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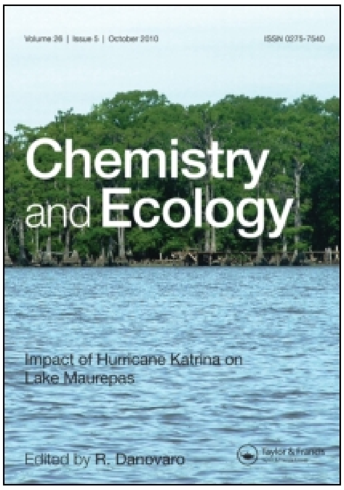

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Effects of Cd, Cr, and Zn on growth and metal accumulation in an aquatic macrophyte, *Nitella graciliformis*

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The effect of heavy metals (Cd, Cr, and Zn) on the growth of the freshwater macrophytic *Nitella graciliformis* J. was observed under laboratory conditions and their accumulations in the plant were measured. The experimental plant was exposed to three different concentrations of Cd (25, 50, 150 $\mu g\,L^{-1}$), Cr (150, 500, 1000 $\mu g\,L^{-1}$), and Zn (150, 500, 1000 $\mu g\,L^{-1}$) for 35 days. The heavy metal concentrations in the plant increased with the increasing Cd, Cr, and Zn concentrations in the mediums. The highest concentration of Zn that accumulated in the plant tissues was 2540 $\mu g\,g^{-1}$ Zn at 1000 $\mu g\,L^{-1}$ medium compared to 547 $\mu g\,g^{-1}$ Cr at 1000 $\mu g\,L^{-1}$ and 290 $\mu g\,g^{-1}$Cd at 150 $\mu g\,L^{-1}$ mediums. As a result, negative growth occurred and the internode elongation was reduced when exposed to these metals at any concentration. We concluded that under experimental conditions, intracellular green alga *Nitella graciliformis* has a potential for accumulating Cd, Cr, and Zn.

Keywords: cadmium; chromium; zinc; accumulation; *Nitella graciliformis*; growth

1. Introduction

Heavy metals are important environmental pollutants, and many of them are toxic even at low concentrations. For many years, macro-algae have been used to remove heavy metals from contaminated water [1], since they have a high capacity to accumulate dissolved metals [2]. Algae are the basis of the food chain in all aquatic ecosystems. Apart from their role in trophic systems, heavy metal uptake and accumulation have been established in numerous freshwater green algae [3]. Recent studies have focused on the use of some macro-algae as a biosorbent material for removing metals from solutions, demonstrating their potential application in technological processes; in particular, for industrial and mining waste treatments [4–6].

Cadmium (Cd), a non-essential toxic heavy metal, can have significant effects on algae [3,7]. Several species of green algae have been shown to bioaccumulate cations (such as Cd or Zn) from the water column [4]. According to Allen [8], the range of Cd concentrations in freshwater plants is 0.01–0.3 $\mu g\,g^{-1}$. Chromium (Cr) is one of the most important heavy metals and is considered

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to cause serious environmental pollution. Toxicity of Cr to plants depends on its valence state: Cr(VI) is an anion which is highly toxic and mobile, whereas cation Cr(III) is less toxic. Cr(VI) is actively taken up and is a metabolically driven process, in contrast to Cr(III), which is passively taken up and retained by cation-exchange sites of the cell wall [9]. Toxic effects of Cr on plant growth and development include alterations in the germination process as well as in the growth of roots, stems and leaves, which may affect total dry matter production and yield [9]. A previous study showed that chromate reduced growth, photosynthesis, and chlorophyll synthesis in green alga *Chlorella pyrenoidosa* 251 [10]. It is reported to be toxic to most of the higher plants at $100\,\mu\text{g}\,\text{g}^{-1}$ dry weight [11]. Zinc (Zn) is a structural and catalytic component of proteins and enzymes; however, at elevated concentrations, Zn is extremely toxic to plant cells [12], which react by defensive mechanisms such as metal accumulation in vacuoles [13] and synthesis of phytochelatins [1]. Zinc concentrations in plant biomass range from $10–150\,\mu\text{g}\,\text{g}^{-1}$; nevertheless in benthic macrophytes, Zn levels less than $100\,\mu\text{g}\,\text{g}^{-1}$ are reported as background for non-polluted areas [14].

The macro-algae Charophyceae, commonly known as stoneworts or brittleworts, are a group of nonvascular hydrophytes with worldwide distribution. In the last few decades, anthropogenic inputs of metals have exceeded natural inputs, and the increasing pollution of water systems reduced the frequency of occurrence of charophyta [15]. However, few laboratory studies have demonstrated the importance of charophytes in the accumulation of heavy metals [15]. In order to establish a cause-effect relationship, the present laboratory experiment was carried out by exposing a common Charophyta, *Nitella graciliformis* J. Groves, to sub-lethal concentrations of three important heavy metals, Cd, Cr, and Zn, chosen because of their importance from both a biological and ecotoxicological point of view.

2. Materials and methods

2.1. *Experimental aquatic plant*

Nitella graciliformis J. Groves is a cosmopolitan species of the family Characeae that grows in small ponds to large lakes, and in freshwater to brackish water ecosystems. There are about 200 species of *Nitella* in the world. They are bright green, submerged, rather slender, monocious freshwater aquatic plants which can grow 30 cm in height. Their internodes somewhat exceed the length of branchlets or are twice the length of the branchlets, and whorls of forked branches are attached at regularly spaced intervals along the stem. They have yellowish to dark brown oospores. These long, delicate, smooth-textured algae lie on the bottom of a lake or pond anchored by rhizoids. In the absence of vascular tissue, the above-ground part of the plant plays an important role in acquiring nutrients from the water column.

2.2. *Plant cultivation and analysis*

The stock of green alga *Nitella graciliformis* was cultured axenically in a 50-litre tank for approximately 1 year at a controlled temperature of 24–25 °C. Apical tips of the stock *Nitella graciliformis* (2–3 internodes, 2–3 cm length) with similar morphological features were harvested and planted in 1 litre glass beakers containing distilled water (pH ∼7), for 10 tips each, positioning 2–2.5 internodes (∼2 cm) above the substrate. The substrate in the experimental beakers consisted of commercially available river sand (90% <1 mm; DIY, Doite®, Japan) approximately for each 400 g. The sand was washed with tap water to remove dust particles and it was further washed with distilled water. All beakers were kept in a water bath at a constant temperature of 24 °C. Three heaters (IC AUTO NEO type 180, NISSO, Japan) were used to maintain the desired temperature, and the water was mixed mechanically to provide a homogeneous temperature. The illumination was supplied using 4 × 20 W fluorescent lamps maintaining a photoperiod of 12:12 h (light:dark). Pre-cultivation extended over 14 days to acclimatise to the laboratory conditions before adding the contaminants. An exposure experiment was carried out in duplicate for each metal within the range of sub lethal concentrations. Cd and Zn were added as nitrate and Cr was added as chromate to the media to give final concentrations of 25, 50, and 150 μg Cd L^{-1}; 150, 500, and 1000 μg Cr L^{-1}; and 150, 500, and 1000 μg Zn L^{-1}, respectively. The metal concentrations were chosen in order to obtain a reduction of algal growth based on the literature [16,17].

The experiment was carried out for a period of 35 days between December 2007 and January 2008. The growth of algae was measured as the increase in the length of the internodes on a weekly basis [15]; this was determined by digital slide calipers (Fujiwara Sangyo Co. Ltd., Japan). The plants were harvested after 35 days of being exposed to the Cr and Zn media and after 28 days to the Cd, and oven dried at 65 °C for 24 h. The total Cd, Cr, and Zn of the whole plant tissue was analysed following dry ashing (at 450 °C for Cd; at 550 °C for Cr and Zn) in a muffle furnace for 3 h. The residue was dissolved in 1 M HNO_3 solution (double distilled purified, Sigma-Aldrich®, Tokyo, Japan) and the sample volume was adjusted to 10 mL using distilled water [18]. Concentrations of Cd, Cr, and Zn in the plant tissue were determined using air/acetylene flame atomic absorption spectrophotometer (AAS; Shimadzu AA-6300, Kyoto, Japan). Deuterium background correction was employed throughout the measurements.

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2.3. *Statistical analyses*

Plant growth was compared by two-way repeated measures ANOVA with metal doses (treatment) as the main factor and sampling dates as the repeated measures factor [19]. If the main effects were significant, differences among the treatments were tested with Tukey's multi-comparison test of means. A regression analysis was performed to establish the cause effect relationships between metal concentrations in the medium and accumulation rates in the plants. All data are presented as mean ± standard deviation (SD). Analyses were run at 5% significance level using statistica.

3. Results and discussion

3.1. *Effects of metals on plant growth*

The length of the internodes of the plant throughout the experiment is shown in Figure 1. Cadmium had an adverse significant effect ($p < 0.001$) on the growth of the experimental species (Figure 1(a)). At 50 and 150 μg L^{-1} concentrations it reduced the growth of the internodes by 21% and 34%, respectively, compared to the internode growth obtained at 25 μg Cd L^{-1} after 28 days. Further, growth differences were more obvious at the last sampling date when the length was inhibited by 52% at 150 μg Cd L^{-1}, as compared to the control (without Cd). There were also significant metal dose-time interactions ($p < 0.001$), indicating that the growth did not follow a similar trend in different sampling dates. The plant was very sensitive to the presence of Cd, as the cells began to die after just 14 days of exposure at 50 and 150 μg Cd L^{-1}. This finding is in agreement with Heumann [15], who observed that the green alga *Chara vulgaris* died after 7 days of exposure at 56 μg Cd L^{-1}. A cell was judged to be dead when picked up if there was a loss in the turgor pressure, a condition in which a cell bends on the spatula and loses its cylindrical shape [20]. Considering the similarities in the structural organisation of internodal cells of *Chara* and *Nitella* species, due to belonging to the same Charophyceae family [15], the effect on growth

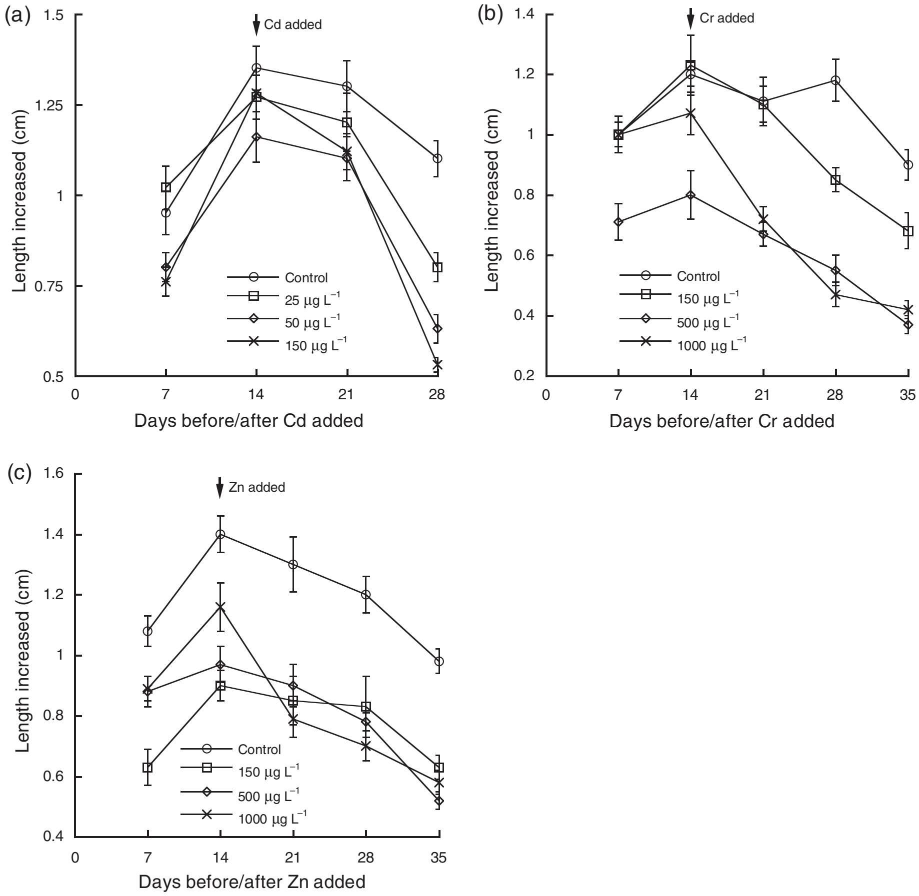

Figure 1. Changes in length of *Nitella graciliformis* exposed to increasing concentrations of Cd (a), Cr (b), and Zn (c).

in these species probably follows a similar pattern. The mechanisms for reduced plant growth due to heavy metal exposures are established. Overnell [21] reported that the Cd concentrations ranging from 10–100 $\mu g\, L^{-1}$ reduced the concentrations of ATP and chlorophyll and decreased oxygen production. Cd has also been reported to inhibit biosynthesis of chlorophyll through targeting –SH groups of several enzymes in the functional mitochondrial system, leading to a growth inhibition in freshwater green algae [22].

Likewise, the experimental plants exhibited significant growth reduction ($p < 0.001$) at all concentrations of Cr (Figure 1(b)) compared to that of the control plants during the 35 day period. At 1000 $\mu g\, Cr\, L^{-1}$ concentrations, a decrease in the growth was greater until the 28 days experimental period (Figure 1(b)). The medium dose (500 $\mu g\, Cr\, L^{-1}$) reduced plant height by 59% as compared to control plants in 35 days. However, there were no differences ($p > 0.05$) in growth reduction between 500 and 1000 $\mu g\, Cr\, L^{-1}$ concentrations on the last three sampling dates (Tukey test). The adverse effects of Cr on plant height and shoot growth have been reported in different plant species. In a laboratory experiment, Gomes and Asaeda [6] observed that Cr(VI) addition at 200 and 400 $\mu g\, L^{-1}$ concentrations inhibited the growth of *Nitella pseudoflabellata*. Shanker et al. [9] noted 11%, 22%, and 41% reductions in plant height over the control when Cr was added at 2, 10, and 25 $mg\, L^{-1}$ to nutrient solutions in sand cultures with oats, respectively. Chromium transport to the aerial part of the plant had a direct impact on the cellular metabolism of shoots, thereby leading to a reduction in plant height [9].

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On the other hand, internodal lengths were significantly shorter ($p < 0.001$) in different concentrations of Zn when compared to the control without heavy metal. At a medium dose ($500\,\mu g\,Zn\,L^{-1}$), the plant height was inhibited by 47% of the control plants at the end of the experiment. The highest growth reduction was observed in the presence of the highest concentration ($1000\,\mu g\,Zn\,L^{-1}$) after 7 days of exposure, beyond which algal growth decreased gradually over the next two weeks. However, there were no significant differences ($p > 0.05$) in growth among the three Zn concentrations, indicating that the experimental plant did not respond to the higher doses. Information on the adverse effects of Zn on freshwater macrophytes are scarce in the literature. However, a similar pattern of growth inhibition due to the effects of Zn has been reported in the green seaweeds *Ulva lactuca* and *Enteromorpha flexuosa* at Zn concentrations ranging from $10–5000\,\mu g\,L^{-1}$ [16]. Although Zn is an essential plant microelement, at higher concentrations Zn interacts with the donor side of PS II to inhibit photosynthetic CO_2 fixation and the Hill reaction, leading to a decrease in the quantum yield [23].

3.2. *Metal accumulation by the experimental plant*

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Algae have the capacity to accumulate heavy metals to several orders of magnitude as compared to the surrounding medium [24], but it is generally accepted that their concentrations are proportional only to the concentrations of metals in the solution from the ambient water mass [25]. Besides, the factors affecting the elemental levels in aquatic plants are most likely the bioavailability of metals in the surrounding water and the uptake capacity of the algae [26,27]. The present study showed a concentration-dependent accumulation of Cd inside *Nitella* tissue (Table1; Figure 2(a)). The Cd accumulation in plants increased linearly with increasing concentrations in the medium ($R^2 = 0.95$). In the control treatment, the Cd concentration of the plants was $27.2\,\mu g\,g^{-1}$. At the lowest ambient concentrations ($25\,\mu g\,Cd\,L^{-1}$), the plant Cd concentration was $58\,\mu g\,g^{-1}$ within 14 days of harvest. The uptake was increased further ($177\,\mu g\,g^{-1}$) with an increase in the concentration to $50\,\mu g\,Cd\,L^{-1}$ in the ambient solution. A maximum of $290\,\mu g\,g^{-1}$ Cd was accumulated in *Nitella* at the highest concentration of $150\,\mu g\,Cd\,L^{-1}$ after the same exposure period. These observations accord with those of other investigators [28], who stated that an increase in Cd in the culture medium resulted in an increase in the concentrations of Cd in the submerged plant *Ceratophyllum demersum* (accumulating $151\,\mu g\,g^{-1}$ Cd at an ambient Cd concentration of $50\,\mu g\,L^{-1}$ after 7 days). Tripathi et al. [29] reported that submerged plants took up Cd by both

Table 1. Metal (Cd, Cr, and Zn) accumulation in *Nitella graciliformis* exposed to different concentrations.

Heavy metals	Treatments ($\mu g\,L^{-1}$)	Accumulation ($\mu g\,g^{-1}$)
Cd	Control	27.2
	25	58.0
	50	176.7
	150	290.0
Cr	Control	67.6
	150	205.3
	500	373.5
	1000	546.6
Zn	Control	90.6
	150	983.3
	500	1492.5
	1000	2540.0

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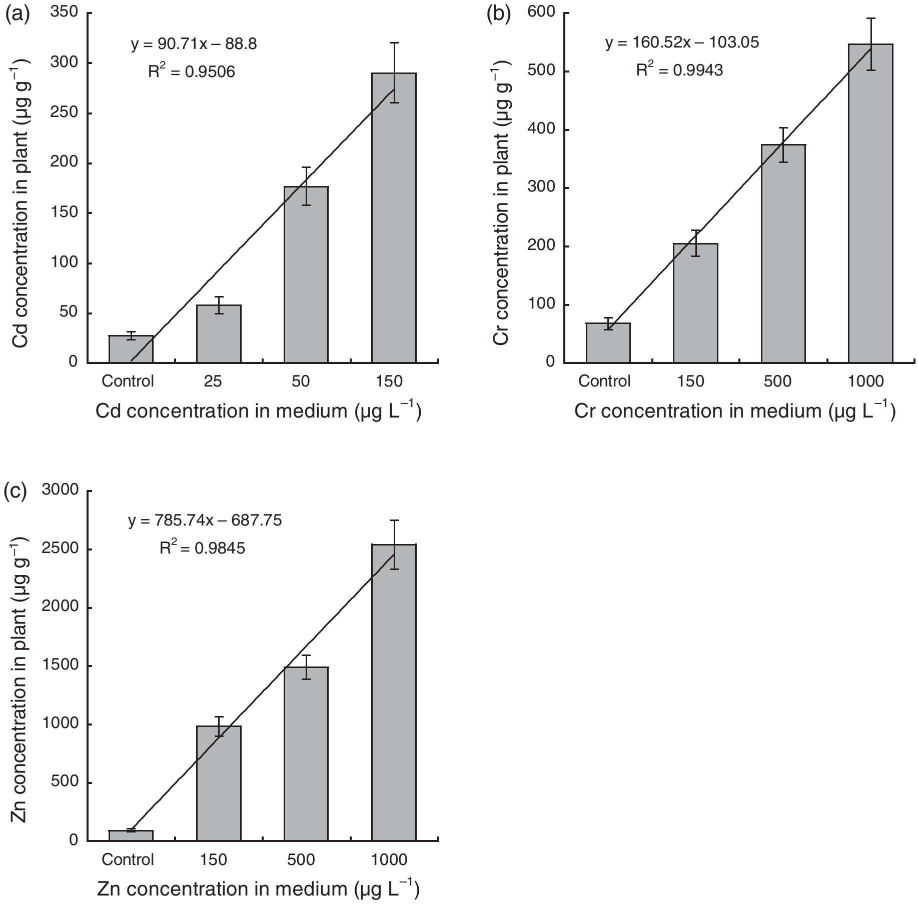

Figure 2. Cadmium (a), chromium (b) and zinc (c) concentrations ($\mu g\,g^{-1}$ dry weight) in *Nitella graciliformis* under controlled conditions.

adsorption and energy-dependent transport. However, the sorptive capacities are species specific and depend on the growth rate and physiological condition of the individual plant [30].

The concentration of Cr in *Nitella* samples increased as the concentration of Cr in the medium increased ($R^2 = 0.99$; Figure 2(b)). The total Cr contents were 205, 374, and 547 $\mu g\,g^{-1}$ for algae grown in 150, 500, and 1000 $\mu g\,Cr\,L^{-1}$ medium, respectively, while the concentration of Cr obtained from the control medium was 68 $\mu g\,g^{-1}$ (Table 1). A similar trend of incline with increasing Cr dose was observed in another study [31], with Cr contents in roots and shoots of water lettuce and soybean increasing when the concentrations in the medium were increased. However, the metal accumulation is not linear in correlation with the increase in concentration. This is probably due to the fact that heavy metals are bound in the tissue, causing saturation that is governed by the rate at which the heavy metal is conducted away [32].

Similar to the other two metals, Zn content in the plant increased as Zn concentrations in the medium increased from 150 to 1000 $\mu g\,L^{-1}$ ($R^2 = 0.98$; Figure 2(c)). Compared with the control, Zn concentration in the algal cultures, for example, was 10 times higher when the medium contained 150 $\mu g\,Zn\,L^{-1}$, and the amount increased to about 30 times as the dosage was raised to 1000 $\mu g\,Zn\,L^{-1}$. At the lowest Zn levels in the ambient solution (150 $\mu g\,Zn\,L^{-1}$), the plant Zn concentration was 983 $\mu g\,g^{-1}$, followed by 1493 $\mu g\,g^{-1}$ (medium of 500 $\mu g\,Zn\,L^{-1}$) and 2540 $\mu g\,g^{-1}$ (1000 $\mu g\,Zn\,L^{-1}$), respectively. However, in the control medium, the concentration of Zn accumulated by the algae was 91 μg g^{-1}. Schlacher-Hoenlinger and Schlacher [33] reported that Zn mainly adsorbed to the surfaces and subsequently transported into the intercellular space by passive diffusion. Among the three metals (Cd, Cr, and Zn) assayed in the present study, Zn is an essential trace element for plants [4,23] and is shown to be accumulated at relatively higher rates when compared with Cr and Cd, and this is in agreement with the findings of other studies [34,35]. Again, as compared to the other trace metals analysed, Zn exhibited higher levels in alga, and this may reflect, firstly, the metabolic requirements of the plant for metals and, secondly, the capacity of the algae to take them up from the environment [26,36].

The result obtained in this study showed that heavy metals affect the macrophytic alga *N. graciliformis* in concentration ranges which might occur in natural environments. The highest concentrations used in this experiment might only rarely occur in unpolluted natural water conditions but they were useful for understanding the plant response to the toxicity. In summary, the results presented in this study demonstrate that the macrophytic alga *N. graciliformis* is sensitive to low dose, long term exposure to heavy metals.

4. Conclusions

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The results show that the uptake of Cd, Cr, and Zn by *N. graciliformis* was increased with increasing metal concentrations. Concentrations of Zn accumulated in the plant tissues were higher than those of Cr and Cd, with the highest concentrations of 2540 μg g^{-1} Zn at 1000 μg L^{-1} medium compared to 547 μg g^{-1} Cr at 1000 μg L^{-1} medium and 290 μg g^{-1} Cd at 150 μg L^{-1} medium, respectively. These metals also reduced plant growth at all concentrations. The present study demonstrated the capability of plant species to take up heavy metals from ambient solution and accumulate them in the above-ground biomass. Therefore, such studies should be an integral part of the sustainable development of ecosystems and pollution assessment programmes.

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