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ORIGINAL PAPER

Characterization of *Desmodesmus pleiomorphus* isolated from a heavy metal-contaminated site: biosorption of zinc

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Abstract Microalgae have been proven efficient biological vectors for heavy metal uptake. In order to further study their biosorption potential, a strain of *Desmodesmus pleiomorphus* (L) was isolated from a strongly contaminated industrial site in Portugal. Under different initial Zn^{2+} concentrations, metal removal by that strain reached a maximum of 360 mg Zn/g biomass after 7 days, at 30 mg Zn/l, after an initial rapid phase of uptake. Comparative studies were carried out using a strain of the same microalgal species that is commercially available (ACOI 561): when exposed to 30 mg Zn/l, it could remove only 81.8 mg Zn/g biomass. Biosorption experiments using inactivated biomass of the isolated strain reached a maximum Zn^{2+} uptake of 103.7 mg/g. Metal removal at various initial pH values was studied as well; higher removal was obtained at pH 5.0. The microalga strain L, isolated from the contaminated site, exhibited a much higher removal capacity than the commercial strain, and the living biomass yielded higher levels of metal removal than its inactivated form.

Keywords Microalgae · Accumulation · Bioremediation · pH · Metal uptake

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Introduction

Contamination by heavy metals has received increased attention in recent years, because this form of pollution may produce adverse biological effects in aquatic environments; these effects will eventually lead to structural changes in local planktonic communities, hence reducing biodiversity, and consequently disturbing the food chain and the internal ecosystem balance (Pérez-Rama et al. 2002).

The use of microbial biomass to remove heavy metal cations from aqueous media has attracted considerable interest, because it is a technically feasible and an economically suitable alternative to physicochemical treatments; these include precipitation, ion exchange and membrane processes (Tien 2002). Microalgae have indeed been found to be potential biosorbents of heavy metal cations from wastewater, owing to a favourable combination of features: low cost, prompt availability, relatively high specific surface area and good binding affinity (Gong et al. 2005; Tien 2002). In addition, they use light as energy source, which allows metabolism in the absence of organic carbon sources unlike what is strictly required by bacteria and fungi (Dönmez and Aksu 2002). Additionally, when compared to such biological materials as fungi, bacteria and yeasts, the heavy metal uptake capacity of microalgae has proven the highest in some cases (Tüzün et al. 2005), owing mainly to their cell walls: these are constituted by polysaccharides, proteins and lipids,

which offer a number of functional groups, e.g. carboxyl, hydroxyl, sulphate, phosphate and amine moieties, all of which are known to bind metal ions tightly (Gong et al. 2005; Kaduková and Virčíková 2005; Rangsayatorn et al. 2002). Hence, both living and dead algal cells have been increasingly used as biosorbents, especially to backup large scale bioremediation strategies (Abu Al-Rub et al. 2004; Deng et al. 2007; Han et al. 2006; Tam et al. 1998). However, non-living cells are more advantageous for industrial applications, because they are not affected by the toxicity of the metal ions and by adverse operating conditions (Chu and Hashim 2004); furthermore large quantities are readily and economically available as by-products of biotechnology industries (Cruz et al. 2004). For instance, Costa and França (2003) described the accumulation of Cd ions by the microalga *Tetraselmis chuii* with both growing and dead cells; Doshi et al. (2007) and Bayramoğlu and Arica (2008) reported the bioremediation capacity of the cyanobacterium *Spirulina* sp. and of dead biomass of the fungus *Lentinus edodes* to remove Cd ions from aqueous solutions.

Of the metallic elements, Zn is one of the most abundant besides being a well-known essential micronutrient (Omar 2002). Since the industrial revolution, and mainly due to a crescent exploration of mining and industrial activities, pollution with this metal is widespread (Nriagu and Davidson 1979), not only in soils and sediments but also in freshwater sources. In aquatic environments, Zn toxicity is often associated with direct toxicity because of high concentrations of Zn in the water, and also with food chain cumulative toxicity, especially to a number of species of algae, crustaceans and salmonids (Irwin et al. 1997). Microalgae constitute the basis of the aqueous food chain, so said toxicity of heavy metals becomes particularly relevant to the whole aquatic food chain and eventually poses a serious threat to human health (Pérez-Rama et al. 2002). High concentrations of Zn in microalgae can hamper cell division and decrease total chlorophyll content (Omar 2002), as well as seriously affect ATPase activity (Labyntseva et al. 1998) and carotenoid/clorophyll ratio (Rai et al. 1981).

Metal removal using microalgal species isolated from polluted environments, taking advantage from their high resistance to toxic pollutants and high binding affinity, has been reported. *WW1*, an isolate identified as *Chlorella miniata* (Tam et al. 2001), and *Chlamydomonas acidophila* (Nishikawa and Tominaga 2001) are examples of two microalgal species isolated from polluted environments, that demonstrated to be highly resistant to toxic metals present in their surrounding habitat.

Removal of heavy metal cations by microalgae is affected by several environmental factors, including the specific surface properties of the microorganism, and the physicochemical properties of the surrounding solution; these encompass pH, metal ion content, temperature and biomass concentration (Özer et al. 1999). Despite the strong effect of the initial metal ion concentration, the solution pH is an important factor for the biosorption of heavy metals, affecting the protonation of the binding sites on the biomass cell wall and the speciation of the metal ions themselves (Deng et al. 2007). In general, an increase in biosorption levels occurs as solution pH increases, due to the strong relation of the extent of biosorption to the number of surface negative charges (which depends on the dissociation of functional groups). The low biosorption capacity observed at low pH values has been attributed to hydrogen ions, that compete with metal cations for the surface sorption sites (Bayramoğlu and Arica 2008).

Desmodesmus pleiomorphus is a green freshwater microalga of the Chlorococcales family, that plays an important role in freshwater ecosystems; it is also frequently found in polluted environments, so it is widely employed in bioremediation studies (Omar 2002).

The central aim of the present work was to study the effect of Zn ions upon growth of, and metal removal by living cells of *D. pleiomorphus*. This wild strain (hereafter denoted as L) was isolated from a heavy metal-polluted site in northern Portugal, containing sediments characterized by levels of metals above the limits established by the European Directive 86/278/EC. Among the metals present at higher levels in those sediments, Zn appears as one of the main contaminants: its levels are as high as 3,620 mg Zn/kg (Oliveira et al. 2001). A commercially available strain of *D. pleiomorphus* (ACOI 561) was included in the study; this permitted conclusion on whether the L strain isolated from the metal contaminated site is more effective in removing the metal from solution, which is an important rationale for its

eventual choice in bioremediation approaches with enhanced performance.

Specific objectives of this research included understanding the adsorption and absorption capacities of the strain that performed better results in metal removal from solution, in both viable and inactivated forms; it was found to be, at high initial metal concentrations and various pH values, the local strain. Although in bioremediation the combination of adsorption and absorption abilities determines the total extent of metal removal, both were characterized in this research effort in order to better comprehend the underlying biosorption phenomena.

Materials and methods

Microalgae

A large industrial complex, composed essentially by heavy chemical facilities, surrounds the area selected as microbial source for the present study. For many years, those plants have disposed off their solid residues on an informal sediment basin located in their surroundings, and released their liquid effluents into a water stream nearby ("Esteiro de Estarreja"). Among the metals detected at higher levels in those sediments—ca. 835 mg Pb/kg, 66 mg Hg/kg, 26 mg Cr/kg, 37 mg Ni/kg and 16,800 mg Fe/kg (Oliveira et al. 2001), Zn appears to be one of the major contaminants: its levels are up to 3,620 mg Zn/kg. The banks of the water stream, characterized by a slope of ca. 45° and a width of ca. 2 m, are periodically flooded with rainwater, from late October to late February, whereas the ditch of the stream, with a depth of ca. 1.5 m, remains almost dry during the remaining months. Sediment samples were taken at random from the ditch; microalgal species were then obtained from those samples via selective techniques, adding 20 mg Zn/l in the medium to create a selective growth pressure, for a period of 40 days (Monteiro et al., unpublished data). Afterwards, microalgae strains were typed as *D. pleiomorphus* and *Scenedesmus obliquus* by the Algae Culture Collection at University of Coimbra (Portugal); the strain labelled hereafter as L was a single clone of *D. pleiomorphus*. The *D. pleiomorphus* (ACOI 561) culture was obtained from the aforementioned Culture Collection (ACOI), and was previously named as *Scenedesmus pleiomorphus*.

Culture conditions

Both strains of *D. pleiomorphus* (L and ACOI 561) were cultivated in Optimal Haematococcus Medium, OHM (Bishop and Senger 1971), without ethylenediaminetetracetic acid (EDTA); this medium contains all nutrients necessary for growth of freshwater microalgal species, and its performance was found to be better than that of other media tested. All chemicals used for culture medium preparation were of analytical-grade: Pronalab (potassium nitrate—KNO_3), Fluka (ferric citrate hydrate—$C_6H_5FeO_7 \cdot H_2O$, chromium oxide—Cr_2O_3, selenium dioxide—SeO_2), Riedel-de-Haen (manganese chloride tetrahydrate—$MnCl_2 \cdot 4H_2O$), Sigma (sodium molybdate dihydrate—$Na_2MoO_4 \cdot 2H_2O$, thiamine hydrochloride and B_{12} vitamin) and Merck (disodium hydrogen orthophosphate dihydrate—$Na_2HPO_4 \cdot 2H_2O$, magnesium sulphate heptahydrate—$MgSO_4 \cdot 7H_2O$, calcium chloride dihydrate—$CaCl_2 \cdot 2H_2O$, cobalt chloride dihydrate—$Cl_2Co \cdot 6H_2O$, copper sulphate pentahydrate—$CuSO_4 \cdot 5H_2O$, biotin). Cultures were maintained at 25°C under continuous light at an irradiance of 29.18 μE/(sm^2), using cool light fluorescent lamps. Microalgal cells were harvested at the exponential phase (after 2–3 days of growth), and were then used as inoculum in all subsequent experiments.

Cell growth was determined by measuring optical density (OD) at 600 nm in a Shimadzu mini 1240 spectrophotometer (Japan), and subsequently converting it to dry weight (DW) via a previously prepared calibration curve; this curve was validated for various stages of microalgal growth, according to Scragg and Bonnet (2002), Schmitt et al. (2001), and Yan and Pan (2002); this indirect analytical approach was mandatory, because of the reduced amount of sample and the (usual) requirement of large amounts for use in gravimetric methods.

A stock solution of Zn ions was prepared by dissolving zinc chloride ($ZnCl_2$) in deionised water, to a final concentration of 5 g Zn/l (note that all zinc concentrations hereafter are expressed as mass of Zn^{2+}, and not as mass of $ZnCl_2$). For each experiment, an appropriate volume of the stock solution was added to the culture medium in order to obtain the desired concentration.

All materials used to handle and grow the microalgae were previously rinsed with nitric acid, and then several times with deionised water.

Determination of cell growth, and adsorption on and absorption by living cells

To asses the effect of the concentration of Zn ions on its removal by the microalgae, metal uptake batch tests were performed in triplicate, with cultures of both strains, at an initial biomass level of 0.02 g/l and using 1 l-glass flasks. The concentrations tested were 0, 1, 5, 15 and 30 mg Zn/l. Samples of 75 ml were collected at the beginning of the experiment, and then daily, in duplicate, for a period of 7 days, and assayed for biomass and Zn concentration. Cell growth was determined by measuring OD at 600 nm, which was subsequently converted to DW. Zinc removal in the supernatant solution was determined as described below, after centrifugation at 4,000 rpm for 15 min at 4°C. Afterwards, the pellet was washed for 20 min with an aqueous solution of 0.02 M EDTA to remove Zn ions adsorbed onto the cell surface, thereby allowing only intracellular Zn to be determined. Cells were then centrifuged, and the pellet was digested overnight with 1 ml of 15 M nitric acid (HNO_3) and 0.5 ml of 70% perchloric acid ($HClO_4$), to determine the amount of metal incorporated in the cytoplasm by microalgal cells. Zinc concentrations in the supernatant solution and in the intracellular fraction were determined using atomic absorption spectrophotometry with flame atomization (FA-AAS), in a Perkin Elmer 3100 (USA) spectrophotometer, with a minimum detection limit of 0.0088 mg Zn/l, according to the method proposed by Matsunaga et al. (1999) and Pérez-Rama et al. (2002). The total Zn taken up by microalgae was assessed by measuring the difference between the initial and the final Zn concentration in the supernatant. The amount of Zn adsorbed onto the cell surface was determined as the difference between the total amount of Zn removed and the intracellular amount of Zn. Blank controls, composed of medium added with metal and no microalgae, were provided for each concentration in all experiments; the total Zn concentration remained constant in those controls for the timeframe of each experiment, so contamination or loss of metal were ruled out. No Zn was found adsorbed on the inner surface nor precipitated on the glass flasks [e.g. as zinc hydroxide (ZnOH) or zinc carbonate ($ZnCO_3$)].

Determination of adsorption on dead cells

To study the effect of Zn concentration on the adsorption capacity of the dead microalga, cells of *D. pleiomorphus* (L) in the exponential growth phase were inactivated, by heating at 100°C in an oven for 24 h. The inactivated cells, at an initial microalgal biomass of 0.02 g/l, were then transferred to the appropriate culture medium (with the desired metal concentration); this was done, instead of transferring to a buffer solution, in order to maintain as similar as possible the initial conditions of the removal experiment using living cells (although in an application setting, a buffer solution would be sufficient).

Metal removal batch tests were performed in triplicate, using 250 ml-glass flasks, in a bath stirred at 100 rpm, and kept at 25°C. The concentrations tested were 1, 5, 15 and 30 mg Zn/l. Samples were collected at 0, 5, 15, 30, 60 and 90 min, and then centrifuged at 6,000 rpm for 15 min at 4°C. The amount adsorbed was evaluated as described above for the viable biomass.

Determination of effect of pH on metal removal

Batch tests were performed in triplicate, using 250 ml-glass flasks, in which the pH of the medium was initially set at given values ranging from 3 to 7. No pH control was provided, as pH did not typically undergo any significant variation within the experiment timeframe. pH was measured (Crison-Micro pH 2001) at the beginning and at the end of each experiment. The initial microalgal biomass employed was 0.32 g/l, and the initial metal concentration was 15 mg Zn/l. A biomass higher than that used in the adsorption and absorption experiments was used in order to improve the discriminating power of the medium pH. Samples of 10 ml were collected at 0, 5, 15, 30, 45, 60, 90 and 120 min, and Zn removal was assessed, as described above.

Statistical analyses

The statistical tests were performed with the SPSS software, v. 16.0 (USA). The experimental data

produced were subject to analyses of variance (ANOVA). To detect the statistical significance of differences between means, Student's *t*-test and Tukey's test were applied (unless otherwise indicated).

Results

Cell growth, and adsorption on and absorption by living cells

The growth curves of *D. pleiomorphus* (L) and *D. pleiomorphus* (ACOI 561) are shown in Fig. 1. For both strains, growth decreased significantly ($P < 0.05$) with increasing Zn concentration in the medium. Two-way ANOVA was performed on data for each strain pertaining to growth vs. initial Zn concentration and incubation time. The *F*-values associated with *D. pleiomorphus* (L) biomass vs. time, vs. initial Zn concentration and vs. their interactions, were 639 ($P < 0.001$), 338 ($P < 0.001$) and 104 ($P < 0.001$), respectively. The *F*-values associated with *D. pleiomorphus* (ACOI 561) biomass vs. time, vs. initial Zn concentration and vs. their interactions, were 367 ($P < 0.001$), 173 ($P < 0.001$) and 56 ($P < 0.001$), respectively. Additionally, statistical analysis of the underlying data (via a Student's *t*-test) indicated that strain L was significantly more tolerant ($P < 0.05$) in the first 2 days of growth, presenting higher growth rates than the commercial strain (ACOI 561). From 3 days on, there were no significant differences between biomass production of the two microalgal strains ($P < 0.05$).

The time courses of Zn removed, adsorbed on and absorbed by the two microalgal strains are

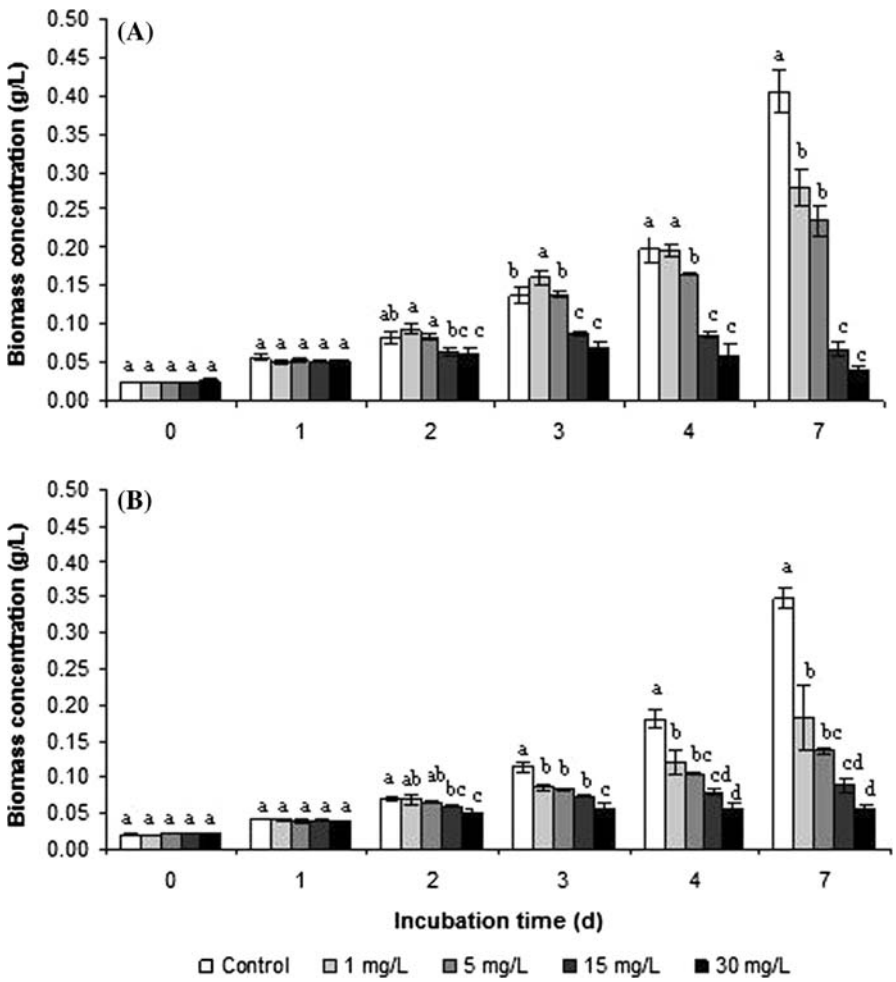

Fig. 1 Growth of **a** *Desmodesmus pleiomorphus* (L) and **b** *D. pleiomorphus* (ACOI 561) throughout incubation time, at various initial concentrations of Zn. Results are expressed as means; *error bars* represent standard deviation ($n = 3$). Means for the same time of collection, labelled with *different letters*, are significantly different from each other ($P < 0.05$)

shown in Fig. 2, for several initial metal concentrations. Significant ($P < 0.05$) differences in total Zn removal between the two microalgal strains were observed at all incubation times, according to Student's *t*-tests. Furthermore, the total metal removal seemed to follow a pattern of initial fast uptake, reaching a maximum by ca. 1 day, with further stabilisation (or only slight variation) in uptake. One-way ANOVA applied to total Zn removal vs. time, for each initial Zn concentration, led to *F*-values for *D. pleiomorphus* (L) of 37 ($P < 0.001$), 67 ($P < 0.001$), 125 ($P < 0.001$) and 21 ($P < 0.001$), for the data in Fig. 2(ia), (ib), (ic) and (id), respectively. In the case of the commercial strain (ACOI 561), the corresponding one-way ANOVA produced *F*-values of 26 ($P < 0.001$), 129 ($P < 0.001$), 62 ($P < 0.001$) and 104 ($P < 0.001$), for the data in Fig. 2(iia), (iib), (iic) and (iid), respectively.

Most Zn was removed via adsorption onto the cell surface, except at the lowest Zn concentration tested with *D. pleiomorphus* (L): in this case, the amount of metal incorporated by the microalga after 3 days was higher than the adsorbed one.

Regression analysis was applied in attempts to unfold the relationship between total amount of Zn removed from solution and those amounts adsorbed and accumulated intracellularly. Positive correlations, characterized by Pearson's correlation coefficients of 1.000 ($P < 0.001$), were found for both strains; this indicated that the adsorption extent is proportional to total removal. Concerning the relationship between total Zn removed and Zn bioaccumulated, no significant correlation was obtained for the ACOI 561 strain, but a negative correlation was obtained for the L one; Pearson's correlation coefficients of -0.061 ($P > 0.05$) and -0.269 ($P < 0.05$), respectively, were accordingly found.

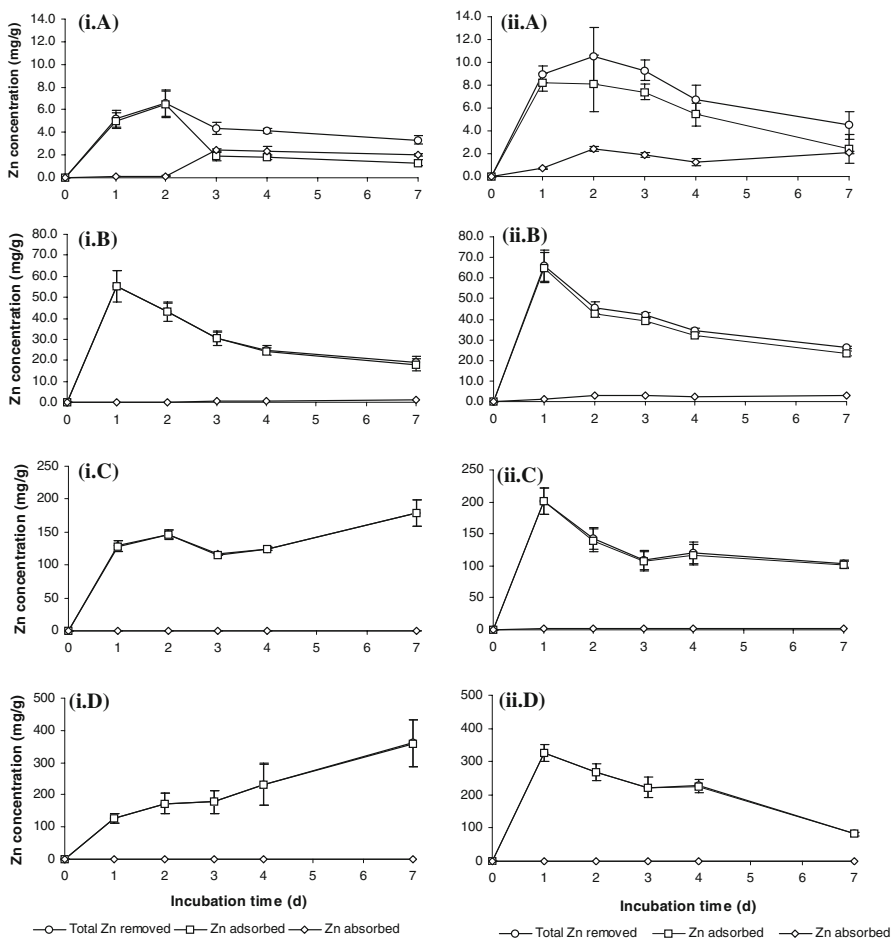

Fig. 2 Total amounts of Zn removed, adsorbed and absorbed by (i) *Desmodesmus pleiomorphus* (L) and (ii) *D. pleiomorphus* (ACOI 561) throughout incubation time, at various initial concentrations of Zn, viz. **a** 1, **b** 5, **c** 15 and **d** 30 mg/l. Results are expressed as means; *error bars* represent standard deviation ($n = 3$)

Cumulative extents of removal of Zn (total, adsorbed and absorbed) were determined by the last day of exposure, and are presented in Table 1. The maximum amount of metal removed was 360 for *D. pleiomorphus* (L) and 103 mg $Zn/g_{microalga}$ for *D. pleiomorphus* (ACOI 561), when exposed to 30 and 15 mg Zn/l, respectively; recall that these were the highest concentrations of Zn considered. The highest percent degrees of removal of Zn took place at the initial concentration of 1 mg Zn/l, and corresponded to 92 and 79% of the total initial Zn, for the L and the ACOI 561 strains, respectively. The level of absorbed Zn decreased with increasing Zn concentration in the supernatant, for both strains (Table 1).

Adsorption on dead cells

The non viable biomass of *D. pleiomorphus* (L) was able to remove appreciable quantities of Zn from solution. For the lower initial concentrations of Zn in solution, most removal occurred during the initial stages, whereas for the higher concentrations (viz. 30 mg/l), the maximum degree of removal was attained later, as apparent in Fig. 3.

The degree of Zn uptake by inactivated cells of *D. pleiomorphus* (L), by the end of the experiment, increased with increasing initial Zn concentration in solution. ANOVA one-way was performed for Zn removal by 90 min vs. initial Zn concentration in solution, with an F-value of 67 ($P < 0.001$). The degrees of removal obtained at 15 and 30 mg Zn/l were not significantly ($P > 0.05$) different from each other, but were all significantly ($P < 0.05$) higher than those obtained at 1 and 5 mg Zn/l, which, in turn, were not significantly ($P > 0.05$) different from each other. The highest value of 103.7 mg $Zn/g_{microalga}$ was observed at the initial concentration of 30 mg Zn/l (Fig. 3).

Effect of pH on metal removal

The effect of pH on Zn adsorption by viable biomass of *D. pleiomorphus* (L) is shown in Fig. 4. By 120 min, the lowest amount of Zn removed (i.e. 13.1 mg $Zn/g_{microalga}$ or 28% removal) was observed at pH 3.0. As pH increased, the biosorption of Zn also increased, and eventually reached a maximum at pH 5.0: 30.7 mg $Zn/g_{microalga}$ (or 67% removal). A two-way ANOVA was performed for Zn removal vs. pH and vs. time. The F-values associated with Zn removal by *D. pleiomorphus* (L) vs. time, vs. pH and vs. time and pH interaction, were 322 ($P < 0.001$), 333 ($P < 0.001$) and 8.54 ($P < 0.001$), respectively. Based on this analysis, *D. pleiomorphus* (L) could be claimed

Table 1 Total amounts of Zn removed, adsorbed and absorbed by 7 days, by the two strains of *Desmodesmus pleiomorphus*, at various initial concentrations of Zn

Initial Zn concentration (mg/l)	Total Zn removed (mg/g)	Zn adsorbed (mg/g)	Zn absorbed (mg/g)
D. pleiomorphus (L)			
Control	$<DL^{c}$*	$<DL^{c}$*	$<DL^{c}$*
1	3.31 ± 0.38^{c}	1.32 ± 0.31^{c}	1.99 ± 0.09^{a}
5	19.17 ± 2.83^{c}	17.86 ± 2.67^{c}	1.31 ± 0.22^{b}
15	179.29 ± 20.45^{b}	178.96 ± 20.30^{b}	0.33 ± 0.15^{c}
30	360.21 ± 71.72^{a}	359.99 ± 71.69^{a}	0.22 ± 0.04^{c}
	$F = 67$ ($P < 0.001$)	$F = 67$ ($P < 0.001$)	$F = 133$ ($P < 0.001$)
D. pleiomorphus (ACOI 561)			
Control	$<DL^{d}$*	$<DL^{d}$*	$<DL^{c}$*
1	4.50 ± 1.19^{d}	2.42 ± 1.28^{d}	2.08 ± 0.09^{b}
5	26.45 ± 0.66^{c}	23.40 ± 1.12^{c}	3.04 ± 0.52^{a}
15	103.38 ± 5.87^{a}	101.26 ± 6.07^{a}	2.12 ± 0.22^{b}
30	81.81 ± 4.67^{b}	81.54 ± 4.69^{b}	0.27 ± 0.08^{c}
	$F = 566$ ($P < 0.001$)	$F = 532$ ($P < 0.001$)	$F = 77$ ($P < 0.001$)

Results are expressed as means ± standard deviation ($n = 3$). Means for the same amount of Zn removed and each microalga, labelled with *different letters*, are significantly different from each other ($P < 0.05$)

* DL detection limit (0.0088 mg/l)

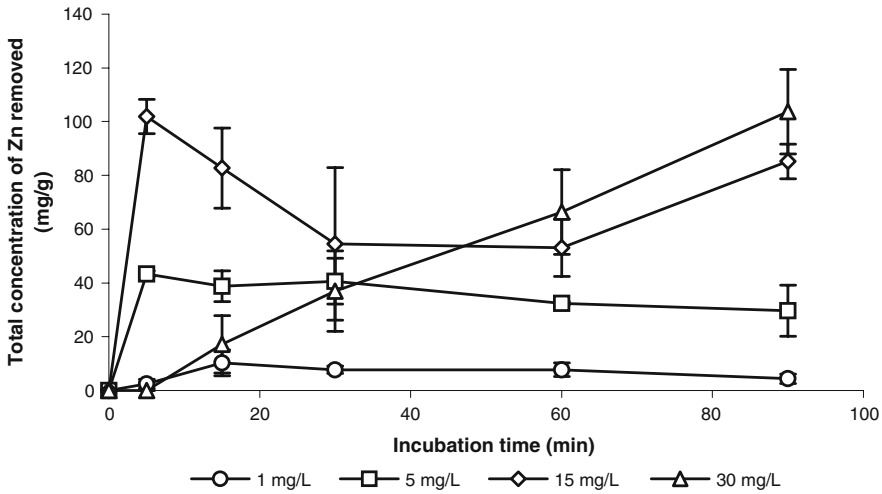

Fig. 3 Total amount of Zn removed by inactivated *Desmodesmus pleiomorphus* (L) throughout incubation time, at various initial concentrations of Zn. Results are expressed as means; *error bars* represent standard deviation ($n = 3$)

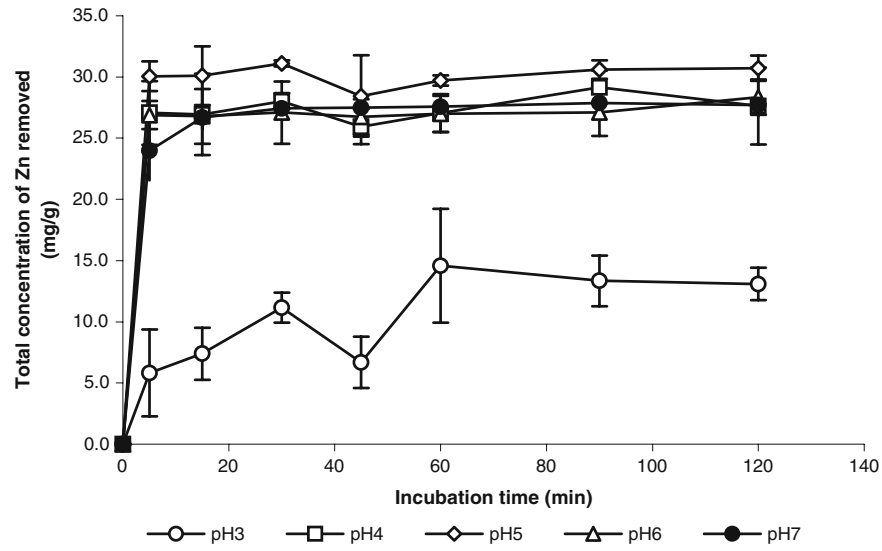

Fig. 4 Total amount of Zn removed by *Desmodesmus pleiomorphus* (L) throughout incubation time, at the initial concentration of Zn of 15 mg/l and at various pH values. Results are expressed as means; *error bars* represent standard deviation ($n = 3$)

as significantly ($P < 0.05$) more efficient at pH 5.0, and less efficient at pH 3.0; the degrees of removal attained at pH 4.0, 6.0 and 7.0 were not significantly ($P > 0.05$) different from each other, but were all significantly ($P < 0.05$) higher than those obtained at pH 3.0, and lower than those obtained at pH 5.0.

Discussion

Cell growth, and adsorption on and absorption by living cells

A native culture (L) isolated from "Esteiro de Estarreja" and a commercial strain were tested in this study, in order to assess topical differences in metal removal capacities in response to similar environmental conditions.

The microalgal cultures showed a significant reduction in biomass levels when exposed to increasing metal concentrations in the culture medium; this decreasing pattern over time is similar to that reported by Omar (2002) for *S. obliquus* and *Scenedesmus quadricauda*, when exposed to Zn.

Zn removal by both microalga strains followed an initial phase of fast uptake, reaching its maximum during the first days of culture, followed by stabilization (or only slight variation) thereafter; maximum Zn removal obtained in this study by the last day of exposure was 360 and 103.38 mg/$g_{microalga}$ for

D. pleiomorphus L and ACOI 561 strains, respectively, at 30 and 15 mg/l (i.e. the highest concentrations tested).

Although, at a first glance, a decrease in biomass should lead to a decrease in Zn sorption, lower biomass density is also equivalent to fewer active sites, each one of which could be more efficient in metal recovery (on a unit basis). In general, increasing biomass increases the overall extent of metal binding; however, as the concentration of the metal decreases along time, metal binding becomes more difficult due to the lower accessibility of the metal. It has been claimed that increasing biomass reduces the intrinsic metal removal capacity, because the solute becomes less available, and unfavourable interference between binding sites and electrostatic interactions is enhanced (Fourest and Roux 1992). Other studies (Ahuja et al. 1999; Fraile et al. 2005) have indeed indicated that electrostatic interactions between cells play an important role in this type of process, and that the shell effect on the external layer at high cell densities constrains the availability of active sites themselves.

After 7 days, at the lowest concentration tested (1 mg/l) for both strains of *D. pleiomorphus*, the amount of metal adsorbed at the cell surface was lower than that incorporated by the cells (Table 1). This pattern may be explained by the need of incorporation of low levels of Zn by microalgal cells to support their growth.

However, for all other Zn concentrations tested, most metal removal was by adsorption onto the cell surface, for both microalgae strains. It has been described that microalgae can protect themselves against the toxicity caused by heavy metals using several approaches: exclusion mechanisms, adsorption to cell surface or intracellular (segregated) accumulation (Abd-el-Monem et al. 1998; Hassler et al. 2005; Omar 2002; Yan and Pan 2002). Because of the lack of published data regarding metal removal by *D. pleiomorphus*, results reported in others studies, with similar microalgae were chosen for the sake of comparison. Hassler et al. (2005) stated that small amounts of cellular Zn were observed in *Chlorella kesslerii* cells, whereas Wilde et al. (2006) described that *Chlorella* sp. accumulated intracellularly the highest proportion of Zn removed from the medium, in the range 0.1–2 mg/l; Yan and Pan (2002) reported, in turn, that *Closterium lunula* cells did not undergo damage in the presence of 50 μg Cu/l, owing to their ability to exclude this metal. Additionally, Ahuja et al. (2001) claimed that the enhanced tolerance of *Oscillatoria anguistissima* to Zn is a consequence of a reduced intracellular uptake. This latter case is the most similar to our evidence encompassing the two strains (as shown in Fig. 2), and it will likely constitute the tolerance mechanism that is characteristic of our species. Regression analysis proved that adsorption extent is proportional to total Zn removal, which is in agreement with Chojnacka et al. (2005).

For Zn removal, decreases were observed in the total and the adsorbed amounts of metal, after initial fast uptakes, especially for the ACOI 561 strain. Such a release of Zn ions back into solution may be due to partial loss of adsorption capacity, as a result of cell intoxication (Danihelka et al. 1997; Kaduková and Virčíková 2005). This phenomenon was in general not observed in strain L, probably owing to an underlying tolerance mechanism, that might have been developed by those microorganisms in response to the pressure posed by their naturally contaminated environment.

The maximum values of total Zn removed by 7 days (as obtained in this study) were higher than those reported by Ahuja et al. (2001) encompassing Zn removal by (the related cyanobacterium) *O. anguistissima*—i.e. 35.7 and 83.4 mg Zn/g D.W., after 10 days of growth under 20 and 30 mg Zn/l, respectively; or by Pawlik-Skowrońska (2003) for (the related green microalga) *Stigeoclonium tenue*—i.e. 8.1 mg Zn/g D.W., after 3 weeks of exposure to 2 mg Zn/l. Omar (2002) and Schmitt et al. (2001) claimed maximum specific adsorption capacities of 5.03 mg/g for *S. quadricauda* (after 24 h incubation in solutions ranging from 0.5 to 11 mg Zn/l) and 72.06 mg/g for *Scenedesmus subspicatus* (when exposed to 0.5 mg Zn/l for 4 days). Romera et al. (2007) reported that the microalga *Spirogyra insignis* removed a maximum of 21.1 mg/g of Zn ions, and 51.5 mg/g when exposed to Pb ions. Another *Spirogyra* sp. has also been described as able to remove a maximum of 141 mg/g, when in the presence of 200 mg Pb^{2+}/l (Gupta and Rastogi 2008). On the other hand, when analysing the removal data as percent of total Zn removed, it decreased with increasing Zn concentrations; however, the total amount removed was higher when cells were exposed

to higher initial concentrations, thus indicating that the adsorption/absorption capacity was not depleted.

Metal incorporation in the cytoplasm is a metabolic-dependent process; since at higher concentrations metals become toxic to microalgae, their normal growth and metabolic performance are likely affected, so absorption will be reduced.

Although the nature of the metal removal process depends on both the metal and the microorganism (Han et al. 2006), rapid initial Zn removal was also reported elsewhere for *Olisthodiscus luteus* and *Tetraselmis suecica* (Leborans and Novillo 1996; Pérez-Rama et al. 2002). Once again, the process of biological removal of heavy metal cations from solution occurred via two parallel routes: adsorption and bioaccumulation. The former involves physical binding of metal ions to functional groups on the cell surface (Matsunaga et al. 1999), whereas bioaccumulation involves intracellular accumulation (or absorption) of metal ions by the cell (Kaduková and Virčíková 2005; Matsunaga et al. 1999). As claimed in previous studies, adsorption is essentially independent of cell metabolism, so it is expected to occur rapidly towards an equilibrium (Chojnacka et al. 2005; Rangsayatorn et al. 2002) and to be essentially reversible (Kaduková and Virčíková 2005). Based on the assumption that metal removal by microalgal cells is mostly via adsorption onto their cell surface, a few authors have tested the efficiency of various desorbing agents to remove the adsorbed metal ions, and thus regenerate the sorbent so as to allow their sequential use. Deng et al. (2007) and Gong et al. (2005) reported that both EDTA and HNO_3 were good desorbing agents, with a recovery of ca. 80% in the case of Pb^{2+} from *Cladophora fascicularis*, and ca. 90% from *Spirulina maxima*. Chojnacka et al. (2005) described, in turn, 0.1 M nitric acid as an efficient desorbing agent, with a 98% recovery of the metal bound to the biomass, without affecting adsorption capacity. The applicability of microalgae in wastewater treatment is thus apparent.

From the data presented in Table 1, adsorption appears to be the dominant mechanism underlying removal of Zn by both *D. pleiomorphus* strains; and Fig. 2 indicates that such a phenomenon occurs rapidly, thus reaching its maximum plateau in a short time span for both strains. In addition, reversibility was also noticed for *D. pleiomorphus* (ACOI 561) grown at 30 mg Zn/l (results not shown).

From inspection of Fig. 2, one notices, for all the concentrations tested on the ACOI strain and for the two lowest ones on the wild strain, that Zn removal went through a maximum in the first days of exposure, and then decreased with time to eventually reach equilibrium toward the end of the experiment. This realisation could be rationalized by a tolerance mechanism of those strains when exposed to toxic metals: almost all metal is adsorbed onto the cell wall in the initial contact time, and then is released back to the culture medium above a given threshold. On the other hand, and for the two highest concentrations tested for the wild strain, Zn removal increased with time; in our case, this is reasonable if we consider that during the time-frame of the experiment, no saturation of the functional groups on the cell wall, involved on the removal of the metal ions, occurred.

In view of the removal capacity attained by *D. pleiomorphus* (L), which is even higher than that exhibited by the commercial strain ACOI 561, there is a logical preference of this species for bioremediation purposes.

Adsorption on dead cells

Although uptake of Zn by the inactivated biomass can be hypothesized (e.g. when only cell walls and membranes are damaged), references available in the literature do not refer to that possibility, so all metal removed from solution was considered to be fully adsorbed onto the dead cells.

The results presented in Fig. 3 reveal that, for lower initial metal concentrations, Zn removal was fast: whereas for higher concentrations, the maximum degree of Zn removal was achieved later in time. These results are consistent with biosorption data reported for other microalgae, such as *Spirulina platensis* (Rangsayatorn et al. 2002) and *Chlorella vulgaris* (Aksu and Kutsal 1990).

For the highest concentration tested, a longer time was required to reach equilibrium between the adsorbed metal ions on the cell surface and the residual metal ions in solution. However, a short exposure time (90 min) was selected based on previous information withdrawn from several published studies. It has been described that metal uptake by inactivated microalgal cells is exclusively due to adsorption onto the cell wall surface (Chu and Hashim 2004), a process that should reach

equilibrium after a few minutes of contact (Özer et al. 1999; Tüzün et al. 2005); hence, equilibrium was not attained under our conditions, but only at the highest initial concentration experimentally tested, a longer contact time would have been necessary to obtain the maximum level of Zn removal.

The maximum amount of Zn removed by inactivated cells of the L strain (103.7 mg $Zn/g_{microalga}$) is ca. one-third of what is sorbed by living cells, for which almost all Zn is adsorbed externally. This might be a consequence of unavailability of functional groups on the surface of the cells (e.g. carboxyl, hydroxyl or sulphate ones) following drastic thermal treatment, in terms of stereochemically adequate positioning and chemical integrity. These adsorption extents are similar to those obtained by Omar (2002) for *S. obliquus* and *S. quadricauda*. Hence, our experimental results suggest that the mechanisms underlying Zn uptake by inactivated *D. pleiomorphus* (L) should be similar to those prevailing in other conventional, non-biological systems of adsorption: as microalgal cells become inactivated, metal uptake occurs only via binding to the cell surface, by physical- or functional group-mediated forces (Costa and França 1998).

Thermally inactivated cells have been proven to accumulate heavy metal ions to the same (or even a greater) extent than growing cells (Özer et al. 1999); however, metal ion binding to non-viable cells is presumed to occur exclusively via surface adsorption (Özer et al. 1999). Conversely, Costa and França (1998) revealed that lower loading capacities by dead cells are observed, thus indicating that many reaction sites may likely be affected by the heating process (and compromising the cell removal capacity).

In view of the above, adsorption via inactivated microalgal biomass of *D. pleiomorphus* (L) is also a valuable method for Zn removal from polluted waters, and appears to be at least as effective as using viable cells of ACOI 561, and certainly more economical, because constrained environmental conditions for maintenance and growth (e.g. light, temperature and nutrients) are not required.

Effect of pH on metal removal

It has been described that pH can significantly influence removal of metal ions (Abu Al-Rub et al. 2004; Deng et al. 2007; Gong et al. 2005). The timeframe of our experiment did not need to be as large as that of the growth experiment, because higher microalgal biomass leads, in principle, to higher degrees of metal removal (Hamdy 2000). The maximum Zn removal obtained was 30.7 mg $Zn/g_{microalga}$, thus indicating that *D. pleiomorphus* (L) is significantly ($P < 0.05$) more efficient at pH 5.0. Similar findings were reported previously: Ahuja et al. (1999) claimed that adsorption of Zn by inactivated cells of *O. anguistissima* is pH-dependent, and reaches maximum levels at pH 5.0. Reports of Fraile et al. (2005) and King et al. (2008) indicated also that an increase in pH had a positive effect on Zn uptake by *C. vulgaris* and *Azadirachta indica*, which was maximized at pH 5.0 and 6.0, respectively. Gong et al. (2005) used *Spirulina maxima* to uptake Pb, and found that to be higher at pH of 5.5; Abu Al-Rub et al. (2004) stated that removal of Ni increased with pH up to 5.0; and Deng et al. (2007) found that *Cladophora fascicularis* exhibited its maximum adsorption capacity of Pb at pH 5.0.

It is largely accepted that the effect of solution pH in metal removal is caused by a change in the number of metal binding sites on the cell surface, coupled with the availability of metal in solution in its various cationic forms (Abu Al-Rub et al. 2004; Gong et al. 2005). Note that microalga cell walls contain polysaccharides that carry charged (cationic or anionic) functional groups (viz. amino, carboxyl, hydroxyl and phosphate groups), the binding performance of which is affected by pH, depending on the groups that are either protonated or deprotonated. At low pH, such functional groups are associated with H^+ ions, thus preventing positively charged metal ions from binding; as pH increases, the sites are mainly in dissociated form, so they can exchange H^+ with metal ions in solution, which is accordingly favourable towards binding of cations (Ahuja et al. 1999; Abu Al-Rub et al. 2004; Chojnacka et al. 2005; Gong et al. 2005). Carboxyl groups are known to possess the highest affinity for metal ions, since they are deprotonated in a wide range of pH values. However, depending on pH, different functional groups will participate in metal ion binding: (1) pH 2–5: carboxyl groups; (2) pH 5–9: carboxyl and phosphate groups; and (3) pH 9–12: carboxyl, phosphate and hydroxyl (or amine) groups. So, at the pH at which maximum Zn recovery took place (pH 5.0), both carboxyl and phosphate groups can be found on the cell walls that

are deprotonated, and which will thus remove Zn ions from solution via electrostatic binding.

Therefore, the capacity of *D. pleiomorphus* (L) for bioremediation depends on the pH of the contaminated waters; that strain will perform especially well at pH values around 5.0, rather than in extremely acid or neutral media.

Conclusions

Biomass of both strains of *D. pleiomorphus* proves efficient toward removal of Zn ions, to extents comparable to those reported for other microalgae, thus unfolding the potential of those strains for water and wastewater treatment processes. However, a better performance was consistently associated with the isolated strain from the polluted environment. Adsorption to the cell surface is the dominant mechanism of metal removal by both microalgae, so inactivated biomass of *D. pleiomorphus* (L) was also able to remove Zn from solution to extents similar to the removal levels obtained with living cells of the ACOI 561 strain; therefore, the local strain is the preferred option for bioremediation of Zn-contaminated waters. On the other hand, pH played a major role upon Zn-removal, the extent of which increased with increasing pH, with a maximum at ca. pH 5.0.

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