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Cadmium and Chromium Toxicity to *Pseudokirchneriella subcapitata* and *Microcystis aeruginosa*

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ABSTRACT

The toxicity of cadmium and chromium to Pseudokirchneriella subcapitata *and* Microcystis aeruginosa *was evaluated through algal growth rate during 96h exposure bioassays. Free metal ion concentrations were obtained using MINEQL$^+$ 4.61 and used for IC50 determination. Metal accumulations by the microorganisms were determined and they were found to be dependent on the concentration of Cd^{2+} and Cr^{6+}. IC50 for* P. subcapitata *were 0.60 μmol L^{-1} free Cd^{2+} and 20 μmol L^{-1} free Cr^{6+}, while the IC50 values for* M. aeruginosa *were 0.01 μmol L^{-1} Cd^{2+} and 11.07 μmol L^{-1} Cr^{6+}.* P. subcapitata *accumulated higher metal concentrations (0.001 - 0.05 μmol Cd mg^{-1} dry wt. and 0.001 - 0.04 μmol Cr mg^{-1} dry wt) than the cyanobacteria (0.001 - 0.01 μmol Cd mg^{-1} dry wt and 0.001 - 0.02 μmol Cr mg^{-1} dry wt). Cadmium was more toxic than chromium to both the microorganisms.*

Key words: Toxic effects, algae sensitivity, metal accumulation, IC50

INTRODUCTION

The release of different pollutants into the environment has increased as result of industrialization, and thereby, lowered the environment quality to alarming levels. Among such pollutants, trace metals are most important because they interact with the biota and may be highly toxic; they accumulate in the environment and represent a potential health hazard for humans. Metals may associate with some ecosystem components resulting in metal complexes with inorganic or organic materials. Considering the amplitude of possibilities for the metal association in the environment, metals bioavailability may vary with environmental conditions and organisms (Lombardi et al. 2002; De Schamphelaere et al. 2005; Töpperwien et al. 2007).

Metals such as cadmium and chromium are often present in industrial wastewaters. Cadmium originates from metal plating, metallurgical alloying, mining, ceramics and other industrial operations (Davis et al. 2000), and chromium from tanning factories, steel works, industrial electroplating, wood preservation and artificial fertilizers (Bagchi et al. 2002).

The toxic effect of heavy metals on aquatic biota is one of the main problems arising from the contamination of natural aquatic ecosystems. Algae and cyanobacteria are the base of the

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detritus and grazing food webs in aquatic systems and in addition, cyanobacteria are important nitrogen fixers. In this sense, studies on the response of these organisms to metals are of particular relevance. Microalgae constitute an important group of photosynthetic organisms that present high sensitivity for the metals, and for this reason they are frequently used for the assessment of the impacts of these elements on aquatic ecosystems. Besides their sensitivity, microalgae are fast growing organisms and relatively simple to follow and maintain under culture conditions, what makes them ideal organisms for the ecotoxicological investigations.

Cyanobacteria are an ancient, large and diverse group of prokaryotic autotrophs. The widespread nature of cyanobacteria in different environments makes them useful as the indicators of environmental pollution (Whitton 1984). Knowledge of the cyanobacteria's response for metal additions is ecologically important due to its high frequency in the aquatic environments in several countries around the world, including Brazil. Cyanobacterial blooms are very frequent episodes partially as a consequence of favorable climatic conditions, but mainly due to the over-enrichment of environment (Sotero-Santos et al. 2008; Figueredo and Giani 2009).

Literature data show that total metal concentration is not a good predictor of bioavailability, toxicity and mobility of metals in the environment (Sunda and Huntsman 1998), whereas the concentration of free ions are closely related to its bioavailability (Campbell et al. 2002; Almas et al. 2006; Worms et al. 2007). Cadmium and chromium have high affinity for the particles (Guéguen et al. 2004), sediment (Murakami et al. 2008) and biological surfaces (Vigneault and Campbell 2005), which may account as significant environmental factors that modify Cd and Cr speciation, thus controlling their availability and toxicity to organisms.

Due to the environmental importance of microalgae and cyanobacteria, studies focusing on the response of these organisms to metals are of particular interest. Both the cadmium and chromium are present in the contaminated aquatic ecosystems and pose risk to the aquatic organisms. At present, few information is available in the literature that show the sensitivity of two different algal species (prokaryote and eukaryotic) to cadmium and chromium (Thompson et al. 2002), as well as their capacity for metal accumulation.

The main objective of this study was to evaluate the toxic effects of the cadmium and chromium on green algae *Pseudokirchneriella subcapitata* and cyanobacteria *Microcystis aeruginosa* using growth rate inhibition as toxicity endpoint. Metal accumulation by microorganisms was also evaluated.

MATERIALS AND METHODS

Microorganisms

Pseudokirchneriella subcapitata was kept in batch cultures in the Laboratory of Ecotoxicology and Ecophysiology of Aquatic Organisms of São Paulo University (São Carlos, Brazil). The inoculum of *P. subcapitata* was obtained from the Phycology laboratory at Universidade Federal de São Carlos, gently donated by Prof. A. Vieira. A strain of the cyanobacterium *Microcystis aeruginosa* that did not produce toxins was gently supplied by Prof. Dr. S. Azevedo (Federal University of Rio de Janeiro, RJ, Brazil).

The green algae was cultured in L.C. Oligo medium that did not contain ethylenediaminetetraacetic acid (EDTA) (AFNOR 1980) and the cyanobacterium in ASM-1 culture medium (Gohram et al. 1964), which contained EDTA. Culture media were sterilized by autoclaving at 121 °C during 15 minutes. Algal cells were grown in 1 L of media in 2 L borosilicate Erlenmeyer flasks under a 12h:12h (light:dark cycle) using "cool-white" fluorescent lamps, 100 µmol photons $m^{-2}s^{-2}$ for the Chlorophyceae and 50 µmol photons $m^{-2}s^{-2}$ for the Cyanobacteria. Controlled temperature was used throughout (24 ± 2 °C). Experimental conditions followed the Brazilian protocol (ABNT 2005) for green algae and American standard practice (APHA 1995) for the cyanobacteria.

Toxicity tests

P. subcapitata and *M. aeruginosa* were exposed for 96 h to a range of chromium and cadmium concentrations. Due to different metal sensitivities (Rodgher 2005), each alga was submitted to a different set of metal concentration range. Filtrates from the algal cultures were used for the determination of total dissolved metal concentrations. The filtrates were obtained by gentle vacuum filtration of 100 mL of algal culture using acid washed cellulose acetate membrane

filters (Schleicher and Schull) with 0.45 μm pore size. Total dissolved cadmium concentrations used for the toxicity tests performed with *P. subcapitata* were 0.06, 0.15, 0.29, 0.68 and 1.29 μmol L^{-1}, and 1.78, 3.24, 7.11, 14.5 and 28.1 μmol L^{-1} for the cyanobacteria. Initial total dissolved chromium concentrations used for the toxicity tests with *P. subcapitata* were 1.82, 3.57, 7.36, 14.9 and 27.4 μmol L^{-1}, and those for *M. aeruginosa* were 1.87, 3.59, 7.41, 15.2 and 29.7 μmol L^{-1}.

Considering that the free metal ions constitute an important metal fraction related to the bioavailability to microalgae and cyanobacteria, the chemical equilibrium software MINEQL$^+$ 4.61 (MINEQL$^+$ version 4.61 2009) was used for the calculation of Cr^{6+} and Cd^{2+} concentrations. In the present study, this was particularly important because the culture medium differed in their composition and chromium and cadmium have different affinities for organic ligands.

The metals were furnished as $Cd(NO_3)_2.4H_2O$ (J. T. Baker) and $K_2Cr_2O_7$ (Merck) titrimetric solutions diluted to $8.9x10^{-5}$ mol L^{-1} Cd and $1.9x10^{-4}$ mol L^{-1} Cr. Tests were carried out in 250 mL borosilicate Erlenmeyer flasks containing 100 mL of medium to which suitable volumes of metal standards were added to achieve the total final concentrations reported above. The cells of *P. subcapitata* and *M. aeruginosa* in the exponential growth phase were inoculated to test flasks to provide initial cell densities of approximately 10^4 cells mL^{-1}. Controls (without added chromium or cadmium) were considered using the same conditions as the experiments with the metals. The flasks were kept on an orbital shaker at 150 rpm. Environmental conditions used for the experiments were the same as described for algal cultures (ABNT 2005; APHA 1995). All the materials used for culture and toxicity experiments were washed with 10% HNO_3 for seven days and rinsed with distilled water prior to use. All the experiments were performed with three replicates per treatment.

The samples (2.0 mL) were taken every 24 h from each test flask, fixed with acid Lugol's iodine solution and used for the determination of cell density. The cells were counted using an Improved Newbauer-Bright Line hemocytometer under optical microscope (Carl Zeiss, Standard model 25). Dry weight of the microorganisms were obtained by filtering a known volume of the culture on a pre-weighed glass-fiber filter. Filters with the algal cells were dried at 60 ^oC for 24 h and weighed to determine the cell mass per volume of the culture (APHA 1995). The microorganisms growth rates ($days^{-1}$) were calculated as described by Fogg (1975) and the data were used to obtain 96 h IC 50 for each metal.

Metal analysis

To determine the total dissolved metals at the beginning of the experiments, the samples were filtered through the membrane filters (cellulose acetate, Schleicher and Schüll) with 0.45 μm pore size and then acidified with concentrated nitric acid (J. T. Baker). Total cell metal (absorbed and adsorbed by the microorganisms) was determined at the end of the experiments using the cells retained in the membrane filters. These were dried and submitted to acid digestion (3.0 mL of concentrated HNO_3 and 1.0 mL of H_2O_2, J. T. Baker). The results are expressed as μmol metal mg^{-1} dry weight of algae (APHA 1995). Three replicates were used per metal determination.

Analytical blank was performed using three clean filters according to Van Loon (1985). All the samples were analyzed by graphite furnace atomic absorption spectrometry (Varian AA 220). The detection limits for Cd and Cr were calculated as described by Miller and Miller (1993) and were $4x10^{-10}$ mol L^{-1} for Cd and $1.6x10^{-8}$ mol L^{-1} for Cr.

Data analysis

The 96 h IC50 values and their 95% confidence intervals were determined by the Trimmed Spearman-Karber method (Hamilton et al. 1977). The result for the dry weight was submitted to tests for normality (Shapiro-Wilk's test) and homogeneity (Bartlett's test). The results were then analyzed through ANOVA and Dunnett's test was applied to detect the significant differences among the controls and metal treatments. Tukey's test (post hoc test) was used in multiple comparisons to detect the significant differences among the total dissolved metal and free metal ion concentrations. The above statistical tests were run using the BioEstat 4.0 program (Ayres et al. 2005).

RESULTS

Metal speciation results are reported in Figure 1. The calculations showed that the presence of EDTA in ASM-1 culture medium significantly

reduced free Cd^{2+} ions concentration in the culture (Tukey's test, $P<0.05$). The concentrations of total dissolved cadmium used in the experiments with the cyanobacteria ranged from 1.78 to 28.1 μmol L^{-1}, with its free ion concentration ranging from 0 to 27%. In relation to chromium, the furnished range of total concentration was 1.87 to 29.7 μmol L^{-1}, which corresponded to a free Cr^{6+} concentration of 100%. Total dissolved metal concentration in L.C. Oligo culture medium, which did not contain EDTA, corresponded to a free Cd^{2+} ion concentration of 96% and 100% for Cr. Considering these different behaviors of metal ions in the culture media and its importance as bioavailable fraction, toxicity evaluation (IC50) was based on the free metal ions concentration and not on their total dissolved concentrations.

The IC50 values for the metals for the microorganisms are shown in Table 1. Based on IC50 values, it was concluded that cadmium was more toxic than chromium to both the algae *P. subcapitata* and cyanobacteria *M. aeruginosa*. For the green algae, values of IC50 were 0.60 μmol L^{-1} free Cd^{2+} and 20 μmol L^{-1} free Cr^{6+}. The values obtained for the cyanobacteria revealed an IC 50 of 0.01 μmol L^{-1} free Cd^{2+} and 11.07 μmol L^{-1} free Cr^{6+}.

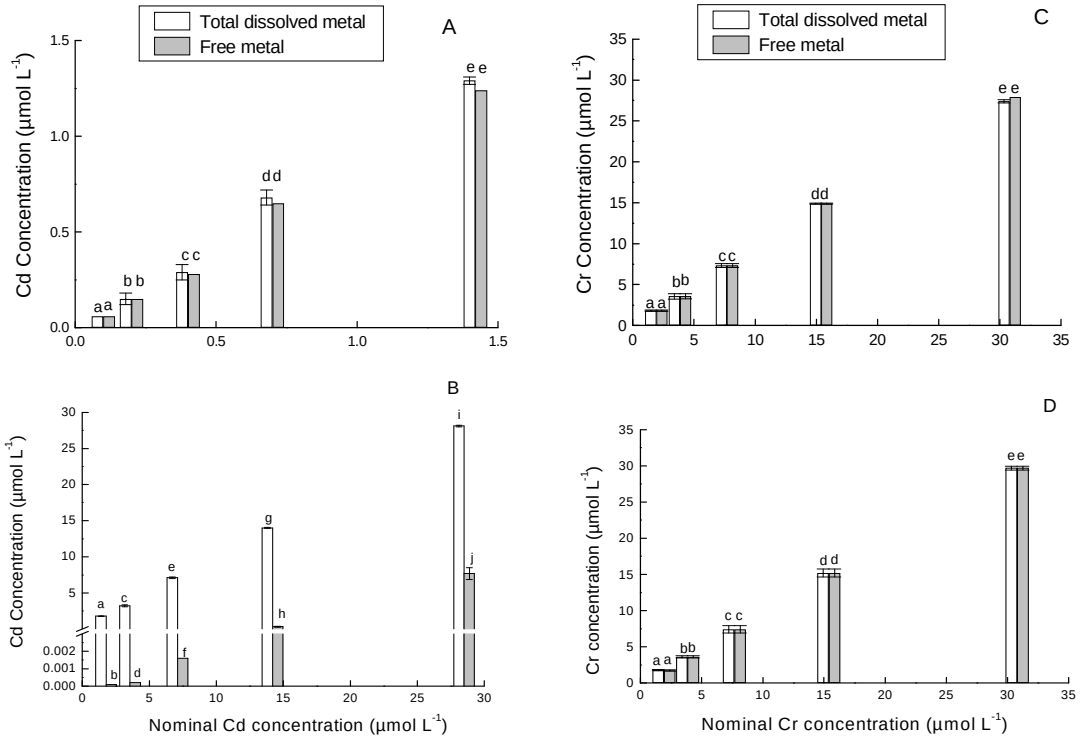

Figure 1 - Initial total dissolved and free metal concentrations for the experimental treatments with *P. subcapitata* (A and C) and *M. aeruginosa* (B and D). Values are mean ± SD of 3 replicates. Means with different letters are significantly different (Tukey's test, $P<0.05$). Error bars denote standard deviation.

Table 1 - Inhibition concentration values (IC50) of free metals for microorganisms. Concentration values are reported in μmol L^{-1}. Values given in parenthesis are 95% confidence limits.

Cd^{2+}		Cr^{6+}	
P. subcapitata	***M. aeruginosa***	***P. subcapitata***	***M. aeruginosa***
0.60 (0.50 - 0.72)	0.01 (0.006 – 0.02)	20 (18 - 23)	11.07 (9.60 – 12.76)

The results of IC50 were supported by the reduction of dry weight for both the microorganisms. A significant decrease of dry weight for the green algae was observed at 0.15 µmol L^{-1} free Cd^{2+} (Dunnett's test, P<0.05). At 14.9 µmol L^{-1} free Cr^{6+}, the dry weight for *P. subcapitata* were lower than that observed in the controls (Dunnett's test, P <0.05). A reduction of dry weight for *M. aeruginosa* was observed at 0.11 µmol L^{-1} free Cd^{2+} and 7.41 µmol L^{-1} free Cr^{6+} (Dunnett's test, P<0.05) (Table 2).

Total cellular metal concentrations are shown in Figure 2. The total cellular metal increased with increasing of the free metal ions in the solution for *P. subcapitata* and *M. aeruginosa*, which continued to accumulate the metal above the 20.0 µmol L^{-1} free Cr^{6+}. Cd accumulated by *M. aeruginosa* did not increase above 0.11 µmol L^{-1} Cd^{2+}, while green algae accumulated above 1.0 µmol L^{-1} free Cd^{2+}. The increase of accumulated Cd by *M. aeruginosa* in relation to free Cd^{2+} was smaller than that verified for the green algae. *P. subcapitata* accumulated higher metal concentrations (0.001 - 0.05 µmol Cd mg^{-1} dry wt and 0.001 - 0.04 µmol Cr mg^{-1} dry wt) than the cyanobacteria (0.001 - 0.01 µmol Cd mg^{-1} dry wt and 0.001 - 0.02 µmol Cr mg^{-1} dry wt).

Table 2 - Dry weight (mg L^{-1}) for microorganisms after exposure to metals. Values are mean ± SD of 3 replicates. Free metals concentrations are reported in µmol L^{-1}. *statistically different from Control (Dunnett's test, P<0.05).

P. subcapitata				***M. aeruginosa***			
Cd^{2+}	**Dry weight**	**Cr^{6+}**	**Dry weight**	**Cd^{2+}**	**Dry weight**	**Cr^{6+}**	**Dry weight**
Control	137.1 ± 23.4	Control	110.7 ± 18.5	Control	22.2 ± 4.4	Control	20.4 ± 4.7
0.06	92.2 ± 27.3	1.82	121.5 ± 29.8	0.0001	21.9 ± 3.0	1.87	21.8 ± 6.3
0.15	89.9 ± 31.0*	3.57	111.9 ± 39.1	0.0002	21.8 ± 6.2	3.59	19.7 ± 5.0
0.28	64.5± 16.9*	7.36	111.2 ± 2.6	0.002	17.9 ± 4.2	7.41	11.3 ± 1.6*
0.65	24.9 ± 6.3*	14.9	49.6 ± 19.8*	0.11	6.8 ± 2.0*	15.20	7.4 ± 1.2*
1.24	9.7 ± 2.7*	27.4	9.8 ± 2.3*	7.68	3.4 ± 1.8*	29.70	7.1 ± 1.6*

DISCUSSION

In unpolluted freshwater environments, dissolved metal concentrations can reach values close to $4x10^{-8}$ mol L^{-1} Cd and $4x10^{-7}$ mol L^{-1} Cr (Ochieng et al. 2008). It has been detected that in the contaminated areas, water may contain dissolved metal concentrations as high as $9x10^{-7}$ mol L^{-1} Cd (Sainz et al. 2004) and $2x10^{-6}$ mol L^{-1} Cr (Bobrowski et al. 2004). In the present study, the concentration of Cd and Cr to which the microorganisms were exposed were similar to the values observed in impacted aquatic systems. However, the concentration of organic and inorganic ligands in the natural waters normally exceeds the trace metal concentrations (Gopalakrishnan et al. 2008), thereby forming the complexes and rending the metal ions less bioavailable to the aquatic organisms. Because free metal concentrations are closely related to the bioavailable fraction of the total dissolved metal, the metals toxicity was related it directly to the free ion species in the culture media.

The IC50 for the cadmium obtained in the present study for *P. subcapitata* (0.60 µmol L^{-1} free Cd^{2+}) was lower than the value obtained by Castañé et al. (2003) for the same metal and same algal species. Using the culture media with EDTA in their algal bioassays, the authors observed that IC50 value for the Cd based on MINEQL^{+} calculated free Cd^{2+} ion was 1.06 µmol L^{-1}, lower than that based on the total metal concentration (2.25 µmol L^{-1} total Cd). The present results were in agreement to those of Errecalde et al. (1998), who found 50% reduction in the growth rate of *P. subcapitata* at 0.65 µmol L^{-1} free Cd^{2+} after 72 h of metal exposure. However, the IC50 value of 20 µmol L^{-1} free Cr^{6+}obtained for *P. subcapitata* in the present study was lower than that found by Brady et al. (1994). Using the test medium with EDTA the authors reported that the growth of *P. subcapitata* was affected only at concentrations of total Cr higher than $2x10^{-4}$ mol L^{-1}.

For *M. aeruginosa*, the results showed an IC50 value of 0.01 µmol L^{-1} free Cd^{2+}. This value was approximately 80-fold lower than the IC50 obtained if total dissolved Cd was considered. This

emphasized the importance of free ions estimation in the toxicity tests with the microorganisms. Neelam and Ray (2003) found 50% inhibition of photosynthetic pigments in *Microcystis* sp. at 0.27 μmol L^{-1} total Cd in the cultures with less EDTA than was used in the present work. Zhou et al. (2006) found inhibition of *M. aeruginosa* growth on the basis of chlorophyll a content at 4 μmol L^{-1} total Cd in the medium with chelator addition (1.78 μmol L^{-1} free Cd^{2+}, MINEQL^{+} calculation).

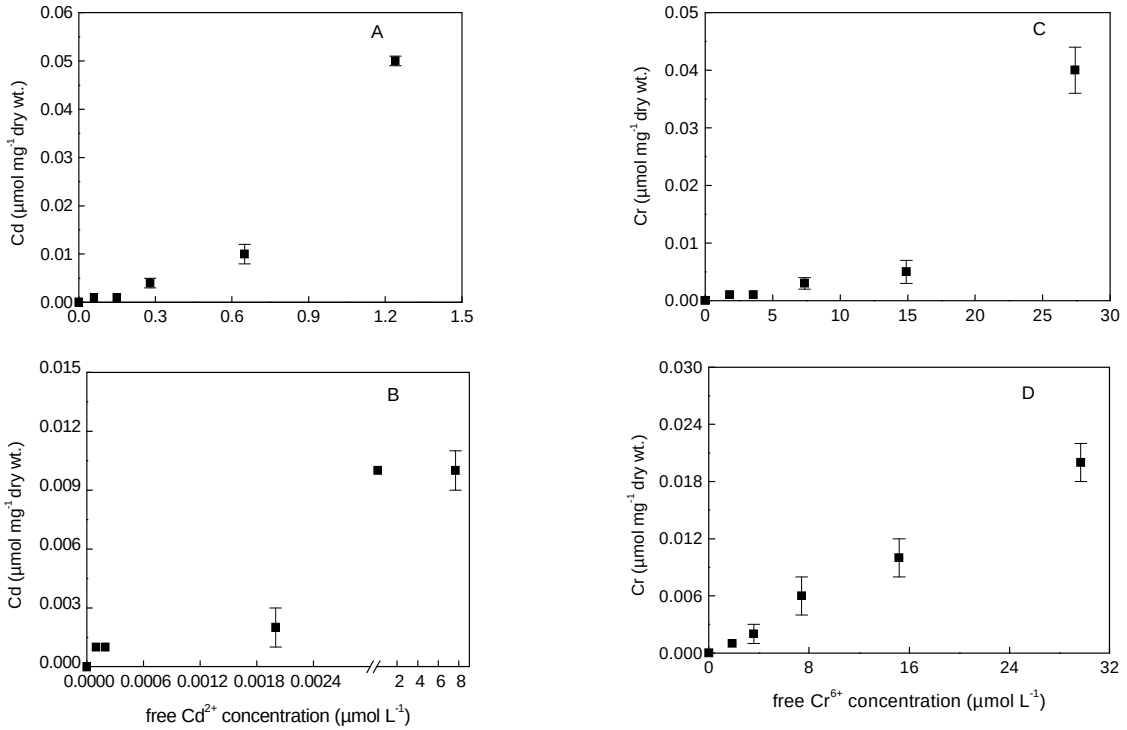

Figure 2 - Metals accumulated in the microorganisms after 96 h of exposure. A and C: Cd and Cr accumulated in *P. subcapitata.* B and D: Cd and Cr accumulated in *M. aeruginosa*. Error bars denote standard deviation.

Only few studies have been reported in literature about Cr^{6+} toxicity to cyanobacteria. Thompson et al. (2002) found growth inhibition of *Synechococcus* sp. and *Nostoc* sp. at $1.0x10^{-3}$ mol $L^{-1}$and 750 μmol L^{-1} total Cr, respectively. Thus, the present study represented an attempt to fill a gap in the information about the toxic effect of chromium on cyanobacteria.

In the present investigation, EDTA was kept in the experiments with *M. aeruginosa* because preliminary studies demonstrated that ASM-1 medium without this metal chelator was not suitable for the growth of the tested cyanobacteria (Rodgher 2005). It is known that EDTA is necessary to maintain Fe in the solution, an important oligoelement for microalgae (Lewis 1995). Chemical speciation modeling of the present results showed that the presence of such ligand reduced free Cd^{2+} ions in the medium, but not Cr^{6+}. Guéguen et al. (2003) also demonstrated that the behavior of metals was influenced by the experimental conditions used for algal bioassays and suggested working with EDTA free medium to avoid complexation with the metals, especially for cadmium. Moreover, as shown by the results, the chelating effect of EDTA varied according to the metal, so special care should be taken when interpreting the results.

The population growth rate is one of the important endpoints used in the toxicity tests since toxic effects are reflected in cell growth. The growth of *P. subcapitata* and *M. aeruginosa* decreased with increasing the cadmium and chromium concentrations. Reduction in the growth rate caused by exposure to metals can be attributed to the loss of cellular constituents and poor nutrient uptake, which may be a consequence of altered membrane permeability and inhibition of the photosynthesis (Pistocchi et al. 2000).

The accumulation of metals by *P. subcapitata* and *M. aeruginosa* was dependent on external free metal ion concentrations, with higher metal accumulation by the green algae when compared to the cyanobacteria. Similarly, a positive correlation between the accumulated Cd by green algae and the concentration of free Cd in the solution has been described by Wang and Dei (2006). The authors showed the accumulation of Cd by *Chlamydomonas reinhardtii* at a medium concentration over 1.0 μmol L^{-1} free Cd^{2+}. Also, Brady et al. (1994) reported that *Pseudokirchneriella* sp. was the most efficient Cr accumulator at $2x10^{-4}$ mol L^{-1} in comparison to *Chlorella* sp. and *Scenedesmus* sp., but all the three species have accumulated the metal.

In relation to the cyanobacteria, the present study disagreed with those of Klimmek et al. (2001) and Zeng et al. (2009). Zeng et al. (2009) observed the accumulation of Cd by *M. aeruginosa* at concentrations higher than 0.20 μmol L^{-1} free Cd^{2+}, while Klimmek et al. (2001) showed that *M. aeruginosa* accumulated Cd at a medium concentration of $9x10^{-4}$ mol L^{-1} total Cd. The present results showed that Cd accumulation by *M. aeruginosa* reached a saturation level at 0.11 μmol L^{-1} free Cd^{2+}, above which no increase was observed. This difference could be explained by the longer exposure time employed in the present study (96 h) as compared to lower exposure time (24 h) by Klimmek et al. (2001) and (48 h) by Zeng et al. (2009) studies.

Cadmium is a major metal pollutant due to its toxicity. In the present study, cadmium was more toxic than chromium to both the microorganisms. *M. aeruginosa* was a more sensitive organism for cadmium than *P. subcapitata*. The results were consistent with those obtained by Guanzon et al. (1994) who have investigated the effects of metals (Cu, Pb and Cd) on the growth rates of three freshwater microalgae and concluded that *M. aeruginosa* exhibited higher sensitivity than other species to cadmium.

In relation to chromium, the cyanobacteria also showed higher sensibility to the metal in comparison to the green algae. Similar to the present study, Chen et al. (2003) found higher chromium tolerance by the green algae *P. subcapitata* (EC50 = $2.40x10^{-4}$ M total Cr) in relation to the cyanophyta *Synechococcus* sp. (EC50 = $1.25x10^{-4}$ M total Cr^{6+}).

The results of the present study demonstrated the importance of using free metal ion concentrations to evaluate the metal toxicity to phytoplankton cells. This is most important when metal ligands are added to the culture media, since it is necessary to optimize the growth of the microorganisms.

Finally, it is important to consider two points: metal accumulation by the microorganisms and difference in sensitivity. The accumulation of the metals by *P. subcapitata* and *M. aeruginosa* could be considered a potential contamination source in the aquatic systems. The metal accumulated by the cell could be interpreted as the total particulate metal (metal species as quantified in this study). For example, the metal that is transported with the cell to other trophic levels, as demonstrated in literature (Fisher and Hook 2002; Wilding and Maltby 2006; Geffard et al. 2008). In addition, *M. aeruginosa* showed higher sensibility to the metals in comparison to the green algae, showing that the cyanobacterium was more suitable for monitoring the contaminated aquatic bodies with Cd and Cr. However, other factors might influence the metal toxicity to the microorganisms in the environment. Future studies should be performed in order to better assess the sensitivity of the green algae and cyanobacteria to the metals.

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