



## Toxicity and Removal of Zinc in the Three Species (*Acutodesmus obliquus*, *Desmodesmus subspicatus* and *Desmodesmus armatus*) Belonging to the Family, Scenedesmaceae (Chlorophyta)

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### Abstract

In this study, the effects of zinc upon growth of three green microalgal species, *Acutodesmus obliquus* (Turpin) Hegewald and Hanagata, *Desmodesmus subspicatus* (Chodat) Hegewald and Schmidt and *Desmodesmus armatus* (Chodat) Hegewald, and the capability of these green algae for removal of zinc were investigated. Growth inhibition of the microalgal cells was determined following exposure for 96 h to five initial concentrations of zinc. The growth of the alga decreased with increasing zinc concentrations. EC<sub>50</sub> values were determined as 2257.824, 1922.049 and 1634.275 µg L<sup>-1</sup> for Zn in the case of *D. subspicatus*, *A. obliquus* and *D. armatus*, respectively. The highest zinc removal percentage from media was determined in *D. subspicatus* (40%), and followed by *A. obliquus* (30%) and *D. armatus* (18%). Phenotypic plasticity in *Scenedesmus* has been documented in response to a wide variety of conditions. The phenotypic plasticity was observed in *A. obliquus* and *D. armatus* in all tested zinc treatments.

**Keywords:** Zinc, algae, *Acutodesmus*, *Desmodesmus*, *Scenedesmus*, removal.

### Scenedesmaceae (Chlorophyta) Familyasına Ait Üç Türde (*Acutodesmus obliquus*, *Desmodesmus subspicatus* ve *Desmodesmus armatus*) Çinkonun Toksikitesi ve Uzaklaştırılması

### Özet

Bu çalışmada; üç yeşil mikroalg türü, *Acutodesmus obliquus* (Turpin) Hegewald ve Hanagata, *Desmodesmus subspicatus* (Chodat) Hegewald ve Schmidt and *Desmodesmus armatus* (Chodat) Hegewald, 'nün gelişimi üzerine çinkonun etkisi ve bu yeşil alglerin çinkoyu uzaklaştırması araştırıldı. Mikroalg hücrelerinin gelişiminin engellenmesi, 5 başlangıç çinko derişimine maruz bırakılarak belirlendi. Alglerin gelişimi, artan çinko derişimi ile azaldı. EC<sub>50</sub> değerleri, *D. subspicatus*, *A. obliquus* ve *D. armatus* 'da sırasıyla 2257,824, 1922,049 ve 1634,275 Zn µg L<sup>-1</sup> olarak belirlendi. Ortamdan en yüksek çinko uzaklaştırma yüzdesi, *D. subspicatus* (%40)'ta belirlendi ve bunu sırasıyla *A. obliquus* (%30) ve *D. armatus* (%18)'un izlediği saptandı. *Scenedesmus*'ta fenotipik plastisiti, değişen çevre koşullarına tepki olarak bildirilmektedir. Fenotipik plastisiti, *A. obliquus* ve *D. armatus*'da test edilen tüm çinko derişimlerinde belirlendi.

**Anahtar Kelimeler:** Çinko, alg, *Acutodesmus*, *Desmodesmus*, *Scenedesmus*, uzaklaştırma.

### Introduction

Zinc is one of the most common elements in the Earth's crust. Most zinc enters the environment as the results of mining, purifying of zinc, lead, and cadmium ores, steel production, coal burning, and burning of wastes. These activities can increase zinc levels in the atmosphere (ATSDR, 2005). Zinc is an essential micronutrient in all biota owing to its involvement in many physiological processes. It is essential in the maintenance of plasma membrane stability, in the activation of more than 300 enzymes (WHO, 2001), DNA and ribonucleic acid (RNA)

synthesis and cell proliferation (U.S. EPA, 2005); however, it becomes toxic when available in higher concentrations (Nalimova *et al.*, 2005) since it decreases cell division, mobility, total chlorophyll content, ATPase activity and carotenoid/chlorophyll ratio in microalgae (Omar, 2002). The toxicity of zinc depends on the external concentration, the zinc speciation, and the pH and hardness of the water (WHO, 2001). In defense against the toxic effects of heavy metals, the algal cells are equipped with a variety of resistance mechanisms. At this respect, phenotypic plasticity is a mechanism commonly documented for *Scenedesmus* in a variety of

ecological conditions (Trainor, 1998; Peña-Castro *et al.*, 2004; Lürling, 2006; Lombardi *et al.*, 2007). For example, in a study of the ecological impact of mine effluents on freshwater microalgal populations, Monteiro *et al.* (1995) found that *Desmodesmus armatus* (R. Chodat) E. Hegewald was the dominant species when heavy metal concentrations were high enough to inhibit the growth of other microalgae. Lombardi *et al.* (2007) also found that *Acutodesmus acuminatus* (Lagerheim) Tsarenko caused phenotypic alterations by the presence of copper. On the other hand, the absorption of heavy metals by green algae affects trophic chains since these producers can contaminate the organisms that depend directly or indirectly on them (Magdaleno *et al.*, 1997). Many chemical methods are currently known for removing heavy metals from aqueous solutions: precipitation, electrolysis, ionic exchange, filtration, evaporation, and others. Disadvantages of these methods are their high cost prize, low economic efficiency, especially during removing small amounts of heavy metals, and a necessity of slag burial. Biological methods of metal detoxication and removal from aqueous solutions lack these disadvantages (Nalimova *et al.*, 2005). Microalgae have been found to be very effective in removing heavy metals from wastewater because of their large surface area and high binding affinity (Chong *et al.*, 2000). In this research, the inhibition effects of zinc on the growth of three species belonging to the family, Scenedesmaceae (Chlorophyta) to were evaluated, and the 96 h removal processes of zinc in microalgae were investigated. Additionally, we recorded the morphological development observed during 96 h static cultures of *A. obliquus*, *D. subspicatus* and *D. armatus*.

## Materials and Methods

### Algal Stock Cultures

Experiments were performed with the green algae *A.* (formerly *Scenedesmus*) *obliquus* (276-3a SAG), *D.* (formerly *Scenedesmus*) *subspicatus* (86.81 SAG) and *D. armatus* (formerly *S. quadricauda*) (276-4d SAG) obtained from Sammlung von Algenkulturen der Universität Göttingen (SAG, Göttingen, Germany). These green algae were grown in a medium recommended by OECD (1984). The pH of culture medium was adjusted to 8 using NaHCO<sub>3</sub>-HCl (0.1 N). Stock cultures were incubated in 250 ml erlenmeyer flasks containing 100 ml of sterilised OECD medium under a photon irradiance of approximately 120 µE m<sup>-2</sup> s<sup>-1</sup> (cool white fluorescent tubes) in constant illumination by constant shaking at 100 rpm and temperature 25±2°C.

### Growth Inhibition Bioassays

96 h growth inhibition bioassays were conducted

according to OECD-guideline 201 (OECD, 1984) (Table 1). To minimize the metal contamination, all laboratoryware in contact with the culture or test medium were soaked for 24 h in 1% HNO<sub>3</sub> and rinsed with deionised water.

Zinc stock solution was prepared from analytical grade zinc chlorid (ZnCl<sub>2</sub>·2H<sub>2</sub>O). The effective concentration range was determined from a range-finding test. Based on the results of the range-finding test, culture media of zinc concentrations 250, 500, 1000, 2000, 4000 µg L<sup>-1</sup> were prepared by diluting a stock of 1000 mg L<sup>-1</sup>. Control cultures were incubated in the same medium without toxicant. There were three replicates for the control and each of the treatment concentrations.

The algal suspension of known cell density was taken from the stock culture during the exponential growth phase. After mixing with synthetic OECD culture medium, the initial cell density was approximately 8x10<sup>4</sup> cells ml<sup>-1</sup>. Different parameters, including growth and removal of zinc were monitored repeatedly, at 24, 48, 72 and 96 hours after the start of the test. The cell number was determined using a Neubauer improved counter (Marienfeld, Germany).

Algal cell densities for each flask were measured daily and calculated as cell numbers (algal cell ml<sup>-1</sup>). The average specific growth rate  $\mu$  (per day) for exponentially growing cultures was calculated using  $\mu = \ln N_n - \ln N_1 / t_n - t_1$  [where  $N_n$  the measured final cell density,  $N_1$  the nominal initial cell density and  $t_n$  the time (day) after the initiation of the test] (OECD, 1984). The percentage inhibition of the cell growth at each test substance concentration ( $I_A$ ) was calculated as the difference between the area under the control growth curve ( $A_C$ ) and the area under the growth

**Table 1.** Summary of standart bioassay protocol for *Acutodesmus* and *Desmodesmus* species growth inhibition bioassay

1. Test type	Static
2. Temperature	25±2 °C
3. Light quality	Cool white fluorescent lighting
4. Light intensity	120 µE m <sup>-2</sup> s <sup>-1</sup>
5. Illumination	Permanent
6. Test chamber size	250 ml
7. Test solution volume	100 ml
8. Renewal of test solutions	None
9. Age of test organisms	3-4 days
10. Initial cell density in test chambers	8x10 <sup>4</sup> cells ml <sup>-1</sup>
11. No. Of replicate chambers/concentration	3
12. Shaking rate	100 rpm
13. Test medium	OECD culture medium
14. Metal concentrations	Minimum of five and a control
15. Test duration	96 h
16. Test endpoint	Growth rate

curve at each test substance concentration ( $A_t$ ) as  $I_A = A_C - A_t / A_C \times 100$  (OECD, 1984).

### Phenotypic Plasticity

The effects of zinc on the phenotypic plasticity of *Acutodesmus* and *Desmodesmus* species were evaluated by quantifying the number of cells per coenobia on each experimental treatment and controls after 96 h exposure (Lombardi *et al.*, 2007). Coenobia were classified into four categories: eight-celled coenobium, four-celled coenobium, two-celled coenobium and free cells (absence of coenobium). Each treatment and control had three replicates.

### Measurements of Zinc

A metal removal experiment was performed under the same conditions as that used in the toxicity tests. The residual metal concentration in the water was measured at 24, 48, 72 and 96 hours. Each sample taken was filtered through milipore syringe filters (pore size 0.45  $\mu\text{m}$ ) to remove the algae. The water samples without algae were acidified (pH<2) with 14 N  $\text{HNO}_3$  and analyzed for the concentrations of residual zinc by flame atomic absorption spectrophotometer (Perkin Elmer AA800). All values obtained were the means of three trials with three replicates and computed following the formula modified from Nacorda *et al.* (2007), as:

Metal Ions Removed in the Medium % = (Initial concentrations – Residual concentration / Initial concentration) x 100

### Statistical Analysis

In the toxicity tests, experiments were conducted three times in one plate, and the average and standart deviation were calculated. The toxicity of Zn was expressed as the effective concentration giving 50% reduction ( $\text{EC}_{50}$ ) in the number of cells over 96 h compared to the controls. This was calculated using the U.S. Environmental Protection Agency (U.S.

EPA) Probit Analysis Program Version 1.5. The dose response equation was  $X^2$  tested with 95% confidence. The data relative to growth and growth rate were analysed by a one-way analysis of variance (ANOVA). All significance levels mentioned in the text are at the  $P < 0.05$  level.

### Results and Discussion

Figure 1 shows the growth inhibition of *Acutodesmus* and *Desmodesmus* species at the selected concentrations of Zn. The growth of the alga decreased with increasing zinc concentrations. A 96 h exposure of *D. subspicatus*, *A. obliquus*, and *D. armatus* to 250 and 4000  $\mu\text{g L}^{-1}$  caused approximately 5.063-66.139, 7.763-69.407 and 11.275-70.588% reduction in cell numbers, respectively. In the absence of added Zn, microalgae density expressed as cell numbers at 96 h was in the order of *D. subspicatus* > *A. obliquus* > *D. armatus*. ANOVA showed that there was a significant effect ( $P < 0.05$ ) upon growth of three species when algae were exposed to different concentrations of zinc (250-4000  $\mu\text{g L}^{-1}$ ).

The growth rates and correlation coefficients of *Acutodesmus* and *Desmodesmus* species at the selected concentrations of Zn were shown in Table 2. Reduction in growth rates was observed with an increase of zinc concentrations. Because of the greatest growth rate of *D. subspicatus* and the greatest sensitivity of *D. armatus* to Zn, the order of growth rate was also *D. subspicatus* > *A. obliquus* > *D. armatus* with all Zn concentrations. The results obtained for *A. obliquus* at control and 2000  $\mu\text{g l}^{-1}$  were similar to those obtained by Magdaleno *et al.* (1997), which had a growth rates of 0.56 at control and 0.40 at 2500  $\mu\text{g L}^{-1}$ .

The 96 h  $\text{EC}_{50}$  values obtained for *D. armatus*, *A. obliquus* and *D. subspicatus* were 1634.275 (95% confidence intervals of 1277.150 - 2211.259), 1922.049 (95% confidence intervals of 1517.647 - 2593.859) and 2257.824 (95% confidence intervals of 1856.212 - 2881.057)  $\mu\text{g L}^{-1}$ , respectively. It can be concluded that *D. armatus* less sensitive to Zn than other two algae. The 96 h  $\text{EC}_{50}$  values obtained were

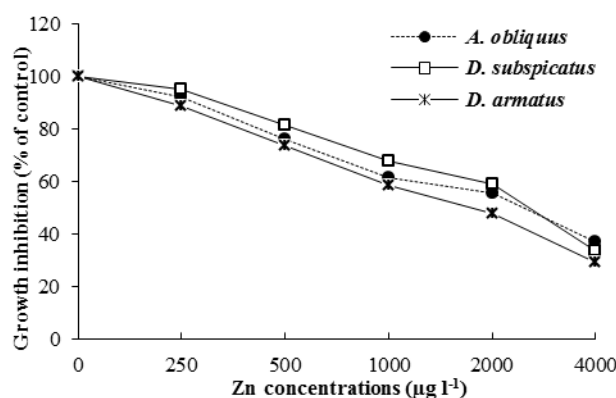


Figure 1. Growth inhibition of *Acutodesmus* and *Desmodesmus* species exposed to Zn for 96 h.

**Table 2.** Growth rates ( $\mu$ ) and correlation coefficients ( $R$ ) of *Acutodesmus* and *Desmodesmus* species exposed to different concentrations of Zn

Zn ( $\mu\text{g L}^{-1}$ )	<i>A. obliquus</i>		<i>D. subspicatus</i>		<i>D. armatus</i>	
	$\mu$	$R$	$\mu$	$R$	$M$	$R$
0	0.553 $\pm$ 0.006 <sup>Ab</sup>	0.972	0.644 $\pm$ 0.006 <sup>Aa</sup>	0.974	0.535 $\pm$ 0.007 <sup>Ac</sup>	0.963
250	0.532 $\pm$ 0.008 <sup>Bb</sup>	0.970	0.631 $\pm$ 0.007 <sup>Ba</sup>	0.984	0.505 $\pm$ 0.002 <sup>Bc</sup>	0.960
500	0.485 $\pm$ 0.009 <sup>Cb</sup>	0.982	0.593 $\pm$ 0.004 <sup>Ca</sup>	0.989	0.458 $\pm$ 0.010 <sup>Cc</sup>	0.958
1000	0.432 $\pm$ 0.000 <sup>Db</sup>	0.974	0.547 $\pm$ 0.008 <sup>Da</sup>	0.989	0.402 $\pm$ 0.000 <sup>Dc</sup>	0.968
2000	0.406 $\pm$ 0.009 <sup>Eb</sup>	0.976	0.512 $\pm$ 0.004 <sup>Ea</sup>	0.976	0.352 $\pm$ 0.009 <sup>Ec</sup>	0.959
4000	0.304 $\pm$ 0.009 <sup>Fb</sup>	0.933	0.374 $\pm$ 0.004 <sup>Fa</sup>	0.974	0.228 $\pm$ 0.025 <sup>Fc</sup>	0.930

tested for not significance with the  $X^2$  test ( $\alpha=0.05$ ).  $EC_{50}$  values obtained for *D. subspicatus* were similar to those obtained by Rojičková-Padrťová and Maršálek (1999):  $EC_{50}$  values for Zn to *Chlamydomonas reinhardtii* Dangeard and to *Chlorella kesleri* (Fott and Nováková) Krientz et. al., were 2171.5 and 2305.2  $\mu\text{g L}^{-1}$ , respectively. Likewise, Tripathi and Gaur (2006) described an  $EC_{50}$  value more similar to that obtained for *D. armatus* here: 1640  $\mu\text{g L}^{-1}$  of Zn for *Scenedesmus* sp. by 48 h. Our strains were more tolerant from those obtained by Rojičková-Padrťová and Maršálek (1999) for *D. subspicatus* (767.4  $\mu\text{g L}^{-1}$ ) and *D. armatus* (812.8  $\mu\text{g L}^{-1}$ ). Toxicity studies reported by a few authors have revealed higher  $EC_{50}$  values for Zn than those obtained in our study, showing that our strains are more sensitive to this toxic metal. For instance, an  $EC_{50}$  of 2432  $\mu\text{g Zn L}^{-1}$  was reported by Magdaleno et al. (1997) in the case of *Ankistrodesmus falcatus* (Corda) Ralfs after exposure for 96 h; Wilde et al., (2006) found an 2700  $\mu\text{g L}^{-1}$  for  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at pH 8 in the case of *Chlorella* sp. by 48 h; Monteiro et al. (2011) obtained an  $EC_{50}$  values of 16990 and 4870  $\mu\text{g L}^{-1}$  for  $\text{ZnCl}_2$  in the case of *A. obliquus* and *Desmodesmus pleiomorphus* (Hindák) Hegewald by 96 h, respectively. Comparison of  $EC_{50}$  values found in this study with literature data is difficult, as differences in test procedures and test conditions affect algal sensitivity to metals. In literature, many data on the effects of zinc on the growth of these species belonging to the family, Scenedesmaceae (Chlorophyta) are reported (Abd-El-Monem et al., 1998; Tripathi and Gaur, 2006). Zinc toxic effects are shown to be related to metal binding to SH-groups of proteins, in the plasma membranes in particular (Nalimova et al., 2005; Tripathi and Gaur, 2006). Zinc effects practically all physiological processes: cell division, membrane functioning, photosynthesis, and respiration (Nalimova et al., 2005). Inhibition of algal cell division by zinc was not related to the intracellular zinc concentration. Rather, zinc toxicity was related to extracellular zinc. Once inside the cell, zinc may be detoxified by binding to thiol-containing proteins such as phytochelatins (Wilde et al., 2006). 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 (Monteiro et al., 2011).

*Scenedesmus*, one of the most common genera of freshwater green algae, has been shown to have high phenotypic plasticity. Many factors may influence the formation of unicellular or colonial morphology in species of *Scenedesmus*, including abiotic and biotic factors (Liu et al., 2010). Figure 2 shows the phenotypic plasticity observed for *A. obliquus* and *D. armatus*, but not observed for *D. subspicatus*, where a reduction on the number of cells per coenobium occurs as zinc increased in the culture medium. When the algae were grown at these zinc concentrations, changes in the morphotypes were registered in comparison to the control cultures. The number of cells per coenobium diminished as the zinc concentration increased. All the zinc concentration tested, free cells were dominant and coenobia were absent for *A. obliquus*. For *D. armatus*, at control and 250  $\mu\text{g L}^{-1}$  Zn, coenobia with four cells were dominant (100%). At 500  $\mu\text{g L}^{-1}$  Zn, coenobia with four cells decreased to 30% and two cells started to increase (30%), at 1000, 2000 and 4000  $\mu\text{g L}^{-1}$  Zn, two celled and four celled coenobia were to 50%-50%, respectively. Among the parameters we evaluated, the number of cells per coenobium was highly sensitive, promptly responding to low metal concentrations and variations even before effects on cell number had been detected. The phenotypic plasticity of *A. obliquus* and *D. armatus* were correlated to zinc concentrations in the cultures. A decrease in the number of cells per coenobium with increase in copper concentrations has also been shown by Peña-Castro et al. (2004) and Lombardi et al. (2007). With respect to field studies on heavy metals pollution-induced phenotypic plasticity, only Whitton (1980) has described observations on two sites where unicellular cells of *Scenedesmus* were predominant; one site was polluted by a combination of sewage and Zn, Pb and Cd, whereas the other was polluted by mill effluents, where metals were likely the sole pollutants (Peña-Castro et al., 2004). Under our experimental conditions coenobial structure was readily affected at lower zinc concentrations than cell numbers. These results indicate that coenobium structure is a very sensitive parameter that promptly responds to low zinc variations in the culture medium.

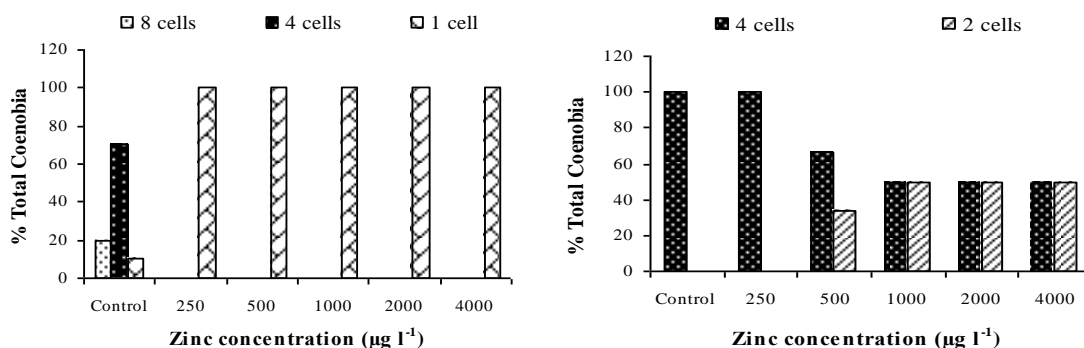
In the next part of our tests, the ability to take up

the zinc from test medium by *Acutodesmus* and *Desmodesmus* species were confirmed. The changes of removal amount of Zn by these algae were shown in Figure 3. Concentrations of Zn remained in the medium were first rapidly and then slowly reduced during the 96 h experimental period. When initial Zn concentration was  $250 \mu\text{g L}^{-1}$ , 40%, 30% and 18% of Zn were removed by *D. subspicatus*, *A. obliquus* and *D. armatus*, respectively. *D. subspicatus* removed the most Zn from the medium. Radway *et al.* (2001) reported that zinc was the best removed by red alga *Cyanidium* in Doemel's *Cyanidium* medium while it was not well enough removed by *S. quadricauda* in Bold Basal medium, and the choice of algal material would be particularly crucial in the cases of Al and Zn. Similarly, results in the present study indicated that Zn was not well enough removed by *D. armatus*

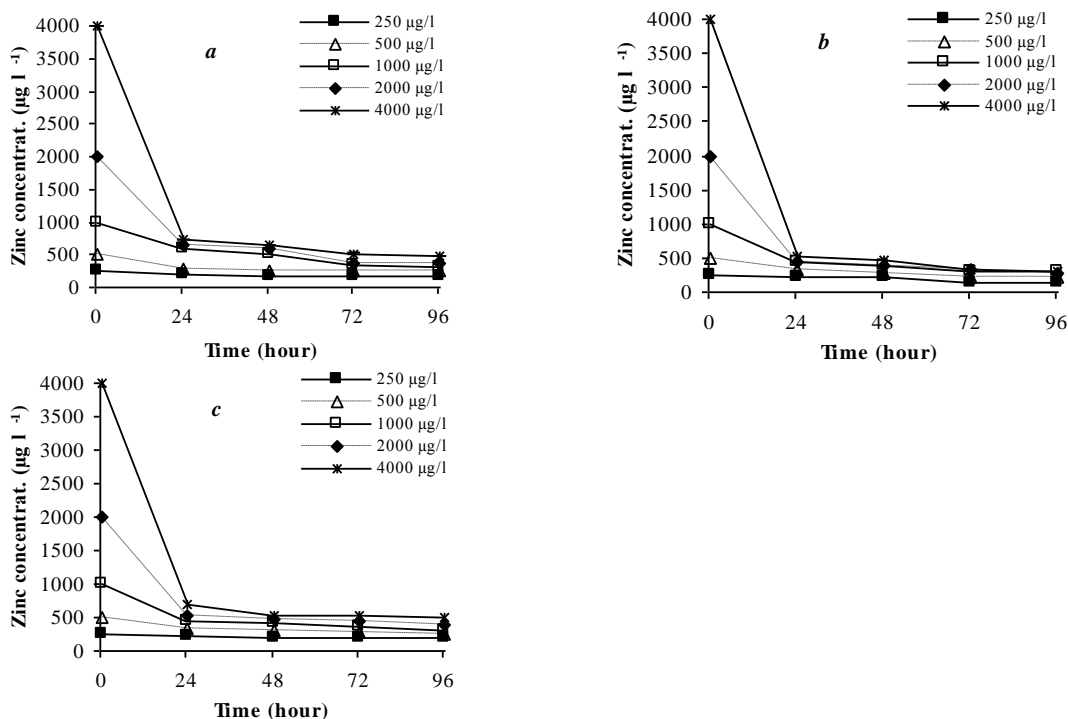
in OECD medium, and that the potential exists for optimizing zinc bioremoval process by judicious algae strain selection dictated by the zinc to be removed.

## Conclusions

As mentioned above, zinc toxicity responses are different according to the *Acutodesmus* and *Desmodesmus* species considered. The most commonly used alga *D. armatus* appears very sensitive to zinc when compared with our other species results, and therefore, can be considered as a good model for toxicity testing. Considering that we used environmentally relevant zinc concentrations, we suggested that the number of cells per coenobium be further investigated as a potential tool for



**Figure 2.** Bar graphs showing the percentage of coenobia with the specified number of cells per coenobium as function of initial zinc concentration in *A. obliquus* (a) and *D. armatus* (b) culture media after 96 h exposure to the zinc.



**Figure 3.** The changes of removal amount of Zn by *Acutodesmus* and *Desmodesmus* species [*A. obliquus* (a), *D. subspicatus* (b), *D. armatus* (c)].

environmental monitoring programs, particularly those focused on detecting chronic contamination. However, the present study shows that *D. subspicatus* can be satisfactorily used for removing Zn at concentrations of 250-4000  $\mu\text{g L}^{-1}$ .

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