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Modification of cell volume and proliferative capacity of *Pseudokirchneriella subcapitata* cells exposed to metal stress

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ABSTRACT

The impact of metals (Cd, Cr, Cu and Zn) on growth, cell volume and cell division of the freshwater alga *Pseudokirchneriella subcapitata* exposed over a period of 72 h was investigated. The algal cells were exposed to three nominal concentrations of each metal: low (closed to 72 h-EC_{10} values), intermediate (closed to 72 h-EC_{50} values) and high (upper than 72 h-EC_{90} values). The exposure to low metal concentrations resulted in a decrease of cell volume. On the contrary, for the highest metal concentrations an increase of cell volume was observed; this effect was particularly notorious for Cd and less pronounced for Zn. Two behaviours were found when algal cells were exposed to intermediate concentrations of metals: Cu(II) and Cr(VI) induced a reduction of cell volume, while Cd(II) and Zn(II) provoked an opposite effect. The simultaneous nucleus staining and cell image analysis, allowed distinguishing three phases in *P. subcapitata* cell cycle: growth of mother cell; cell division, which includes two divisions of the nucleus; and, release of four autospores. The exposure of *P. subcapitata* cells to the highest metal concentrations resulted in the arrest of cell growth before the first nucleus division [for Cr(VI) and Cu(II)] or after the second nucleus division but before the cytokinesis (release of autospores) when exposed to Cd(II). The different impact of metals on algal cell volume and cell-cycle progression, suggests that different toxicity mechanisms underlie the action of different metals studied. The simultaneous nucleus staining and cell image analysis, used in the present work, can be a useful tool in the analysis of the toxicity of the pollutants, in *P. subcapitata*, and help in the elucidation of their different modes of action.

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1. Introduction

The water pollution due to the presence of metals is a wide-world problem. The anthropogenic activities are the main responsible for the contamination of the environment with metals. Industries such as energy production, battery manufacturing, mining, metallurgical and electroplating, produce effluents containing Cd(II), Cr(III), Cr(VI), Cu(II), Ni(II) and Zn(II). These effluents should be pre-treated before being discharged in rivers or oceans. Metals are not metabolically degraded. This characteristic, associated with the solubility and mobility of metals, makes their concentration and transfer through the food chain possible.

Metals exhibit short- and long-term toxic effects from microorganisms to higher organisms, including humans (Gadd, 2009). Cd, Cr, Cu and Zn affect photosynthesis of the alga *Scenedesmus obliquus* (Mallick and Mohn, 2003). Copper and zinc, at sublethal concentrations, inhibit photosynthesis, respiration, nitrate uptake, nitrate reductase activity and reduce the protein, carbohydrate and photosynthetic pigment levels in *Scenedesmus* sp. (Tripathi and Gaur, 2006). Cd, Cu and Zn produce ultrastructural changes (increase in number and volume of starch grains and vacuoles), evaluated by electron microscopy, in the alga *Chlamydomonas acidophila* (Nishikawa et al., 2003). Different metals [Cd(II), Cr(III), Cr(VI), Cu(II), Pb(II) and Zn(II)] induce the production of reactive oxygen species in *Chlamydomonas reinhardtii* (Szivak et al., 2009). Also Cu-induced ROS production on *P. subcapitata* and *Chlorella vulgaris* was described (Knauert and Knauer, 2008).

Short-term toxicity assays using microorganisms have gained a paramount importance in toxicity studies due to their simplicity, cost-effectiveness and reproducibility (Blaise and Férard, 2005; Wadhia and Thompson, 2007). Among the different microorganisms, microalgae are usually included in hazard assessment as representative of the aquatic community, and considered an important tool in the evaluation of physiological changes induced by metals (Torres et al., 2008). The alga *P. subcapitata* is particularly

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suited to toxicity testing due do it ecological relevance. In addition, it displays a higher sensitivity than invertebrates, fish and other standard test organisms to a wide range of hazardous substances, which favours its use as a reliable indicator of toxicity (Blaise et al., 1986; Geis et al., 2000).

The cell size is an essential characteristic of all organisms. It is associated with cell cycle progression and is influenced by internal and external stimuli (Bryan et al., 2012). Copper caused an increase in cell size of freshwater (*P. subcapitata* and *Chlorella* sp.) and marine (*Phaeodactylum tricornutum*) algae (Cid et al., 1996; Franklin et al., 2001). Similarly, cadmium increased the cell volume of the alga *Scenedesmus vacuolatus* (Le Faucheur et al., 2005). It was also described that Zn-treated cells of the marine diatom *Nitzschia closterium* were larger than control cells (Stauber and Florence, 1990). In the case of the alga *Chlorella* sp., copper had a bigger effect than cadmium on cell size (Franklin et al., 2002; Wilde et al., 2006).

At present, the tools that can be used for measuring cell volume are basically limited to image analysis, resistive-pulse technique (Coulter) and light scatter (Bryan et al., 2012). Flow cytometry gives the mean cell volume, based on forward light-scatter (FSC), which is dependent of the cell size and its refractive index. Generally, the larger cell size, the more forward scatter light is generated as the cell passes through a laser. Cell volume is determined assuming that all cells are spherical and have identical optical properties; deviations in cell shape and content introduce error to FSC measurements (Bryan et al., 2012). Therefore, these equipments can give erroneous results when the biovolume of nonspherical algae (Hillebrand et al., 1999), such as the alga *P. subcapitata*, is determined. In such algae, the determination of cell volume using microscopic measurements (by microscopic image analysis) and an appropriate mathematical equation seems to be an alternative.

Although the alga *P. subcapitata* is one of the most frequently used standard organism (American Standards for Testing Materials, American Public Health Association, Organization for Economic Cooperation and Development, International Organization for Standardization and United States Environmental Protection Agency) in toxicity tests (Janssen and Heijerick, 2003), limited information is available regarding the impact of cadmium, chromium, copper and zinc on alga morphology and proliferation capacity, despite the fact that these metals are generally present in domestic and industrial effluents.

In the present work, we have examined the impact of Cd(II), Cr(VI), Cu(II) and Zn(II), at different growth inhibitory concentrations on cell proliferation capacity and biovolume. Additionally, the impact of these metals on cell cycle progression of the algae *P. subcapitata* was evaluated.

2. Materials and methods

2.1. Strain, media and culture conditions

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

The algae were 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, sterilized and stored 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 4300 K), with an intensity of 4000 lux 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 $\sim 5 \times 10^4$ 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 400 mL of OECD medium in 1 L Erlenmeyer flasks, with an initial cell concentration of $\sim 5 \times 10^4$ cells mL^{-1} from the pre-culture, in the absence or presence of metals. Taking into account a previous study using different ranges of metals, three total nominal concentrations (for each metal) were selected as exposure concentrations. Appropriate volumes of $ZnCl_2$, $Cu(NO_3)_2$, $CdCl_2$ and $K_2Cr_2O_7$ solutions were added from standard solutions (Merck) or from primary standard $K_2Cr_2O_7$ solution, respectively. Cells were incubated under the conditions described above for the starter cultures for 72–96 h.

At defined intervals of time (given in the figures) samples were withdrawn and cell number determined using an automated cell counter (TC10-Bio-Rad). At low algal concentration ($<1 \times 10^5$ cells mL^{-1}) growth was measured by direct cell counting using a microscope and a counting chamber. Algal cell concentration was also evaluated, indirectly, by measuring the absorbance at 750 nm, according to US-EPA (2002); a calibration curve (number of cells versus absorbance) was first constructed. For low biomass concentration it was used a cuvette with a light path of 4 cm.

2.2. Calculation of EC values

The 72 h-EC_{10}, 72 h-EC_{50} and 72 h-EC_{90} values, represent the concentration of the toxicant that caused the inhibition of 10%, 50% and 90% of cell count, respectively, after 72 h, compared to the positive control (cells not exposed to the toxicant). EC values were calculated using linear interpolation method (TOXCALC version 5.0.32, Tidepool Scientific Software).

2.3. Calculation of specific growth rate and doubling time

The specific growth rates (μ) were calculated by least-square fitting to the linear part of the semilogarithmic growth plots of the number of cells mL^{-1} versus time. The time it takes the algal population to double in cell number (doubling time or generation time) (g) was calculated using the following equation:

$$g = \frac{\ln 2}{\mu}$$

2.4. Cell size analysis

Photos of non-treated (control) and treated algal cells, with the different metal concentrations, were acquired using a Leica DC 300F camera and processed with Leica IM 50-Image manager software. In order to achieve accuracy in the measurement of width and length of algal cells, several photos were taken in randomly selected fields, in phase-contrast microscopy, using an N plan X100 objective.

For biovolume determination, a minimum sample size of 200 cells was used in each metal concentration and in each experiment. The cell volume was calculated based on the assumption that *P. subcapitata* generally conforms to the shape of a sickle-shaped cylinder (Sun and Liu, 2003). Cell volume (V) is defined as:

$$V \approx \left(\frac{\pi}{6}\right) \cdot a \cdot b^2$$

where a and b are cell apical section view (length) and transapical section (width), respectively.

For each metal and concentration it was determined the frequency (i.e., the number of times) of each biovolume.

2.5. Nucleus staining

Algal cells were exposed to 1.9 μmol L^{-1} Cd(II), 41 μmol L^{-1} Cr(VI), 1.3 μmol L^{-1} Cu(II) or 2.5 μmol L^{-1} Zn(II), for 72 h. As control, cells were grown in the absence of the metals. After metal treatment, cells were centrifuged, washed and resuspended in OECD medium at 3×10^6 cells mL^{-1}. Then, cells were treated with 1-pentanol (70%, v/v), for 1 h, in order to permeabilize cell membrane, and stained with SYTOX Green as previously described (Machado and Soares, 2012). Briefly, algal cells were incubated with 0.5 μmol L^{-1} SYTOX Green, for 40 min, at 25 °C, in the dark, and were observed using an epifluorescence microscope, equipped with a HBO-100 mercury lamp and filter set GFP from Leica. Images were acquired and processed as described above.

For the determination of cell distribution among the different phases, 200 cells were counted, in each experiment. For the determination algal biovolume, at least 75 cells were measured for each metal and cell stage.

2.6. Reproducibility of the results and statistical analysis

Data presented are mean values of two-four independent experiments carried out under identical conditions. Unless otherwise specified, in the legend of the figures, results were expressed as the mean ± standard deviation, presented with 95% confidence limits. In Fig. 4, the statistical difference between control and metal-treated cells were tested using unpaired *t* test.

3. Results

3.1. Growth inhibition

A previous study was carried out exposing algal cells for 72 h to seven total nominal concentrations of each metal, arranged in a geometric series, in the following ranges: 0.041–4.1 μmol L^{-1} Cd(II), 0.88–88 μmol L^{-1} Cr(VI), 0.072–7.2 μmol L^{-1} Cu(II) and 0.1–10 μmol L^{-1} Zn(II). Based on this study, three total nominal concentrations were selected as exposure concentrations: a low concentration (0.12 μmol L^{-1} Cd, 2.7 μmol L^{-1} Cr, 0.080 μmol L^{-1} Cu and 0.15 μmol L^{-1} Zn), close to 72 h-EC_{10} values, but where a growth inhibition was detectable; an intermediate concentration, close to 72 h-EC_{50} values (0.47 μmol L^{-1} Cd, 11 μmol L^{-1} Cr, 0.32 μmol L^{-1} Cu and 0.60 μmol L^{-1} Zn) and a concentration higher than 72 h-EC_{90} values (1.9 μmol L^{-1} Cd, 41 μmol L^{-1} Cr, 1.3 μmol L^{-1} Cu and 2.5 μmol L^{-1} Zn). The cell concentration in the control cultures (without metals) increased by a factor larger than 16 within 72 h, and displayed a coefficient of variation less than 7%, which is in agreement with the validation conditions of "alga, growth inhibition test" guidelines (OECD, 2011).

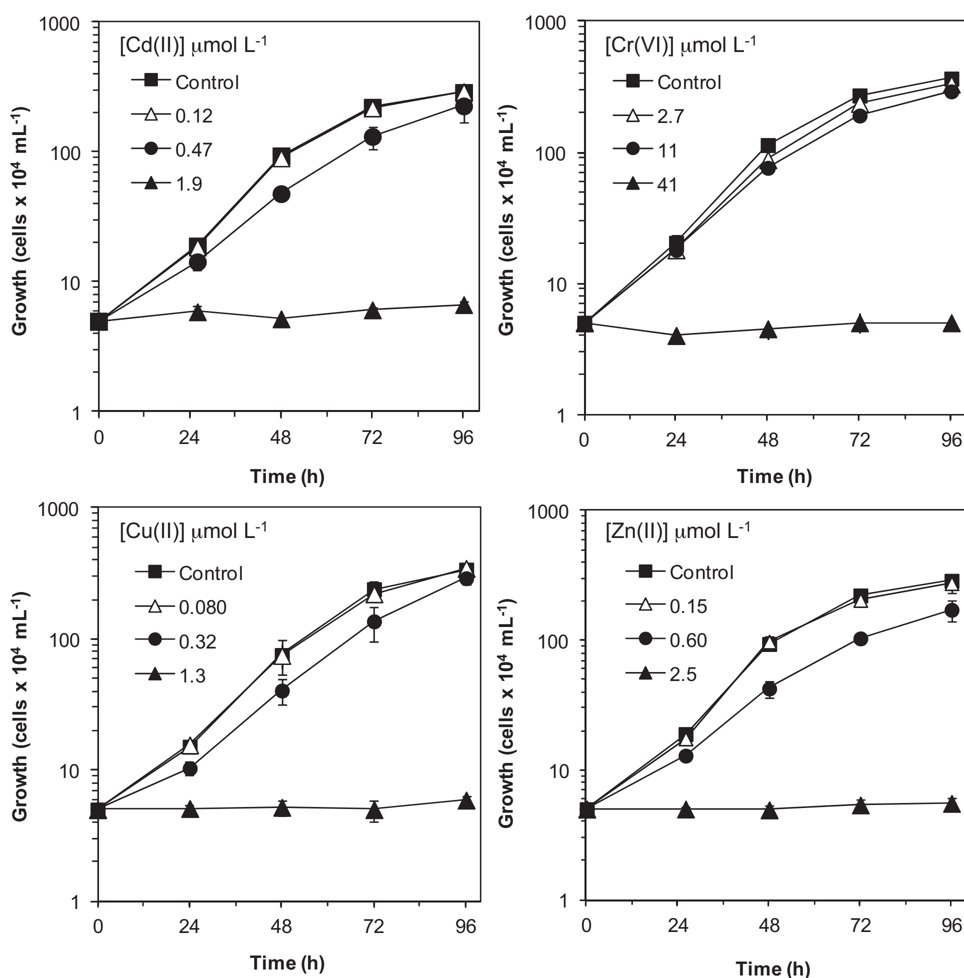

Fig. 1. Impact of the presence of different metals on the growth of *P. subcapitata*. Algal cells in exponential phase of growth were inoculated at 5×10^4 cells mL^{-1} in OECD medium containing different metal concentrations, and were incubated at 25 °C on an orbital shaker at 100 rpm under continuous "cool white" fluorescent light. These experiments were performed two times in duplicate. Each point represents the mean of four determinations. The error bars represent the SD calculated with 95% confidence limits; where no error bars are shown they are within the points.

The growth curves of *P. subcapitata* algal cells revealed that, with the exception of the metal concentrations corresponding to the highest values (where the growth was arrested), no important modification of latency phase was observed (Fig. 1). Control cultures (no metals added) grew exponentially during 48–72 h, with an average specific growth rate (μ) of 0.06 h^{-1}, which corresponded to a doubling (generation) time of ~12 h, and reached the stationary phase after 72–96 h (Fig. 1). The increase of metal concentration reduced the growth rate, and consequently, increased the doubling time in a concentration-dependent manner. The exposure of algal cells to metals in a concentration closed to EC_{50} values caused an increase of the doubling time from ~12 h (control cultures) to 14–16 h.

3.2. Effect of metals on algal morphology and cell division

The impact of metals on algal morphology was examined by microscopy image analysis. For this purpose, the width and length of algal cells grown during 72 h, in the absence (control) or presence of metals were measured. The biovolume was calculated assuming that *P. subcapitata* generally conforms to the shape of a sickle-shaped cylinder (Sun and Liu, 2003). The algal biovolumes were grouped in six classes (i.e., the cell volumes which fall within a given range) of 40 μm^3. In the case of Cd(II) an additional distribution with seven classes with a width of 80 μm^3 was considered (figure inset), due to the big increase of volume of the cells exposed to 1.9 $\mu mol\ L^{-1}$ Cd. Fig. 2 shows the relative frequency distribution of algal biovolumes (the percentage of biovolumes falling in each class) for all the metals and concentrations studied.

Non-treated cells (control) displayed an average size (length × width) of 9.1 × 3.2 μm which corresponds to a mean cell volume of 49 μm^3. The analysis of the relative frequency distribution of population non-treated with heavy metals revealed that >85% had a cell volume ≤85 μm^3 (Fig. 2). The exposure of algal cells to the lower concentrations of the metals studied, resulted in a decrease of cell volume comparatively with the control. In these conditions, 65–80% of the cells displayed a volume <45 μm^3, while in non-treated cells, 56% presented this volume (Fig. 2). When exposed to concentrations close to EC_{50} values, two behaviours were found: Cu(II) and Cr(VI) induced a reduction of cell volume, while Cd(II) and Zn(II) provoked an opposite effect (Fig. 2). At the highest concentrations, where the growth was arrested metal-induced increase of cell volume was observed; a shift to the right of the population distribution can be seen: 30–72% of metal-treated cells had a cell volume >85 μm^3. The increase of algal cell size is less pronounced in Zn-treated cells. In the case of Cd-treated cells two populations were observed: one population comprised of ~28% of cells with a normal morphology (≤85 μm^3), and another population comprised of ~57% of cells with a cell volume of 165–565 μm^3 (Fig. 2, insert).

P. subcapitata reproduces asexually via autospores. The staining of the nucleus with SYTOX Green, combined with the measuring of cell volume allowed distinguishing three phases in *P. subcapitata* cell cycle: (a) growth of mother cell; (b) cell division, which includes two divisions of the nucleus; and, (c) release of four autospores (daughter cells). Four stages can be found in algal population (Fig. 3).

The determination of the biovolume of algal cells belonging to each of the 4 stages, shown in Fig. 3, revealed that cells of stage

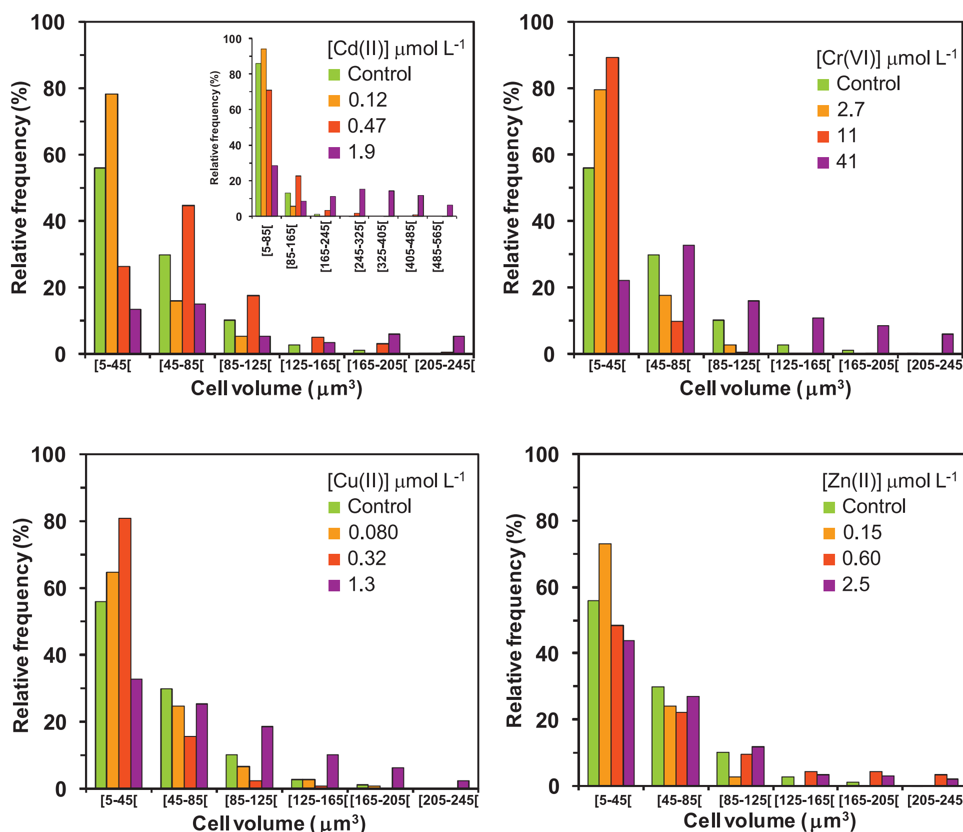

Fig. 2. Influence of metals stress on *P. subcapitata* cell volume. Algal cells were incubated during 72 h, in OECD medium, in the absence (control) or the presence of different metal concentrations, as described in Fig. 1. Cell volume was calculated based on the assumption that *P. subcapitata* generally conforms to the shape of a sickle-shaped cylinder. The algal biovolumes were grouped in six classes of 40 μm^3 width. In the case of Cd(II) an additional distribution, with seven classes, with a width of 80 μm^3 was considered (figure inset). Relative frequency is the percentage of biovolumes falling in each class. The values of cell volumes were obtained from three independent experiments. In each experiment, for each metal concentration, at least 200 cells were measured.

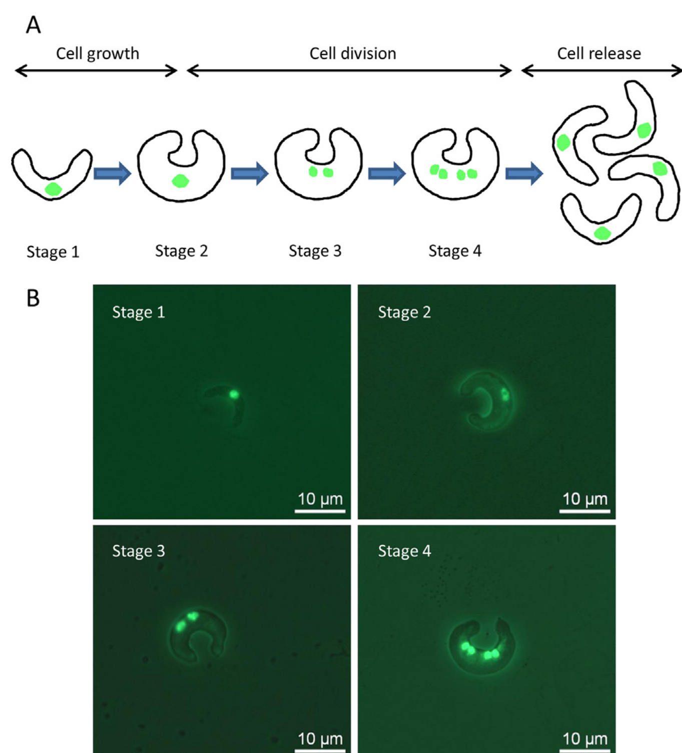

Fig. 3. Cell proliferation in *P. subcapitata*. (A) Diagrammatic representation of the cell cycle of the algae, which comprises of three phases: (1) growth of mother cell; (2) cell division; (3) release of daughter cells (autospores). (B) Photomicrographs of fluorescence plus phase contrast images of algal cells at different stages. For visualization of nucleus, cells were permeabilized with 1-pentanol (70%, v/v) for 1 h, and subsequently stained with 0.5 μmol L^{-1} SYTOX Green for 40 min.

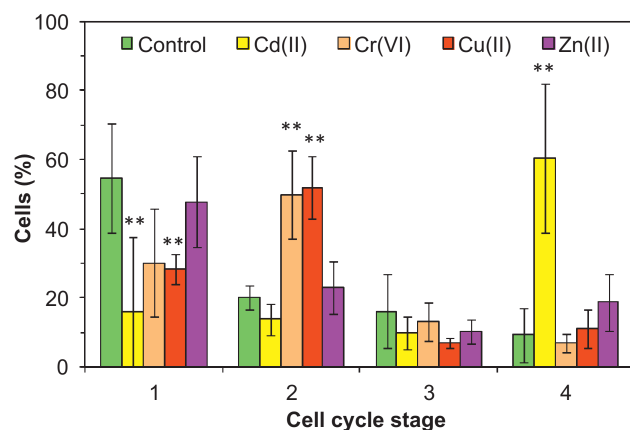

Fig. 4. Distribution of *P. subcapitata* algal cells through the cell cycle. The algal cells were incubated in the absence (control) or the presence 1.9 μmol L^{-1} Cd(II), 41 μmol L^{-1} Cr(VI), 1.3 μmol L^{-1} Cu(II) or 2.5 μmol L^{-1} Zn(II), for 72 h, as described in Fig. 1. The cell cycle stages considered are presented in Fig. 3. Values are obtained from three independent experiments. In each experiment, for each metal, at least 200 cells were measured. The error bars represent the SD calculated with 95% confidence limits. For each cell cycle stage statistical differences between control and metal-treated cells were tested using unpaired *t* test. The means with (**) are very significantly different ($P < 0.01$) from the control.

1, 2, 3 and 4, displayed a biovolume of 22 ± 1 μm³, 45 ± 4 μm³, 65 ± 4 μm³ and 81 ± 6 μm³, respectively; standard deviations were calculated with 95% confidence limits. Thus, the analysis of daughter (stage 1) and mother cell (stage 4) subpopulations in control cultures not exposed to metals, showed that the mean volume of mother cells is ~4 times higher than the mean volume of the daughter cells.

P. subcapitata algal cells, not exposed to metals (control), were mainly (55%) in stage 1 (Fig. 4). Compared to the control, algal cells exposed to the highest values of Cd(II), Cr(VI) and Zn(II) displayed a very significantly different ($P < 0.01$) stage distribution. Cr(VI) and Cu(II)-treated cells were accumulated mainly (~50%) in stage 2 and Cd(II)-treated cells in stage 4 (60% of the cells). In the case of Zn(II), the distribution through the different cell cycle stages did not differ very significantly ($P < 0.01$) from the control (Fig. 4). These results strongly suggest that cell growth of Cr(VI) and Cu(II)-treated cells was arrested before the first division, while cell growth in Cd(II)-treated cells was arrested before the release of the daughter cells.

4. Discussion

The chlorophyte (green alga) *P. subcapitata* is unicellular, non motile, easily cultivated in the laboratory, representative of eutrophic and oligotrophic freshwater environments and displays a higher sensitivity to a variety of hazardous substances (Blaise and Vasseur, 2005). These characteristics make this alga well suited for to be used as cell model in toxicity studies.

In the present work, the impact of three nominal concentrations of different metals (Cd, Cr, Cu and Zn), usually used as reference toxicants (Blaise and Vasseur, 2005; US-EPA, 2002), on proliferation capacity, biovolume, and cell cycle progression of the algae *P. subcapitata* was studied.

Non-treated cells (control) displayed an average size (length × width) of 9.1 × 3.2 μm which corresponds to a mean cell volume of 49 μm³; these values are within the range described by OECD, measured with electronic particle counter: cell size (length × width) of 8–14 × 2–3 μm and a cell volume of 40–60 μm³ (OECD, 2011). Algal cell volume changed due to the presence of metals. Thus, algal cells exposed to lower concentrations of Cd, Cr, Cu and Zn reduced their cell volume (Fig. 2). An intermediary behaviour (reduction for Cr and Cu and increase of cell volume for Cd and Zn) was found in the exposure to intermediary concentrations, close to 72 h-EC_{50} values. The highest exposure concentrations of Cd, Cr, Cu and Zn resulted in an increase of cell volume (Fig. 3). Franklin et al. (2001), also described a copper concentration-dependent increase in cell size of *P. subcapitata* evaluated by flow cytometry, based on forward light-scatter. In the present work, a detailed analysis of cell population, exposed to different metal concentrations was carried out by microscopic image analysis, making it possible to evaluate subtle metal-induced changes of cell volume. These results showed that the presence of metals, although at sub-inhibitory concentrations, can modify algal cell volume.

The classical model of the cell-division cycle comprises the following phases: G1 (cells increase in size), S (DNA replication), G2 (gap between DNA synthesis and mitosis) and M (mitosis) (Smith and Fornace, 1996). Eukaryotic organisms present control points in the cell cycle. One of these checkpoints in unicellular algae is related with the attainment of a critical cell volume, an equivalent to START in budding yeasts (Vitova and Zachleder, 2005). In fact, the control of cell volume is a determining issue in the regulation of cell cycle in order to maintain the cell size (Oldenhof et al., 2004). *C. vulgaris* (Rioboo et al., 2009) and *C. reinhardtii* (Matsumura et al., 2003) algal cells grow during G1 phase until duplicating their size; after reaching a critical threshold size, algal cells begin to divide. A similar observation was done in *P. subcapitata* (Fig. 3). It was found that mother cells (stage 4), in control cultures, displayed a volume ~4 times higher than daughter cells (stage 1). The nuclear staining combined with the cell measurement allowed discriminating different cell stages within a population of *P. subcapitata* and perform the analysis of the volume of each subpopulation.

In the case of the algal cells that divide by multiple fission, resulting in 4 or 8 daughter cells, such as *Scenedesmus quadricauda*, the

growth is associated with more than one checkpoint within the cell cycle (Hlavova et al., 2011). In these cells multiple DNA replications, nuclear division rounds and, at the end, a multiple cleavage of the cell occur (Hlavova et al., 2011). Due to the fact that nuclear and cellular division are temporally separated during cell cycle progression, it was suggested that there is a gap phase, called G3, between nuclear and cell division (Zachleder et al., 1997). The division process in *P. subcapitata* includes two divisions of the nucleus (Fig. 3). Thus, algae cell cycle can be arrested in two or more points when exposed to toxicants. Consistent with this possibility, the exposure of asynchronous cultures of *P. subcapitata* to metals resulted in the stop of the growth at different cell division stages. As can be observed from Fig. 4, the exposure of algal cells to the highest Cr(VI) and Cu(II) concentrations resulted in the accumulation of cell population in stage 2, before the first nuclear division. Cells exposed to Cd(II) accumulated in cell stage 4, after the second nucleus division but before the cytokinesis (release of daughter cells).

In conclusion, morphological alterations of the alga *P. subcapitata* were observed when the algae were exposed for 72 h to sub-inhibitory, and, particularly, inhibitory growth concentrations of Cd(II), Cr(VI) and Cu(II). The simultaneous nuclei staining and cell image analysis allowed assessing the impact of metal pollutants on cell morphology and cell cycle progression, which can be helpful to identify and elucidate the different modes of action of pollutants, in *P. subcapitata* exposed to chronic or acute toxicity.

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