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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.

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.