Toxicity of cadmium and zinc on two microalgae, *Scenedesmus obliquus* and *Desmodesmus pleiomorphus*, from Northern Portugal

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Abstract Aquatic environments often contain toxic heavy metals that may enter the food web via uptake by microalgae and eventually cause severe poisoning problems at higher trophic levels. The effects of Cd and Zn cations upon growth of two native green microalgal species, *Scenedesmus obliquus* and *Desmodesmus pleiomorphus* (previously isolated from a polluted site in Northern Portugal), were accordingly evaluated. Growth inhibition of the microalgal cells was determined following exposure for 96 h to several initial concentrations of aqueous solutions of either of those two metals. At the higher end of Cd and Zn experimental concentration ranges, a significant reduction in cell density was observed in the cultures; EC_{50} values, calculated after fitting a Weibull model to the experimental data, were 0.058 and 1.92 mg L^{-1} for Cd and 16.99 and 4.87 mg L^{-1} for Zn in the case of *S. obliquus* and *D. pleiomorphus*, respectively. One observed that *S. obliquus* can tolerate higher Zn concentrations than *D. pleiomorphus*, but the reverse holds regarding exposure to Cd.

Keywords Microalgae · Heavy metals · Cd · Zn · EC_{50} · Weibull model

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Introduction

Aquatic ecosystems have become increasingly contaminated by heavy metals as a consequence of release of wastewaters containing such pollutants generated by anthropogenic sources; this poses a serious threat to human beings owing to their toxicity (even at minute concentrations), bioaccumulation and biomagnification in the food web (Herpin et al. 1996; Mohammed and Markert 2006; la Rocca et al. 2009). In fact, cadmium induces a wide spectrum of toxic effects upon plant physiology: it alters enzymatic activities via binding to functional groups or by displacing the metal therein (Báscik-Remisiewicz et al. 2009; Sanitá di Toppi and Gabbrielli 1999). On the other hand, zinc is an essential micronutrient for several organisms including microalgae and acts as an important enzyme cofactor (e.g. in carbonic anhydrase, superoxide dismutase and RNA polymerase); however, it becomes toxic when available in higher concentrations (viz., 100–500 mg day^{-1}; Báscik-Remisiewicz et al. 2009; Omar 2002a) since it decreases cell division, mobility, total chlorophyll content, ATPase activity and carotenoid/chlorophyll ratio in microalgae (Omar 2002b).

Among the organisms more frequently used in vitro for toxicity tests, freshwater microalgae (Moreno-Garrido et al.

2000) are particularly sensitive in detecting the potential toxic effects of pollutants (la Rocca et al. 2009); moreover, results of microalgal toxicity tests are relatively reliable and repeatable; finally, microalgae are ubiquitous in aquatic environments, and toxicity tests resorting to these organisms are relatively quick and inexpensive (Lam et al. 1999; Torres et al. 2000). Green algae and diatoms are the microalgae most commonly used for toxicity tests (Moreno-Garrido et al. 2000), which, in their most standard form, measure the decrease in growth rate or in final cell biomass brought about by exposure to the appropriate metal for 48 up to 96 h; growth endpoints are particularly relevant because changes in population growth may influence species succession, as well as community structure and function (Franklin et al. 2002).

The sensitivity of a toxicity test depends on the initial cell density (Franklin et al. 2002; Moreno-Garrido et al. 2000), and there is evidence indicating decreases in sensitivity upon increases in cellular levels (Moreno-Garrido et al. 2000). Since the surface of microalgal cells is negatively charged, it provides a set of binding sites for metal cations (e.g. Zn^{2+} and Cd^{2+}); hence, higher initial cell densities entail more surface ligands available and thus less ions per cell basis, which will likely lead to a lower toxic effect by the metal(s) onto the microalgal cells. For most protocols, the recommended initial cell density lies within 10^3–10^5 cells mL^{-1} (OECD 2006; Wong and Couture 1986), yet Blaise and Ménard (1998) recommended a microalgal inoculum of 10^6 cells mL^{-1}.

Besides useful as the basis for assay methods, assessment of the toxicity of heavy metals in soluble form upon wild microalgae from contaminated sites is of particular relevance in ecotoxicology studies—especially because such wild strains are normally exposed to highly polluted environments, and are consequently more prone to conveying admission of metals into the food web. Although metal toxicity to microalga cells has sometimes been claimed to depend on the speciation of the metal, only divalent cationic forms are usually assumed by either Zn or Cd. In addition, experimental evidence indicates that toxicity depends more strongly on the activity of the free metal ion rather than the total metal concentration: for example, Knauer et al. (1997) and Mbabazi et al. (2010) claimed that Zn and Cd were predominantly present in their cationic form, otherwise no toxicity was observed, whereas Allen et al. (1980) referred that the main forms of zinc in solution are Zn^{2+} and $ZnOH^{+}$, both of which can be considered as free zinc divalent cations.

Toxicity studies encompassing microalgal species have for long been produced owing to their importance as crucial components at the basis of the food chain. However, only a minor fraction of those studies considered microalgae isolated from polluted locations, yet such unique sources entail a potentially high resistance to toxic pollutants. Hence, a major significance of the ecotoxicity tests described in this paper derives from consideration of wild microalga strains that had systematically been exposed to severely stressing conditions of heavy metal contamination.

In this study, the effects of Cd and Zn cations upon the growth of two green microalga species, *Scenedesmus obliquus* and *Desmodesmus pleiomorphus*, which had previously been isolated from a polluted zone in Northern Portugal, were thus studied. To this deed, 96-h EC_{50} was calculated using nonlinear regression of a Weibull model to actual experimental data. The Weibull model is a rather flexible one, containing very few parameters, which, owing to its flexible shape, has been successfully used in many applications where an empirical model suffices (Christensen and Nyholm 1984; OECD 2006; Weibull 1951).

Materials and methods

Microalgal culture

Scenedesmus obliquus and *Desmodesmus pleiomorphus* unialgal cultures were isolated from a heavy metal-polluted region of Northern Portugal—"Esteiro de Estarreja". This site has for long been contaminated with heavy metals, chiefly Pb (approx. 835 mg kg^{-1}), Hg (approx. 66 mg kg^{-1}) and Zn (up to 3620 mg kg^{-1}; Oliveira et al. 2001); the Cd concentration in those sediments was below detection limit.

Both species were cultured in PHM medium (Borowitzka 1988) containing 1 g L^{-1} of Tris–HCl buffer and trace levels of Zn (19.7 μg L^{-1}) in the absence of EDTA, and were maintained at 25°C under continuous light. Inocula for all experimental batches were obtained from exponentially growing cultures and resuspended in test medium at the desired initial cellular density. All materials and culture media were previously autoclaved for 15 min at 121°C and 1 atm. Toxicity experiments were conducted in glass flasks, and the samples taken for cell measurements used glass tubes; all these materials were previously washed with nitric acid, and then several times with deionised water, so as to rule out any possibility for unwanted complexation of the target heavy metal cations (which would jeopardize the analytical assays).

Toxicity assessment

To determine the effect of Cd and Zn cations upon growth of both microalgae, cells were grown (in quadruplicate) in

250-mL glass flasks on a rotary shaker set at 100 rpm for a period of 96 h. Aiming at the best compromise between maximizing bioassay sensitivity and having sufficient viable cells, the initial cellular density was set to 1.0–1.2 $\times 10^5$ cells mL^{-1}. Determination of cell numbers from each test flask was performed, in duplicate, using a Neubauer Improved bright-line haemocytometer. The culture medium was further supplemented with Zn (to levels higher than those usually considered for micronutrients) or Cd (herein referred to as initial metal concentrations), which were taken from stock solutions previously prepared with the corresponding chloride salts $ZnCl_2$ and $CdCl_2$, respectively, dissolved in deionized water. The initial Zn concentrations tested were 1, 5, 10, 20, 30 and 45 mg L^{-1} for both microalgae, whereas the initial Cd concentrations were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg L^{-1} for *S. obliquus* and 0.5, 1, 2.5, 5, 10 and 20 mg L^{-1} for *D. pleiomorphus*.

Although Cd was not listed as a major pollutant in the contaminated site from where microalgae cultures had been obtained, it is normally associated with Zn contamination owing to the classical nature of its industrial sources; hence, its effect upon growth of both microalgae was also assessed.

By the last day of the experiment, a sample of the culture medium was taken to determine the amount of metal left in the supernatant and thus quantify the metal removed by either microalga; that determination was according to Matsunaga et al. (1999) and Pérez-Rama et al. (2002), and resorted to atomic absorption spectrophotometry with flame atomization using a Perkin Elmer 3100 (USA) spectrophotometer.

A control experiment was also considered using culture medium (with 19.7 μg L^{-1} Zn as trace metal requirement for micronutrient towards healthy cell grow) plus the desired microalga, and with no extra addition of either metal, in order to determine the maximum cell density reached in the cultures. A negative control, i.e. non-inoculated culture medium with the desired metal concentration, was considered as well in order to double check whether the concentration of metal in solution remained constant for the whole time frame of the experiment.

Inhibition parameter estimation

The toxicity of both metals was expressed as percent cell inhibition, calculated as:

$$\%\ \text{Cell inhibition} = \frac{(\text{biomass of control}) - (\text{biomass of treatment})}{\text{biomass of control}} \times 100. \quad (1)$$

A Weibull model was fitted to such growth inhibition data by nonlinear regression, using SPSS software v. 16.0 (USA), according to:

$$\%\ \text{Cell inhibition} = C_\infty \times \left[1 - \exp\left(-\left[\frac{C_{metal}}{\beta}\right]^\alpha\right)\right] \quad (2)$$

where C_∞ is the maximum value of cellular inhibition and α and β are the shape and concentration parameters, respectively.

The effective concentration of metal that inhibits 50% of the microalga population by 96-h of exposure thereto (i.e. 96-h EC_{50}) was calculated afterwards by interpolation of the aforementioned model.

Results

Toxicity assessment

Exposure of either *D. pleiomorphus* or *S. obliquus* to all initial concentrations of Zn or Cd, in terms of growth over a 96-h period, leads to clear differences in cell number between controls and experiments in which microalgae were exposed to toxic metals. Furthermore, growth inhibition was essentially proportional to metal concentration.

The *D. pleiomorphus* biomass was higher than that of the control, at the lowest Zn concentration considered (1 mg L^{-1}), by 2 and 3 days of contact time; however, higher initial Zn concentrations reduced cell density significantly ($p<0.05$, data not shown).

Analysis of growth of either microalga when exposed to Cd indicated that this metal is much more toxic than Zn to both species: *D. pleiomorphus* tolerated higher Cd concentrations, with its cell growth being fairly inhibited above 2.5 mg L^{-1}, whereas the detrimental effect upon growth of *S. obliquus* was obvious even at as low as 0.1 mg L^{-1}.

ANOVA showed that there was a significant effect of the metals tested upon growth of both microalgae. Tukey's test suggested that sorting of Zn initial concentrations by toxic effect was control $<1<5<10=20\leq30<45$ mg L^{-1} for *S. obliquus* and control$=1<5<10=20\leq30=45$ mg L^{-1} for *D. pleiomorphus*. Likewise, the toxic effects of the initial Cd concentrations upon growth should be sorted as: control$=0.01=0.025=0.05<0.1=0.5=0.25=1$ mg L^{-1} for *S. obliquus* and control$<0.5=1<2.5<5<10=20$ mg L^{-1} for *D. pleiomorphus.*

The amounts of Zn removal (by both microalgae) and of Cd removal (only by *D. pleiomorphus*) from solution during the experimental time frame are plotted in Fig. 1.

The highest amounts of cations removed from solution, by 96 h, were 29.2±0.6 and 17.9±1.7 mg L^{-1} for Zn in the case of *S. obliquus* or *D. pleiomorphus*, respectively, at an initial concentration of 45 mg L^{-1}, and 5.7±0.1 mg L^{-1} for Cd in the case of *D. pleiomorphus* at an initial concentration of 20 mg L^{-1}.

Inhibition parameter estimation

Nonlinear regression of the parameters in Weibull model to cell inhibition data, pertaining to both metals and both microalgae (Table 1), led to good fits (Fig. 2), and no major trend in the residuals could be perceived. The fact that full inhibition (100%) is never reached is a consequence of the intrinsic scatter of the data and of the unavoidable bias resulting from extrapolation to an asymptotic behaviour when not high enough metal concentrations were actually tested. However, that accuracy in modelling would be redundant because this model was used with the sole purpose of interpolating data to calculate EC_{50} values rather than describing asymptotic trends of cell inhibition. Likewise, comparison with alternative models based on lack-of-fit statistical analyses was also not relevant given our purpose, so it was not pursued.

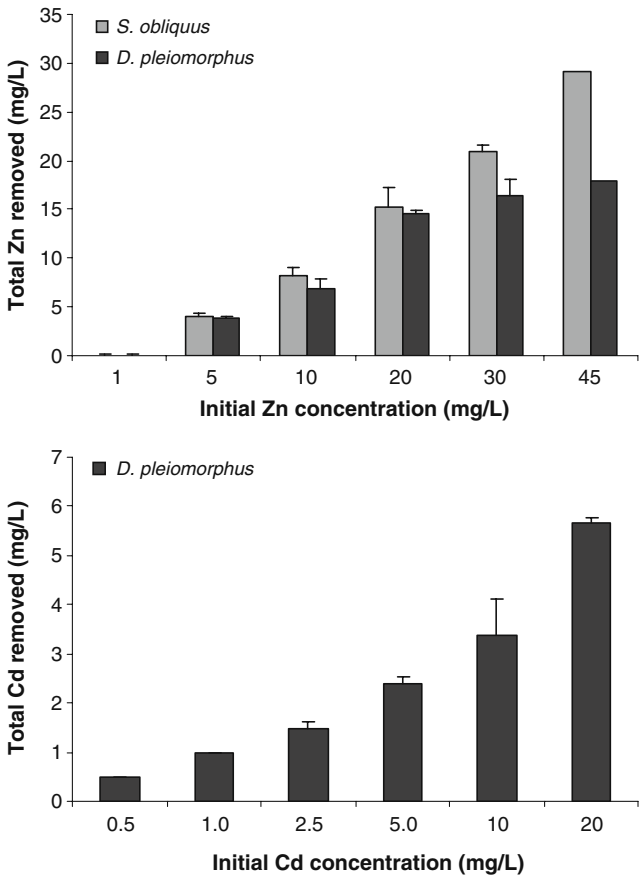

Fig. 1 Extent (average ± standard deviation, $n = 4$) of Zn and Cd removal by 96 h of exposure of the microalgae to various initial concentrations

Table 1 Best estimates (mean ± standard deviation) of Weibull model parameters, pertaining to *D. pleiomorphus* and *S. obliquus*, by 96 h of exposure of the microalgae to various initial Zn and Cd concentrations

Microalga	Metal	Weibull model parameter		
		C_∞ (%)	β (mg/L)	α
D. pleiomorphus	Zn	74.0±1.5	4.5±0.3	1.5±0.2
	Cd	89.5±1.6	2.2±0.1	1.6±0.1
S. obliquus	Zn	97.1±2.0	35.6±2.9	0.44±0.03
	Cd	91.9±1.7	0.076±0.005	0.92±0.07

The 96-h EC_{50} values obtained for *S. obliquus* and *D. pleiomorphus*, after exposure to the heavy metals at stake, were 16.99 and 4.87 mg L^{-1} for Zn and 0.058 and 1.92 mg L^{-1} for Cd, respectively. It can be concluded that Cd is highly toxic for both species, although *D. pleiomorphus* is less sensitive to Cd than *S. obliquus*. On the other hand, the EC_{50} values pertaining to Zn and *S. obliquus* suggest that this microalga tolerates significantly higher Zn concentrations than *D. pleiomorphus*.

Discussion

The capacity of a microalga to resist the toxic action of a heavy metal may be assessed via its EC_{50} value (Torres et al. 2000). In fact, the effective concentration of a heavy metal that causes 50% inhibition of microbial growth by 96 h is widely used as an index of toxicity (Yan and Pan 2002). Its calculation based on biomass instead of growth rate was elected because the latter requires data generated exclusively during the exponential phase; hence, calculation of EC_{50} based on biomass has fewer sources of variation associated therewith (Moreno-Garrido et al. 2000).

Recall that in order to assess the toxicity of Zn on the growth of microalgae, *D. pleiomorphus* and *S. obliquus* were exposed to various initial concentrations of this metal. In the former case, its biomass was higher than that of the control by 2 and 3 days of contact time at the lowest Zn concentration considered (1 mg L^{-1}). This can easily be rationalized since Zn is a micronutrient required for microalgal metabolism (Báscik-Remisiewicz et al. 2009; Vallee and Auld 1990) as it is a part of prosthetic moieties of some of its relevant enzymes. Our strains were more tolerant to Zn than others described in the literature, as concluded from their higher EC_{50} values: 16.99 and 4.87 mg L^{-1} for *S.*

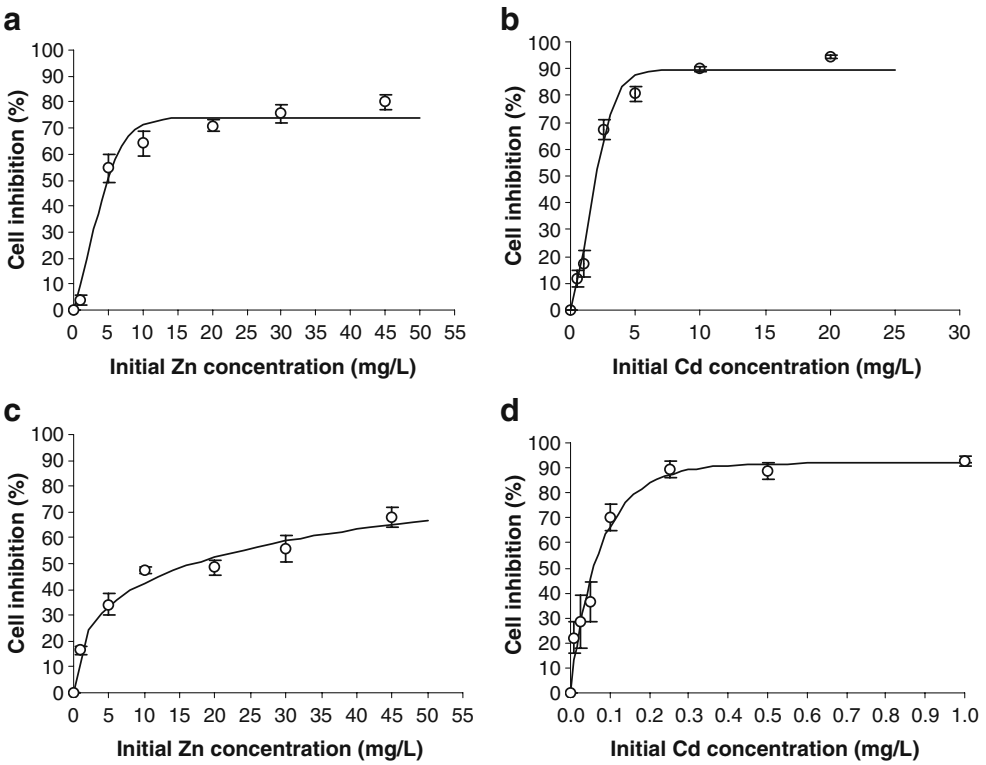

Fig. 2 Extent of cell inhibition of *D. pleiomorphus* (**a**, **b**) and *S. obliquus* (**c**, **d**) by 96 h of exposure of the microalgae to various initial concentrations of Zn (**a**, **c**) and Cd (**b**, **d**): experimental data (*open symbols*, average ± standard deviation, $n = 4$) and theoretical prediction using Weibull model (*solid line*)

obliquus and *D. pleiomorphus*, respectively. Tripathi and Gaur (2006) obtained a 50% reduction in cell number after exposure of *Scenedesmus* sp. to 1.64 mg L^{-1} Zn by 48 h, whereas EC_{50} values of 0.81 and 2.3 mg L^{-1} for *S. quadricauda* and *Chlorella kessleri*, respectively, were reported by Rojíčková-Padrtová and Maršálek (1999). Toumi et al. (2007) reported an EC_{50} of 0.34 mg L^{-1} after exposure of *Micratinium pusillum* to Zn for 72 h. The fact that this metal was an abundant contaminant in the original source site of our microalgae strains may account (at least partially) for our outstanding observations.

Growth inhibition of microalgae in response to increasing heavy metal concentrations in the medium has been reported elsewhere (Báscik-Remisiewicz et al. 2009; Costa and França 1998; Torres et al. 2000); such toxic effects apparently depend on both microalgal species and metal concentration, besides contact time. The exposure period chosen for our study has frequently been used in similar toxicity tests and is considered to be sufficient to unfold the putative toxic effect of metals (Torres et al. 2000; Toumi et al. 2007). In particular, Omar (2002b) reported that growth of *S. obliquus* and *S. quadricauda* decreased as Zn concentrations increased in the culture medium; low Zn concentrations when growing *S. obliquus* (i.e. 0.5 and 1.5 mg L^{-1}) and *S. quadricauda* (i.e. 0.5 mg L^{-1}) permitted a gradual increase in their growth rates, whereas sudden exposure to high Zn concentrations of *S. obliquus* (i.e. 4.5 and 8 mg L^{-1}) or *S. quadricauda* (i.e. 1.5, 4.5 and 8.0 mg L^{-1}) suppressed growth, with the most notable inhibition being observed at the highest concentration, and leading to 24% and 33% growth inhibition of *S. obliquus* and *S. quadricauda*, respectively. Nalimova et al. (2005) also described growth inhibition of *Spirulina platensis* cultures following exposure to higher and higher Zn concentrations.

Comparing the amount of Zn taken out from solution by both microalgae, by the last day of exposure, *S. obliquus* revealed a higher capacity to remove Zn than *D. pleiomorphus* at all initial concentrations experimented with (Fig. 1). The highest level of metal removal (29.2 mg L^{-1}) was attained at the highest initial metal concentration (45 mg L^{-1}).

Concerning exposure of both microalgae to Cd, growth inhibition was noticed right from the lowest metal concentration tested, with strong inhibition following exposure to the highest levels. Likewise, *S. obliquus* was more sensitive to Cd than *D. pleiomorphus* one. Toxicity studies reported by a few authors have revealed higher EC_{50} values for Cd than those obtained in our study (0.058 and 1.92 mg L^{-1} for *S. obliquus* and *D. pleiomorphus*, respectively), showing that our strains are more sensitive to this toxic metal. For instance, an EC_{50} of 22.39 mg L^{-1} for Cd was reported by Torres et al. (2000) in the case of *P. tricornutum* after exposure for 96 h; Báscik-Remisiewicz et al. (2009) found an EC_{50} of 16.8 mg L^{-1} for $CdCl_2$ in the case of *Desmodesmus armatus* by 24 h; Tukaj et al. (2007) obtained an EC_{50} of 10.45 mg L^{-1} for $CdCl_2$ in the case of *Scenedesmus armatus* by 24 h; and Lam et al. (1999) claimed an EC_{50} of 2.48 mg L^{-1} for Cd in the case

of *Chlorella vulgaris*. On the other hand, Visviki and Rachlin (1994) described an EC_{50} value much more similar to that obtained here: 0.5 mg L^{-1} of Cd for *Dunaliella salina* by 96 h. Finally, Toumi et al. (2007) exposed cells of *M. pusillum* to different levels of Cd and reported an EC_{50} value of 0.28 mg L^{-1} by 72 h.

Cain et al. (1980) found that growth of *S. obliquus* was markedly affected by concentrations of Cd above 1 mg L^{-1} and that the extent of inhibition correlated directly with that concentration. Visviki and Rachlin (1991) attributed the reduction of microalgal growth, as driven by toxic metals, to the inhibition of normal cell division because of metal binding to sulfhydryl groups that are important in regulating such a metabolic process. Costa and França (2003) reported that Cd in soluble form markedly affected cell growth of *Tetraselmis chuii*, with a 60% inhibition when exposed to 10.0 mg L^{-1}. Yan and Pan (2002) also reported a decrease in the growth of *S. obliquus* following exposure to increasing initial concentrations of the (related transition) metal Cu, whereas Mohammed and Markert (2006) found that the growth rate of *S. quadricauda* decreased upon addition of Cd; these authors claimed that this inhibition should be attributed to toxic effects exerted mainly on the respiratory process.

Comparing the amount of metal taken out from solution at similar initial metal concentrations (e.g. 20 mg L^{-1}), Zn was removed to a larger extent. A plausible explanation for this is that small amounts of Zn could be used by the cell as actual micronutrients (e.g. for enzyme synthesis) besides the amount of metal adsorbed onto the cell surface, whereas Cd is not necessary for cell metabolism at all, so it is solely (or mainly) removed by adsorption. The higher toxicity, to both species, of Cd than Zn may thus derive from the former being a nonessential element for living organisms, with no known biological function (Tukaj et al. 2007). Therefore, the reduction observed in the growth of microalgal cells, when in the presence of increasing concentrations of Zn and Cd, does apparently result from interference with basic physiological processes (e.g. cell division, membrane assembling, photosynthesis and respiration) owing to their great affinity for biological structures that contain –SH groups; occurrence of severe toxic effects at high concentrations would then cause cell enlargement and structural damages (e.g. thylakoid disorganization in the chloroplasts) that will eventually cause cell death (Nalimova et al. 2005; la Rocca et al. 2009). As a self-defence mechanism against the toxic effects of said metal ions, microalgal cells are equipped with machinery for extracellular adsorption or intracellular complexation, the efficiency of which is reflected on their EC_{50} value for each specific metal ion (Nacorda et al. 2007).

Microalgae are some of the most important organisms in our ecosystem because they are the main producers lying on the base of the food chain. Microalgae are characterized by rapid growth rates and ubiquitous distribution throughout natural environments, yet they show a critical sensitivity to environmental variations (e.g. nutrient levels and presence of pollutants); this is why the use of microalgae as test organisms is gaining importance. Furthermore, microalgal toxicity tests are quick and inexpensive, and can be effectively used to assess those toxic substances that are found in concentrations too low for conclusive detection via higher trophic organisms (Wong and Couture 1986). Finally, there is a likely application of microalgae in determining toxicity of metal ions in situ because the microalgal species used already exist in a contaminated environment, so any change in the levels of the toxic metals will be directly reflected upon the size of its population.

Conclusions

The 96-h EC_{50} values obtained were 0.058 and 1.92 mg L^{-1} for Cd and 16.99 and 4.87 mg L^{-1} for Zn, in the case of *S. obliquus* and *D. pleiomorphus*, respectively. Cd was toxic to *S. obliquus* and *D. pleiomorphus* at much lower concentrations than happened with Zn. Cd and Zn significantly inhibited growth of both microalga species at the highest concentrations tested, whereas lower concentrations caused only a slow decline in microalgal biomass; at the lowest Zn concentration considered (i.e. 1 mg L^{-1}), an increase in the growth of *D. pleiomorphus* cells was actually observed. The Weibull model provided a good fit to data pertaining to the inhibition of both microalgae upon exposure to either Zn or Cd ions, so it may be useful in attempts to calculate EC_{50} values associated with toxic effects in aquatic ecosystems at large. In view of the increasing contamination of aquatic ecosystems by toxic metals, and given the placement of microalgae at the basis of the food chain, ecotoxicological studies (as the one reported here) encompassing wild strains are of greatest importance towards the design of bioremediation strategies and integrated environmental monitoring.

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