification of Chl *a* autofluorescence has been used for monitoring photosynthetic capacity (Hyka et al. 2013). Thus, the measurement of the Chl *a* fluorescence of green algae can be used for the detection of compounds with negative effects on photosynthesis (Franklin et al. 2001b; Fai et al. 2007).

Our objective in the present study was to use fluorescent dyes for the evaluation of heavy metal-induced metabolic and structural changes in the alga *P. subcapitata* after short-term exposure (6 h). For this purpose, algal cells were exposed to different heavy metals [Cd(II), Cr(VI), Cu(II) and Zn(II)], and the toxic effects were assessed using SG (membrane integrity), FDA (esterase activity) and Rh123 (mitochondrial membrane potential). Additionally, the impact of heavy metals on algal autofluorescence was also investigated. The usefulness of these fluorescent markers as an early diagnostic tool for heavy metal toxicity is discussed.

Materials and methods

The freshwater green alga *Pseudokirchneriella subcapitata* (strain 278/4) obtained from the Culture Collection of Algae and Protozoa (CCAP), UK, was used. The alga was maintained in OECD algal test medium (OECD 2011) with 20 g L^{-1} agar (Merck), in the dark, at 4 °C. Medium stock solutions were prepared according to OECD guidelines (OECD 2011).

The starter cultures were prepared weekly by inoculating a loop of algal cells (from agar slant) in 20-mL OECD medium, in 100-mL Erlenmeyer flasks. The cells were incubated for 2 days, at 25 °C, on an orbital shaker at 100 rpm, under continuous "cool white" fluorescent light (fluorescent lamps with a colour temperature of 4,300 K), with an intensity of 55 μmoL photons m^{-2} s^{-1} at the surface of the flask, verified using an illumination meter. The pre-cultures were prepared by inoculating 40-mL OECD medium, in 100-mL Erlenmeyer flasks with an initial cell concentration of ~5×10^4 cells mL^{-1} from the starter cultures. The cells were incubated for 2 days under the conditions described above for the starter cultures. 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 ~5×10^4 cells mL^{-1} from the pre-culture. Cells were incubated under the conditions described above for the starter cultures.

Cell concentration was determined using an automated cell counter (TC10, Bio-Rad) or by measuring the absorbance at 750 nm, according to US-EPA (2002); a calibration curve (number of cells versus absorbance) was first performed. For low cell concentration, a cuvette with a light path of 4 cm was used.

Exposure to heavy metals

Algal cells in exponential phase of growth (2 days) were harvested by centrifugation (2,500×*g*, 5 min) and suspended at 5.5×10^6 cells mL^{-1} in deionised water. Three nominal concentrations of each metal [Cd(II), Cr(VI), Cu(II) and Zn(II)] were used, as previously defined (Machado and Soares 2014): low concentrations [0.12 μmol L^{-1} Cd(II), 2.7 μmol L^{-1} Cr(VI), 0.080 μmol L^{-1} Cu(II), 0.15 μmol L^{-1} Zn(II)], medium concentrations [0.50 μmol L^{-1} Cd(II), 11 μmol L^{-1} Cr(VI), 0.32 μmol L^{-1} Cu(II), 0.60 μmol L^{-1} Zn(II)] and high concentrations [1.9 μmol L^{-1} Cd(II), 41 μmol L^{-1} Cr(VI), 1.3 μmol L^{-1} Cu(II), 2.5 μmol L^{-1} Zn(II)]. The low and medium concentrations corresponded, approximately, to the values of the heavy metals that induced the inhibition of 10 and 50 % of algal growth, during 72 h (72-h EC_{10} and 72-h EC_{50} values), respectively, when compared to the control (cells not exposed to the metals). For the highest concentrations (>72 h EC_{90} values), the complete arrest of algal growth was observed.

The assays were carried out in 1-L Erlenmeyer flasks containing OECD algal test medium. A convenient volume of metal was added from stock solutions (Merck) of $CdCl_2$, $Cu(NO_3)_2$ and $ZnCl_2$ or from a primary standard $K_2Cr_2O_7$ solution. The flasks were inoculated with the algal suspension (final cell concentration 5×10^4 cells mL^{-1}). The final volume of the assay was 400 mL. As a control, the cells were inoculated under the same conditions used for the assays; however, metals were omitted. The flasks were incubated at 25 °C for 6 h on an orbital shaker at 100 rpm under continuous "cool white" fluorescent light.

After contact with metal, the algal cell suspensions were centrifuged (2,500×*g*, 5 min, 4 °C) and resuspended in fresh OECD culture medium or in phosphate-buffered saline (PBS; 0.1 mol L^{-1}, pH 7.0) at a concentration of 1×10^6 cells mL^{-1}. Toxicity was evaluated using four parameters: membrane integrity, esterase activity, mitochondrial membrane potential and Chl *a* fluorescence.

Staining with fluorochromes

Plasma membrane integrity was determined using the fluorescent probe SYTOX Green (SG) (Molecular Probes, Invitrogen). The staining was carried out as previously described (Machado and Soares 2012). Briefly, 10 μL of a working solution of SG (50 μmol L^{-1}) was added to 1 mL of each suspension, followed by incubation in the dark for 20 min; the final dye concentration was 0.5 μmol L^{-1}. The stock solution of SG (5 mmol L^{-1}) was provided in dimethyl sulphoxide (DMSO) and stored at −20 °C. Intermediary and working solutions were prepared before use by diluting the stock solution in OECD medium. In the assay, the final concentration of DMSO was ≤0.04 % (*v*/*v*). As positive control, algal cells were heat-treated to permeabilise the plasma membrane, as previously described (Machado and Soares 2012). The cells were observed using a Leica DLMB epifluorescence microscope equipped with an HBO-100 mercury lamp and the GFP filter set from Leica (excitation filter, BP 470/40; dichromatic mirror, 500; suppression filter, BP 525/50). In each assay, three samples of ~200 cells were scored in randomly selected microscope files.

Esterase activity was assessed using fluorescein diacetate (FDA) (Sigma-Aldrich). Due to the strong fluorescent signal produced by FDA, algal cells were diluted to 5×10^5 cells mL^{-1} in OECD medium. Subsequently, the cells were incubated with FDA at a final concentration of 20 μmol L^{-1} in the dark at 25 °C for 40 min, as previously described (Machado and Soares 2013). Cells that were not exposed to metals (metabolically active, positive control) and heat-treated cells (65 °C for 1 h) (negative control) were also used. The assays were carried out in sterile 96-well flat microplates (Orange Scientific), in which 200 μL of each sample was dispensed in quintuplicate. Fluorescence intensity (in relative fluorescent units, RFUs) was measured in a Perkin-Elmer (Victor^3) 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 normalised (considering the cell concentration). The percentage of esterase inhibition (EI %) was calculated using the following equation:

$$\mathrm{EI}\ (\%) = 100-[(F_a/F_{\max}) \times 100] \quad (1)$$

where F_a is the fluorescence of algal cells in the assay (metal-treated cells) and $F_{\max}$ is the mean fluorescence of the samples in which all the cells are metabolically active (live cells, positive control).

Mitochondrial membrane potential (ΔΨm) was evaluated using Rhodamine 123 (Rh123) (Sigma-Aldrich). Algal cells were harvested by centrifugation (2,500×*g*, 5 min), suspended in 0.1 mol L^{-1} PBS buffer (pH 7.0) at a final concentration of 1×10^6 cells mL^{-1} and stained with a 2.5-mmol L^{-1} working solution of Rh123 (prepared in PBS buffer before use) for 30 min at 25 °C in the dark; the final concentration of Rh123 was 5 μmol L^{-1}. A 25-mmol L^{-1} Rh123 stock solution was prepared in water and stored at −20 °C. After staining, the cells

were washed twice and suspended in PBS. For each metal concentration, the cell suspensions were dispensed in quintuplicate into a microplate (200 μL per well), and the fluorescence intensity was measured as described above (evaluation of esterase activity). Fluorescence was corrected and normalised as described above. As a negative control, algal cells were treated for 10 min with 20 mmol L^{-1} sodium azide (NaN_3) (Sigma-Aldrich). Subsequently, the cells were stained with Rh123. The toxic effect of heavy metals was expressed as the ratio of fluorescence in the assay/fluorescence in the control (cells not exposed to heavy metals).

Chl *a* fluorescence (autofluorescence) was assessed in 96-well microplates; ten 200-μL aliquots (1×10^6 cells mL^{-1} in OECD medium) were dispensed into the wells. As a control, cells were incubated under the same conditions used in the assays and metals were omitted. Fluorescence intensity (RFU) was measured in a microplate reader at a fluorescence excitation wavelength of 485 nm and an emission wavelength of 680 nm. Fluorescence was corrected (by subtracting culture medium autofluorescence) and normalised (considering cell concentration). The toxic effect was expressed as described above for the evaluation of ΔΨm.

Reproducibility of the results and statistical analysis

Data presented are mean values of three to six independent experiments carried out under identical conditions. The data reported are the mean±standard deviation, presented with 95 % confidence value. Statistical differences among non-treated and heavy metal-treated algal cells were tested by one-way ANOVA followed by Tukey-Kramer multiple comparison method.

Results

Cd(II), Cr(VI), Cu(II) and Zn(II) are considered priority pollutants by the US-EPA (2006). However, little is known about the specific cell targets in the alga *P. subcapitata* that are affected due to short-term exposure to metals. At present, fluorescent dyes are available that can be used to monitor distinct biochemical properties. These dyes provide the ability to monitor the physiological responses of algal cells.

The labelling of algal cells exposed to heavy metals with SYTOX Green (SG) allowed cells with an intact membrane to be distinguished from the cells with a damaged or permeabilised plasma membrane. The incubation of algal cells with 0.32 and 1.3 μmol L^{-1} Cu(II) provoked the permeabilisation of the cell membrane in 10 and 23 % of the cells, respectively. A ≤5 % loss of membrane integrity was observed for 0.08 μmol L^{-1} Cu(II). Similarly, Cd(II), Cr(VI) and Zn(II) did not damage the cell membrane at the concentrations tested (Table 1).

Table 1 Loss of cell membrane integrity of the alga *P. subcapitata* exposed to heavy metals for 6 h.

Metal (μmol L^{-1})	SYTOX Green positive cells (%)
Cd(II)	
1.9	0.9±0.6
Cr(VI)	
41	2±2
Cu(II)	
0.08	5±3
0.32	10±4
1.3	23±3
Zn(II)	
2.5	1.7±0.6

The mean values were obtained from three experiments performed in duplicate (n=6). For each experiment, at least 600 cells were scored in randomly selected microscope fields

Once inside live cells, fluorescein diacetate (FDA) is hydrolysed to form a fluorescent product (fluorescein), which is considered a marker of cytosol esterase activity. A decrease in fluorescence can be used as an indicator of a loss of metabolic activity (esterase activity). The green fluorescence decreased very significantly (P<0.01) in algal cells exposed to all metals, even at low concentrations (Fig. 1). The results presented show that the inhibition of esterase activity, evaluated with FDA, is a very sensitive parameter.

Rhodamine 123 (Rh123) is concentrated by active mitochondria and serves as a marker of mitochondrial membrane potential (ΔΨm). The specificity of mitochondrial staining was verified by incubation of algal cells with 20 mmol L^{-1} NaN_3 (negative control) prior to staining with Rh123. The treatment of algal cells with an electron transport inhibitor reduced the fluorescence very significantly (P<0.01) (data not shown). The exposure of algal cells to Cu(II) at 0.32 and 1.3 μmol L^{-1} provoked a very significant (P<0.01) reduction of Rh123 fluorescence, most likely as a consequence of a ΔΨm reduction. No significant (P<0.01) reduction or increase (hyperpolarisation) of ΔΨm was observed for Cd(II), Cr(VI) and Zn(II) in the concentration ranges tested (Fig. 2).

Chl *a* is a characteristic algal pigment that constitutes approximately 1–2 % (dry weight) of algal biomass and exhibits a characteristic red autofluorescence. A 6-h exposure of algal cells to intermediate or high concentrations of Cr(VI) or Cu(II) very significantly increased (P<0.01) Chl *a* autofluorescence (Fig. 3). Cd(II) and Zn(II) did not significantly (P<0.01) modify Chl *a* autofluorescence (Fig. 3) in the concentration ranges tested.

Discussion

In the present work, specific fluorescent dyes were used to monitor different biochemical properties. The impact of short-

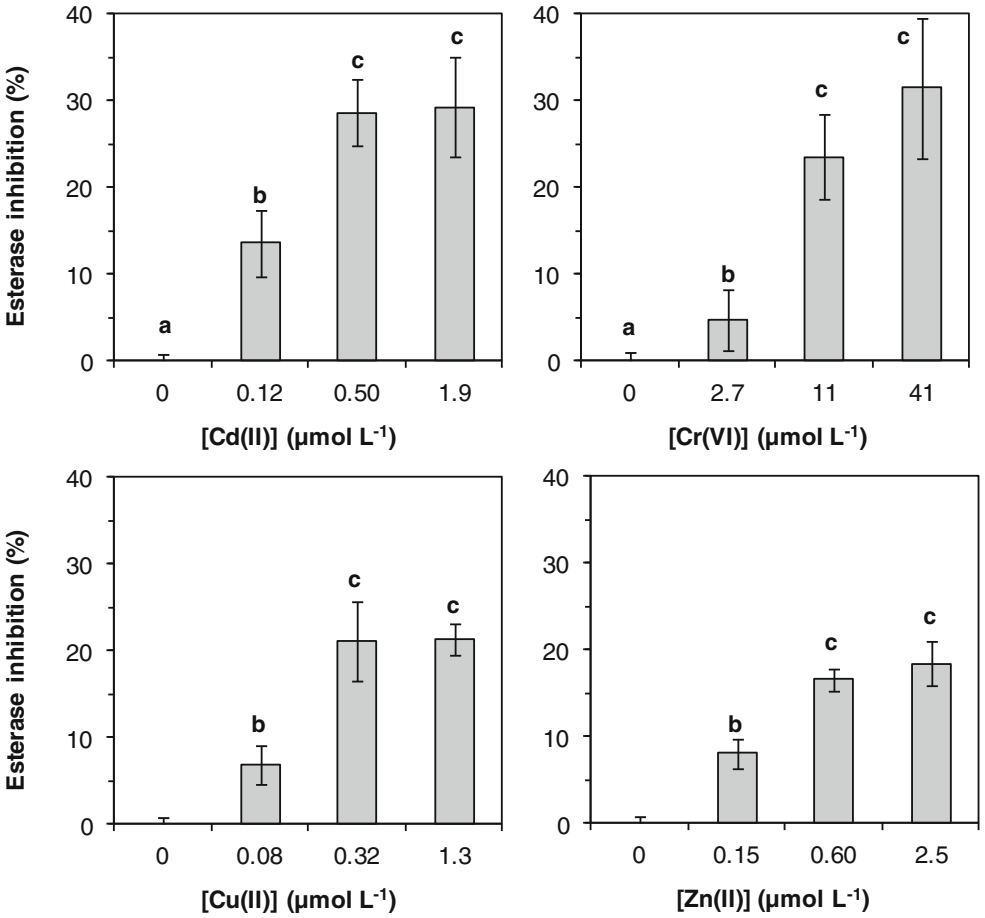

Fig. 1 Effect of heavy metals on esterase activity of the algal cells of *P. subcapitata*. Cells were exposed to different concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II) for 6 h and subsequently centrifuged, suspended in fresh OECD medium and incubated with 20 µmol L^{-1} fluorescein diacetate, for 40 min, at 25 °C. The percentage of esterase inhibition was calculated considering the maximum fluorescence exhibited by the cells not exposed to heavy metals (control). Each *bar* represents the mean of at least three experiments performed in quintuplicate ($n \geq 15$). The *error bars* represent the standard deviation calculated with 95 % confidence limits. Statistical differences among different metal concentrations were subject to ANOVA followed by Tukey-Kramer multiple comparison method; for each metal concentration, the means with different letters are very significantly different ($P<0.01$)

term exposure to heavy metals (6 h corresponds to approximately half the generation time of the alga) on the physiological parameters of *P. subcapitata* algal cells was assessed.

Membrane integrity is particularly important for the normal function of the cell and has been used as a criterion for determining cell viability. This parameter was sensitive to Cu(II). At intermediate (close to the 72-h EC_{50} value) or high (higher than the 72-h EC_{90} value) Cu(II) concentrations, 10 and 23 % of the cells, respectively, were SG positive (cells with damaged membranes) (Table 1). Franklin et al. (2001b) did not observe a modification of the cell membrane potential [evaluated with $DiOC_6(3)$] of the alga *P. subcapitata* exposed to 7 µmol L^{-1} Cu(II) for 1–4 h; nevertheless, the authors described damage to the cell membrane during longer Cu(II) exposure times. Most likely, SYTOX Green (SG) is a more sensitive fluorochrome than $DiOC_6(3)$ for detecting cells with an injured plasma membrane (non-viable cells). When the cells of the alga *P. subcapit*ata were exposed to Cd(II), Cr(VI) and Zn(II), even at higher concentrations, no loss of membrane integrity was observed (Table 1). These results suggest that different mechanisms underlie the toxic action of the different heavy metals under study. The plasma membrane appears to be one of the primary targets of Cu(II) at concentrations close to the 72-h EC_{50} or 72-h EC_{90} values.

Fluorescein fluorescence reflects cellular esterase activity. For all metals studied, a loss of esterase activity was detected at the lowest concentration used. Fluorescence was markedly reduced at high metal concentrations (Fig. 1). These results are in agreement with those described in the literature, which indicated that the esterase activity of marine and freshwater microalgae, including *P. subcapitata*, was inhibited after short-term (1–6 h) exposure to Cu(II) (Radix et al. 2000; Franklin et al. 2001a; Machado and Soares 2013).

Mitochondria are dynamic organelles. Their shape, number and subcellular distribution can be altered according to the physiological requirements of the cell and as a consequence of cytotoxic conditions (Liu et al. 2008; Scholz and Westermann 2013). The mitochondrial membrane potential ($\Delta\Psi m$) reflects mitochondrial function. According to the chemiosmotic theory, the passage of electrons through the electron transport chain in the inner mitochondrial membrane generates a transmembrane electrochemical gradient that is required for ATP production by the enzyme ATP synthase (Jastroch et al. 2010). Rh123 appears to be a sensitive and useful potentiometric dye

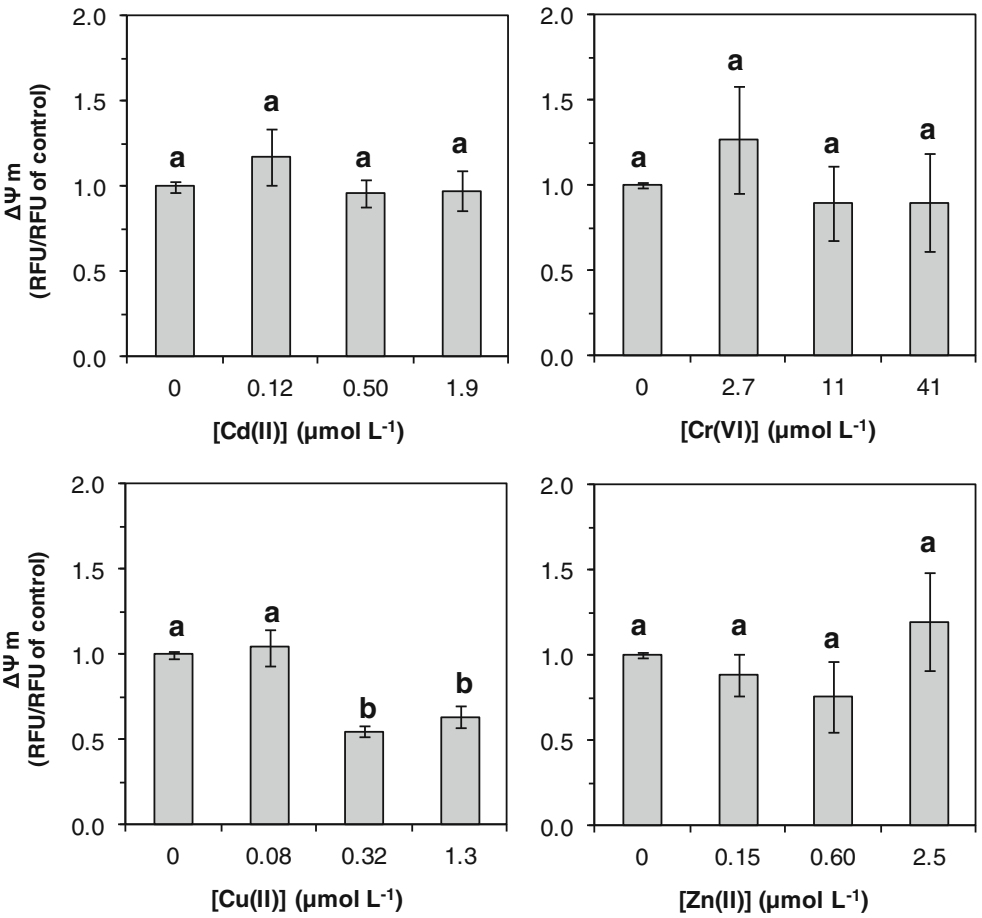

Fig. 2 Effect of heavy metals on mitochondria membrane potential (ΔΨm) of the algal cells of *P. subcapitata*. Cells were exposed to different concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II) for 6 h and subsequently centrifuged, suspended in fresh PBS buffer (pH 7.0) and incubated with 5 μmol L^{-1} rhodamine 123, for 30 min, at 25 °C. After 30 min, cells were washed (two times) and suspended in PBS. Each *bar* represents the mean of at least three experiments performed in quintuplicate ($n \geq 15$). The *error bars* represent the standard deviation calculated with 95 % confidence limits. Statistical differences among different metal concentrations were subject to ANOVA followed by Tukey-Kramer multiple comparison method; for each metal concentration, the means with different letters are very significantly different ($P<0.01$)

for use in the investigation of mitochondrial bioenergetics (Brickley et al. 2012). The complete loss of mitochondrial respiratory function due to the treatment of *P. subcapitata* cells with sodium azide (an electron transport chain inhibitor) led to the collapse of ΔΨm. Therefore, Rh123 was not accumulated by the mitochondria (data not shown). The exposure of algal cells to 0.32 and 1.3 μmol L^{-1} Cu(II) resulted in a decrease in Rh123 fluorescence, most likely as a consequence of a ΔΨm reduction (Fig. 2). Cu(II) at 0.32 and 1.3 μmol L^{-1} damaged the integrity of the cell membrane in *P. subcapitata* algal cells (Table 1). The results obtained with Rh123 suggest that Cu(II) at 0.32 and 1.3 μmol L^{-1} had a similar effect on the inner mitochondrial membrane, leading to the disruption of the transmembrane ion gradient. That is Cu(II) at intermediate and high concentrations appears to have an ionophore-like effect, which reduces ΔΨm and most likely inhibits respiration. A similar effect (collapse of the mitochondrial membrane potential) was reported in *Chlamydomonas reinhardtii* exposed to the herbicide bromoxynil octanoate (Brickley et al. 2012).

The in vivo Chl *a* fluorescence of green algae can be used as a tool to detect negative effects on photosynthesis (Fai et al. 2007). Chl *a* fluorescence is a function of the cell pigment content and the photochemical activity of photosystem II (PSII) in the photosynthetic electron transport chain (Franklin et al. 2001b). An increase in Chl *a* fluorescence can be observed when the acceptor side of PSII is inhibited (Yruela et al. 1993). In the present work, algal autofluorescence was found to be enhanced in cells exposed to intermediate and high concentrations of Cr(VI) and Cu(II). Similarly, an increase in Chl *a* intensity in *C. reinhardtii* exposed to Cd for 48 h was reported (Jamers et al. 2009). Franklin et al. (2001b) reported that Cu(II) can cause both an increase and a decrease in the Chl *a* fluorescence of *Chlorella sp.*, *P. subcapitata* and *Phaeodactylum tricornutum*, depending of the time of exposure and the Cu(II) concentration.

Figure 4 presents an overview of the impact of a 6-h exposure to Cd(II), Cr(VI), Cu(II) and Zn(II) on the cell structure and metabolism of the alga *P. subcapitata*, as evaluated using a fluorescent approach. Thus, SG was used to detect cytoplasmic membrane integrity, FDA provided information regarding the metabolic activity (esterase activity) in the cytoplasm, Rh123 was used to evaluate alterations in mitochondrial membrane potential and autofluorescence was used to identify changes in chloroplasts. At all concentrations tested, Cd(II) induced a loss of esterase activity. At a low

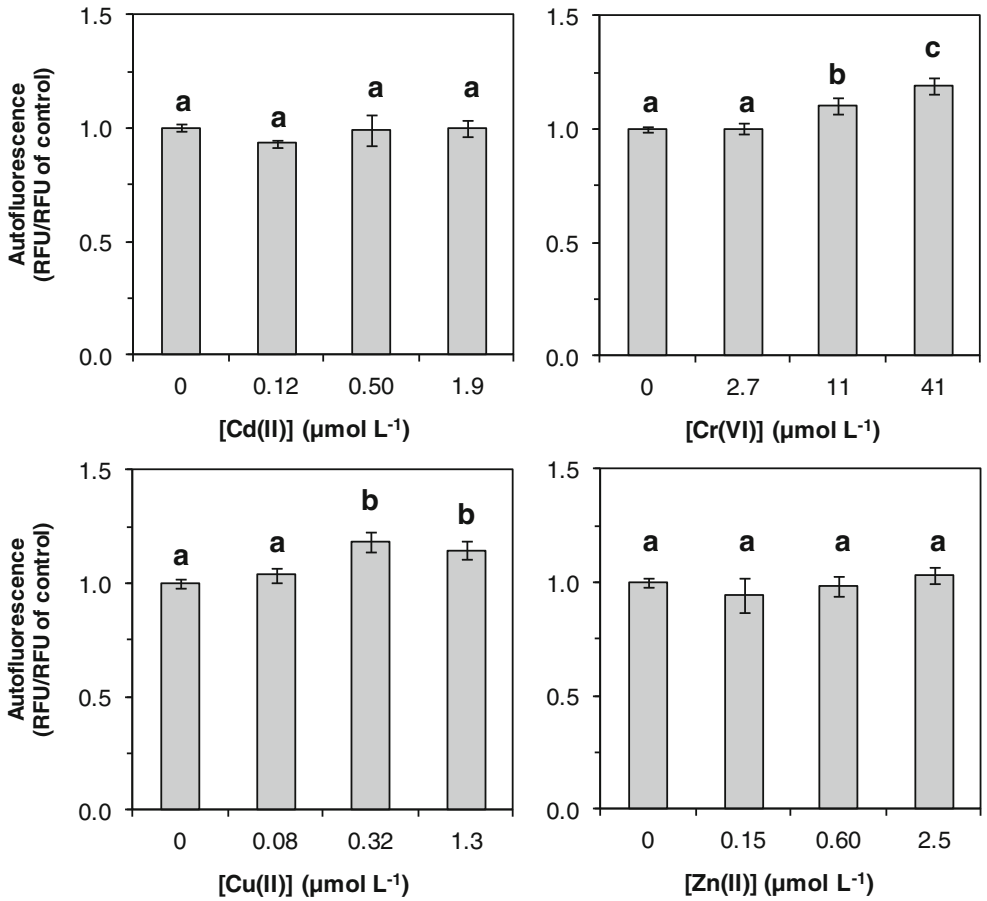

Fig. 3 Comparison of the effect of Cd(II), Cr(VI), Cu(II) and Zn(II) on chlorophyll *a* fluorescence of algal cells of *P. subcapitata*. Cells were incubated with different metal concentrations for 6 h and subsequently centrifuged and suspended in fresh OECD medium. Each *bar* represents the mean of at least four experiments performed in tenfold ($n \geq 40$). The *error bars* represent the standard deviation calculated with 95 % confidence limits. Statistical differences among different metal concentrations were subject to ANOVA followed by Tukey-Kramer multiple comparison method; for each metal concentration, the means with different letters are very significantly different ($P<0.01$)

concentration, Cr(VI) inhibited esterase activity; at moderate and high concentrations, it inhibited esterase activity and modified Chl *a* autofluorescence. Cu(II) was the most toxic metal. At a low concentration, Cu(II) induced esterase inhibition; at intermediate and high concentrations, it induced a loss of cell membrane integrity and esterase activity, compromised mitochondrial function and modified Chl *a* autofluorescence. At all concentrations tested, Zn(II) induced esterase

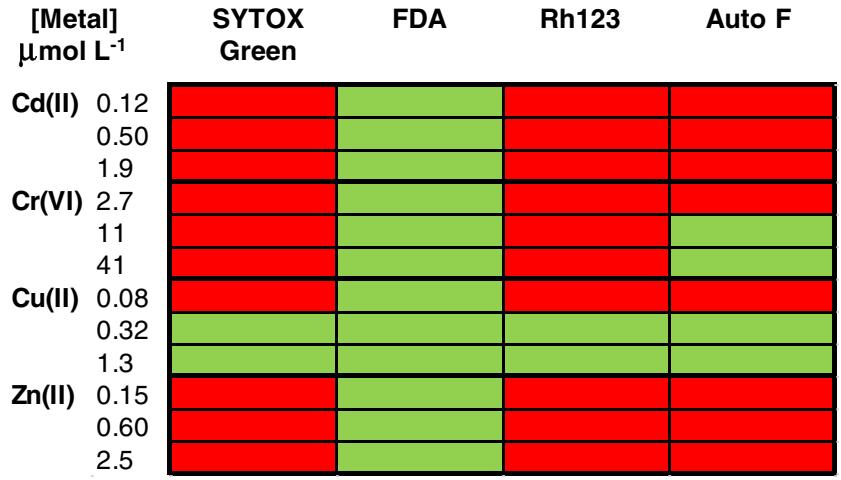

Fig. 4 Overview of the short-term responses of the alga *P. subcapitata* exposed to Cd(II), Cr(VI), Cu(II) and Zn(II). Algal cells of *P. subcapitata* were exposed to different heavy metal concentrations for 6 h. SYTOX Green evaluates membrane integrity. Fluorescein diacetate (FDA) and rhodamine 123 (Rh123) evaluate esterase activity and mitochondrial membrane potential ($\Delta\Psi m$), respectively. Chlorophyll *a* autofluorescence (Auto F). *Green*, response was very significantly different ($P<0.01$) from the control (no treated cells). *Red*, response was not very significantly different ($P<0.01$) from the control. In the case of membrane integrity, it was considered a response when the loss of membrane integrity was >5 %

inhibition (Fig. 4). Esterase activity was found to be the most sensitive marker. Autofluorescence was affected by Cr(VI) and Cu(II). Membrane integrity and $\Delta\Psi m$ were sensitive to the action of Cu(II) (Fig. 4).

In conclusion, exposure to Cd(II), Cr(VI), Cu(II) and Zn(II) for 6 h provoked physiological modifications in the alga *P. subcapitata*. With the use of specific fluorescent probes, theoretically, any cellular parameter can be measured; thus, fluorescence-based assays are an appropriate approach in this case. This study revealed that the inhibition of esterase activity, loss of membrane integrity and modification of mitochondrial membrane potential can serve as useful and specific markers for the evaluation of toxicity in algal cells. Additionally, they can provide useful mechanistic information on the mode of action of particular toxicants.

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