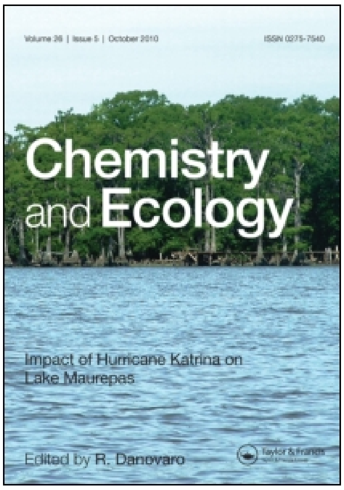

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The aim of this study was to investigate the potential utility of *Allium cepa* L. as a bioindicator organism for measuring copper bioaccumulation and toxicity in laboratory conditions. Onions were exposed to increasing concentrations of the metal (0, 0.1, 0.5, 1, 5 and 10 $\mu g\,mL^{-1}$) for 7 days. Root and leaf development were chosen as biological endpoints, while bioaccumulation was evaluated in roots, bulbs and leaves. Copper caused inhibition of root elongation with increasing effects at the higher doses, growth being reduced by almost 60% at 0.1 $\mu g\,mL^{-1}$ and up to 95% at 10 $\mu g\,mL^{-1}$. The elongation of leaves was significantly lower only in specimens exposed at 0.5 $\mu g\,mL^{-1}$, but a total absence of newly formed tissues was observed at 10 $\mu g\,mL^{-1}$. A marked bioaccumulation of copper was measured in roots, with values increasing up to almost four orders of magnitude compared to controls; only slight or even no significant differences were observed for copper levels in leaves and bulbs of treated *A. cepa*. Multiple linear correlations revealed a significant inverse relationship between copper concentrations and tissue length in both the roots and leaves, evidencing a sensitive responsiveness of this biological model. The overall results suggest the suitability of *A. cepa* as a robust species for easy and simple ecotoxicological bioassays to test the toxic effects and bioavailability of environmental pollutants, especially trace metals.

Keywords: copper; bioaccumulation; toxicity; onion; *Allium cepa* L.

1. Introduction

Anthropogenic activities are causing a constant increase of chemical contamination with possible adverse effects for ecosystems and human health due to the bioaccumulation and trophic transfer of various classes of pollutants [1]. New monitoring approaches have been developed and the use of living organisms as bioindicators of environmental quality has been widely validated [2]; the integration of chemical analyses with the assessment of biological responses allow us to better define the degree and impact of chemical pollution, indicating both the bioavailability of

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chemicals and their cumulative effects on biological systems. Such monitoring programmes have been shown to be ecologically relevant and cost-effective [3].

Several investigations successfully used higher plants as sensitive and rapid bio tools for screening the chemical contamination of the atmosphere, soils, surface and ground waters, landfill leaches and wastewater/sludge [4–7]. The common onion *Allium cepa* is found across a wide range of latitudes and altitudes in Europe, Asia, America and Africa, reaching a world annual production of several million tonnes [8]. The use of *A. cepa* as a biological test system was first introduced in 1938 to evaluate the cytogenetic effects of colchicine [9], and since then this species has been used as a bioindicator for different classes of pollutants, such as trace metals, polycyclic aromatic hydrocarbons, and halogenated pesticides [10,11]. Bioaccumulation of chemicals in specific tissues, inhibition of root and leaf elongation, cytogenetic or mutagenic effects and oxidative stress responses have been characterised in various toxicological studies with *A. cepa*. Practical advantages in the use of this species include the sensibility, reproducibility and rapidity of results, as well as the need of small volumes of samples and low cost [12].

Copper (Cu) has a great environmental relevance, being a natural constituent of the Earth's crust, but is also released by a wide number of anthropogenic activities, including mineral and coal extraction and processing industries [13]. Despite the fact that this element is an essential micronutrient, acting as an enzymatic cofactor in several metabolic processes, the presence of elevated cellular concentrations generates adverse effects, mostly due to its prooxidant potential [14]. In plants copper may induce phytotoxic responses, such as structural and functional alterations of membranes, cell cycle disturbance, genotoxicity, growth inhibition, photosynthesis and chlorophyll content reduction, leading to the onset of oxidative damage [15]. The evaluation of such toxicity responses has been used in various plant species for monitoring copper pollution, while relatively few studies have tested the suitability of *A. cepa* toward this element [16,17].

Coal extraction is an important economic resource for the southern region of Santa Catarina, Brazil. This activity generates acid mine drainage (AMD), characterised by low pH levels (<3.0) and an elevated concentration of heavy metals (i.e. Cu, Al, Fe, Mn), that may adversely affect the environmental quality [18]. In this respect, the possibility of validating easy and reproducible bioassays to test the toxic potentials of AMD may represent an important tool for monitoring and managing these polluted wastes.

The aim of the present work was to investigate the responsiveness of *A. cepa* to evaluate both the bioavailability and toxicity of copper under experimental exposures in laboratory conditions. The bioaccumulation capability was compared in different tissues (roots, bulbs and leaves), while inhibition of root and leaf development was tested as an economic bioassay with *A. cepa* to routinely assess the toxicity of industrial effluents containing copper, such as those deriving from coal mining.

2. Materials and methods

2.1. *Laboratory exposure and measurement of growth inhibition*

The basic toxicological protocol described for *A. cepa* was applied with slight modifications [13]. Roots and leaves were removed immediately before the exposure and bulbs ($n = 10$) were placed on the top of the tests tubes filled with 50 mL of various copper solutions (0, 0.1, 0.5, 1, 5 and 10 $\mu g \cdot mL^{-1}$ as copper, from $CuSO_4$, in 1 mM K phosphate buffer at pH $= 6.5$). The range of nominal copper concentrations were environmentally realistic and similar to those measured in waste waters, industrial effluents and acid mine drainage [19]. Solutions were changed daily and the exposure was carried out for 7 days at 25 °C in darkness. After this period the growth of both roots and leaves was measured.

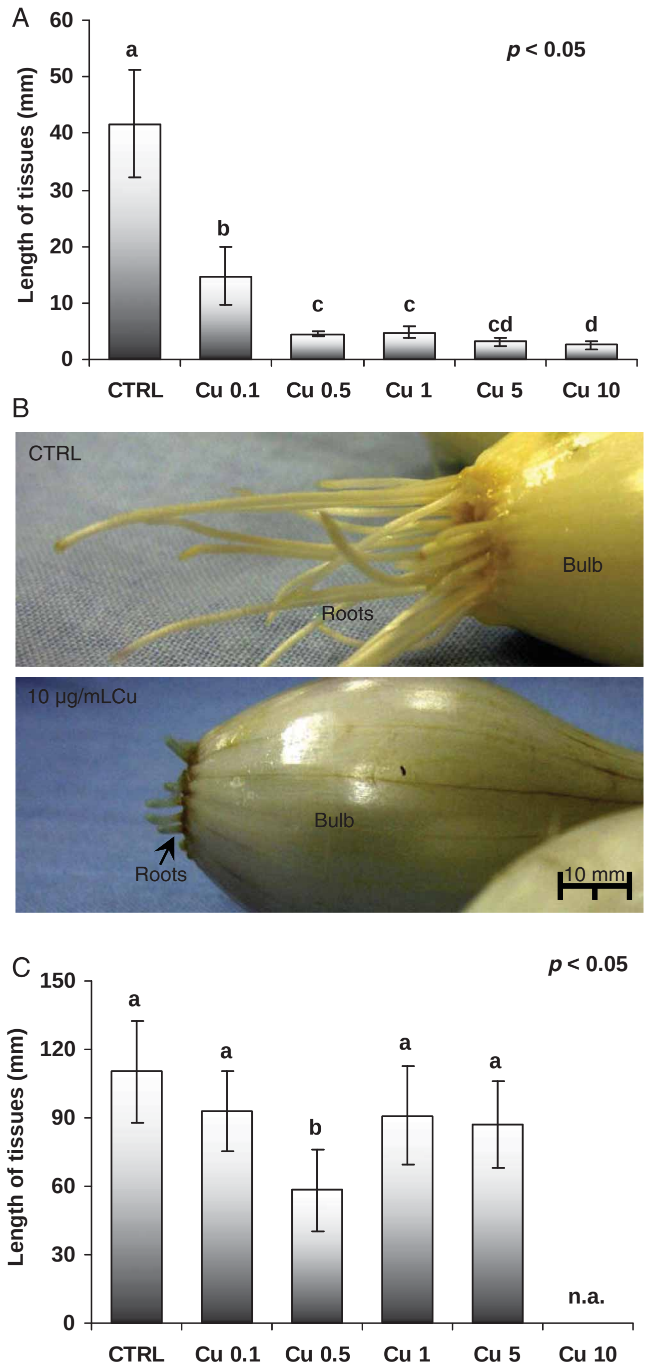

Figure 1. Length of roots (A) and leaves (C) in *A. cepa* exposed to different copper concentrations (0.1, 0.5, 1.0, 5.0 and $10\,\mu g\,mL^{-1}$). Results were expressed as mean ± standard deviation ($n = 5$). Different letters indicate significant differences as determined by ANOVA and *post-hoc* tests. A representative image of root development in control onions and those exposed to $10\,\mu g\,mL^{-1}$ for 7 days is given in (B).

2.2. *Determination of copper*

Roots, bulbs and leaves of *A. cepa* were dried at 60 °C until reaching a constant weight. Approximately 0.2 g of dried samples were digested under pressure with 5 mL nitric acid in a microwave mineralisation system (CEM Mars 5, CEM Corporation), with the following programme: stage 1 at 600 W, 110 °C for 15 min; stage 2 at 600 W, 160 °C for 20 min; stage 3 with cooling at less

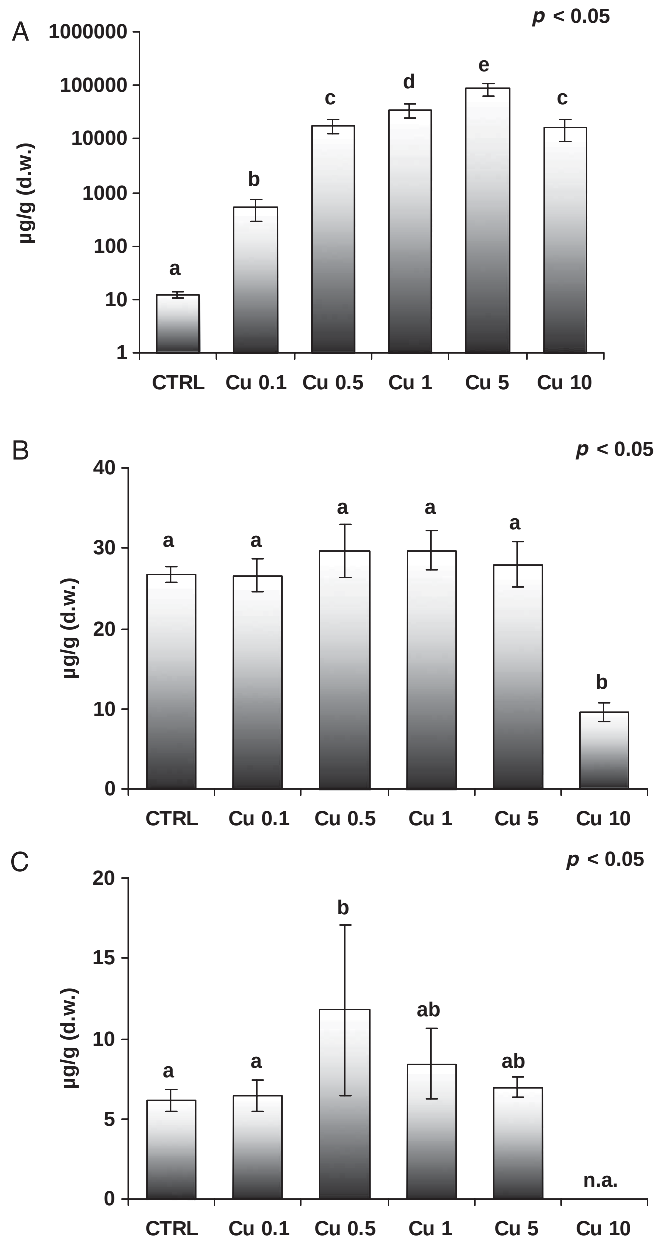

Figure 2. Bioaccumulation of copper in roots (A), bulbs (B) and leaves (C) of *A. cepa* exposed to different concentrations of copper (0.1, 0.5, 1.0, 5.0 and 10 µg mL^{-1}). Results were expressed as mean ± standard deviation ($n = 5$). A logarithmic scale was applied for (A). Different letters indicate significant differences as determined by ANOVA and *post-hoc* tests.

than 50 °C. Copper was analysed by atomic absorption spectrophotometry with electrothermal atomisation and the Zeeman effect (Spectra300 Zeeman, Varian, Mulgrave, VIC, Australia). The concentrations were expressed as μg/g dry weight (d.w.). Quality assurance and quality control were tested by processing blank samples and a standard reference material (SRM 2977, National Institute of Standards and Technology, Gaithersburg, MD, USA). The concentration obtained for the SRM was always within the 95% confidence interval of certified values.

2.3. *Statistical analyses*

Biological and chemical parameters in control and exposed onions were compared by one-way analysis of variance (ANOVA, 95% of confidence interval, $\alpha = 0.05$). The homogeneity of variance was checked by Levene's test and *post hoc* comparisons (Student–Newman–Keuls) were applied to discriminate between means of values. Multiple linear correlations between Cu bioaccumulation in different tissues and biological indexes were performed by a Pearson test ($\alpha = 0.05$, 2-tailed).

3. Results

Copper caused particularly marked inhibition of root elongation with increasing effects at the higher exposure doses (Figure 1A, B): growth was reduced by almost 60% at 0.1 $\mu g\,mL^{-1}$ and up to 95% at 10 $\mu g\,mL^{-1}$. More moderate effects were observed on the elongation of leaves, which were inhibited only in onions exposed to 0.5 $\mu g\,mL^{-1}$, although a total absence of newly formed tissues was observed after treatment at 10 $\mu g\,mL^{-1}$ (Figure 1C).

Results on the bioaccumulation of copper in various tissues of *A. cepa* are reported in Figure 2. Concentrations in newly formed roots were about 12 μg/g for control onions and significantly higher in all the exposure groups, respectively up to 513 ± 234 at 0.1 $\mu g\,mL^{-1}$, $17{,}500 \pm 5000$ at 0.5 $\mu g\,mL^{-1}$, $34{,}000 \pm 9700$ at 1 $\mu g\,mL^{-1}$ and $87{,}300 \pm 22{,}500$ at 5 $\mu g\,mL^{-1}$; levels of Cu did not further increase in *A. cepa* treated with the highest dose (10 $\mu g\,mL^{-1}$), showing a mean content of $15{,}900 \pm 6700$ μg/g (Figure 2A).

Concentrations of copper did not change in bulb tissues of exposed onions compared to the controls, with average values of approximately 25 μg/g (Figure 2B); interestingly, lower concentrations (10.1 ± 1.00 μg/g) were measured in bulbs of onions treated with 10 $\mu g\,mL^{-1}$. Bioaccumulation of copper in leaves of *A. cepa* showed a significant effect only in onions exposed to 0.5 $\mu g\,mL^{-1}$ with values of 12.0 ± 5.0 μg/g compared to 6.0 ± 1.0 μg/g measured in controls (Figure 2C).

Multiple linear correlations revealed a significant inverse relationship ($p < 0.01$) between copper concentrations and length of both the roots and leaves (Table 1), suggesting a mechanistic involvement of this metal in the development of such tissues.

Table 1. Multiple linear correlation (Pearson, 2-tailed) between copper concentration and length of roots and leaves in *A. cepa*.

Correlations Cu concentration/Tissue growth	Roots	Leaves
Pearson coefficient	−0.4792	−0.5762
Significance (2-tailed) p	0.0074*	0.0026*

Note: *Correlation is significant at the 0.01 level (2-tailed).

4. Discussion

Trace metals such as copper represent a relevant and important class of environmental pollutants, which can accumulate in the tissues of living organisms with consequent toxic effects. In this respect, several investigations have described the use of plants as bioindicator organisms in ecotoxicological applications to assess the effects of metals in polluted or disturbed environments [20–22].

The experiment performed in the present work was aimed at evaluating the capacity of *A. cepa* to bioaccumulate copper in different tissues and the possible adverse effects caused by this element on the growth of roots and leaves.

Sensitive and significant toxicological responses were obtained in various tissues of exposed *A. cepa*. The roots exhibited highest sensibility, with significant effects even at the lower copper concentrations, a certain steady-state equilibrium, in terms of growth inhibition and Cu accumulation at the higher doses and lower Cu concentrations in onions exposed at 10 $\mu g\,mL^{-1}$. On the other hand, the leaves showed a significant accumulation and a lower growth only at the dose of 0.5 $\mu g\,mL^{-1}$, but no tissues were newly developed by onions treated at 10 $\mu g\,mL^{-1}$. At the same exposure dose, the bulbs revealed a significant decrease of copper levels which had not been affected compared to controls in the other treated groups. Overall, the lower concentrations measured at the highest dose and the absence of the development of new leaves strongly suggest that the onions were suffering a toxic state with general inhibition of their metabolic status.

This aspect may be particularly important when *A. cepa* is tested in field conditions or with complex environmental matrices, since the presence of elevated levels of pollutants could drastically compromise the general status of the organisms, thus leading to an under-estimation of the bioaccumulation of chemicals. In this view, the possibility of integrating chemical analyses with biological end-points in various tissues (i.e. roots, bulbs and leaves) represents a valid tool for better assessing the toxicity of complex mixtures. Since tissues were shown to be characterised by a different responsiveness, our results strongly suggest that onion roots may be considered the ideal tissue to detect the toxic effects of metals in waste waters.

The utility of onions in ecotoxicological studies has already been described in the scientific literature. Comparable results to those presented in our study were obtained by Palacio et al. [23], who reported a 40% reduction of the mean root length in onions exposed to river waters containing supplemented Cu, Pb and Zn at about 0.03, 0.1 and 1 $\mu g\,mL^{-1}$, respectively. Although inhibition of root development might have also been related to the presence of additional undetermined substances, the authors suggested the validity of this approach to test the toxicity of running river waters [23].

Similarly, Yıldız et al. [24] obtained a 50% reduction of onion root development in specimens exposed for 4 days to 1.5 and 5.5 $\mu g\,mL^{-1}$ of cobalt and copper, respectively. They also observed a significant decrease of the mitotic index in exposed onions and the formation of bridges, stickiness, vagrant chromosomes, fragments, c-anaphase and multipolarity chromosome aberrations in anaphase–telophase root cells.

Inhibition of the mitotic index and appearance of chromosomal aberrations, micronuclei and binucleate cells were also observed by Srivastava et al. [25] after exposure of *A. cepa* for 15 days to municipal sludge lichates containing trace metals such as chromium, copper, nickel and lead. Testing different dilutions of waste sludge, the authors measured a dose-dependent reduction of onion root development, with a maximum of about 50% of mass reduction.

Beside *A. cepa*, other plants such as the garden cress *Lepidium sativum*, the sunflower *Helianthus annuus* and the water lettuce *Pistia stratiotes* were found to be useful in assessing the potential toxicity of metals and organic pollutants [23–28], further supporting the use of these organisms for laboratory ecotoxicological bioassays.

Our results demonstrated a good correlation between copper bioaccumulation and phytotoxic effect in tissues of *A. cepa*. Although the mechanisms of action remain to be fully elucidated, several hypotheses suggest that metals can alter the composition, rigidity and fluidity of membranes, inhibiting water and nutrient fluxes, cellular division, and thus the normal development of the growth of roots and leaves [17].

The bioassay test with *A. cepa* presents some practical advantages compared to other short-term tests, and among these are the sensitivity, reproducibility, low cost, and the possibility of testing several environmental conditions including complex mixtures like waste waters containing metals and other contaminants [29].

The southern region of Santa Catarina is one of the most polluted in Brazil, with surface water and groundwater affected by effluents from coal mining, especially acid drainage, characterised by extremely high concentrations of metals, including copper, and organic compounds. However relatively few studies have been carried out to evaluate the toxic potential of these contaminants to the biota [30]. Therefore, our results on bioaccumulation and toxicity of copper in *A. cepa* represent an important base for future application of such bioassays to test waste effluents and to evaluate the effects of chemicals, including metals, organic pollutants and complex mixtures, typically present in coal mine effluents.

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