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Comparative Response of Lemnaceae Clones to Copper(II), Chromium(VI), and Cadmium(II) Toxicity

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Vascular plants such as aquatic macrophytes have been used as reference organisms in ecotoxicological assessments of environmental toxicants in aquatic systems for more than two decades (Lewis 1995; Wang and Freemark 1995; Lytle and Lytle 2001). Lemnaceae are the most extensively studied family (Wang 1990; Wang 1992; Mohan and Hosetti 1999) and were incorporated to standardized protocols by environmental protection agencies or organizations (USEPA 1996; Environment Canada 1999; OECD 2000). *Lemna gibba* and *Lemna minor* were the selected species among the Lemnaceae family for most of the standarized protocols. A limitation in the selection of these reference species is distribution; *L. gibba* is widely distributed in South America while *L. minor* is not found in the Neotropical region (Landolt 1986; Landolt 1996). The search for reference organisms to be used in ecotoxicological testing with bioassays in the Pampean Region of Buenos Aires Province (Argentina) comprises the use of fish, amphibians, crustaceans and algae from surface water bodies (Ronco et al. 2000a). Previous local reports using vascular plants aimed at assessing toxicity with seeds (Sobrero et al. 1996; Ronco et al. 2000b). The present study reports data on the comparative response of a local clone of *L. gibba* with two collection clones of *L. gibba* and *L. minor* to three environmentally relevant toxic metals using laboratory toxicity tests. Effects of copper, chromium and cadmium were measured on growth rate, complemented with the response on total chlorophyll content and chlorophyll a/ chlorophyll b ratio, and evaluation of frond area development (Mohan and Hosetti 1997; Lytle and Lytle 2001; Prasad et al. 2001). Inhibition of root elongation was also assessed.

MATERIALS AND METHODS

The local clone of *L. gibba* (LgP) was isolated from plants collected in El Pescado stream (Buenos Aires Province, Argentina), which runs along rural areas, and flows into the southwestern coast of the Río de la Plata estuary. Clones of *L. gibba* (G3, collected from Catania, Italy, by Kandeler in 1955, called here LgJ) and *L. minor* (collected in Marburg an der Lahn, Germany by Pirson and Seidel in 1950, called here LmJ) were kindly provided by the Institute of General Botany, Friedrich-Schiller-University of Jena, Jena, Germany. The description of the LgJ and LmJ clones source was given by Landolt (personal communication). Axenic

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stock cultures of *Lemna* clones were maintained in the laboratory under standard growth conditions with sterile, weekly renewed, nutrient solution (125µM NH_4NO_3, 110µM $CaCl_2$, 203µM $MgSO_4$, 15µM K_2HPO_4, 250µM $NaHCO_3$, 0.9µM EDTA-$FeCl_3$, 8.9 µM H_3BO_3, 0.9µM $MnCl_2$, 0.04µM $CoCl_2$, 0.08µM $ZnSO_4$, 0.04µM $CuSO_4$, 0.7µM Na_2MoO_4, pH 7.0). Cultures were kept at 22 ± 2 °C, under 16 hr day with 80 µM $m^{-2}s^{-1}$, cool-white fluorescent light. Toxicity testing was done under the same conditions as the ones used for culturing, but with continuous illumination. Before testing, clones were acclimated for a month at assay conditions. Tests were performed using a modified protocol of Huebert and Shay (1993a) in 500 mL jars, containing 300 mL sterile nutrient solution, starting experiments with 4-8 fronds. Medium and toxicant dilutions were renewed partially every 2-3 days (with a pH adjustment when required) during the 14 days of exposure, using an increasing renewal ratio from 1:6 to 1:1, as a function of biomass increment. Assays were acceptable if negative controls exhibited exponential growth (Huebert and Shay 1993b). Experimental design included duplicates with 3-4 replications per concentration, 6 toxicant concentrations (as the nominal concentration of the metal ion)-Cu(II) from 0.1 to 1.25 mg L^{-1}; Cr(VI) from 0.1 to 5 mg L^{-1} (clones LgP and LgJ) and from 0.05 to 3 mg L^{-1} (clone LmJ); Cd(II) from 0.01 to 0.35 mg L^{-1}- and controls. Toxicant concentration range was obtained from preliminary tests. Assays with all clones were done simultaneously. Toxicant dilutions using nutrient solution were prepared from stock solutions of $CuSO_4x5H_2O$ (Anedra), $K_2Cr_2O_7$ (Anedra) and $CdSO_4$ (Carlo Erba). The total metal concentration was only verified in each stock solution by atomic absorption spectrophotometry (Varian Spectra AA, air-acetylene flame) (APHA 1998) using certified standards (Accu Trace TM). All reagents used were analytical grade. Measured endpoints included effects on the growth rate (GR), frond area (FA), total chlorophyll content (TCC), chlorophyll a/chlorophyll b ratio (Chla/Chlb) and root elongation. Growth rate was calculated as: GR = 1000*(logFt-LogFo)/t, where Ft corresponds to the number of fronds at time t, Fo to the initial frond number, and t is the exposure time in days. The frond count included every visible protruding bud (Huebert and Shay 1991; Environment Canada 1999). Frond area was measured with an area meter (LI-COR, LI-3100) from photographic enlargements of the frond images. Data from area meter were finally adjusted to real size. To reduce the effect of age in the frond area estimate, only fronds with buds or daughters were considered. Chlorophyll content was determined on N,N-dimethylformamide extracts (100 mg Fwt in 5 mL solvent) measuring its absorbance at 661 and 664 nm (Shimadzu UV-1203) (Zscheile and Comar 1941). Effect on root elongation was assessed measuring mean root length of the whole test population in each replicate. Statistical analysis of results for the different endpoints comparing the three clones response included regression analysis and factorial ANOVA. Significant differences in multiple comparisons were tested according to Tukey ($p \leq 0.05$) (Zar 1996; Environment Canada 1999). The IC_{25} and IC_{50} estimates from nominal concentrations were calculated by a non-parametric linear interpolation method and the confidence intervals were obtained by a bootstrap method of random re-samplings from the actual observations (Environment Canada 1999).

RESULTS AND DISCUSSION

Fitted regression lines (Fig. 1a) from both *L. gibba* clones show a slight change in the growth response rate to Cu(II), while the response on LmJ clone is significantly different ($p \leq 0.05$). According to IC_{25} and IC_{50} growth rate values, the sensitivity of the three clones is very similar, though clone LmJ is slightly more sensitive (Table 1). On the other hand, the effect of Cu(II) on the frond area is greater than that observed on the growth rate. Differences in the inhibition of the FA among clones are also similar, though *L. gibba* LgJ is sligthly more sensitive (Fig. 1b and insert and Table 1). Although the effect on total chlorophyll content is severe, it only appears at concentrations higher than 0.75 mg L^{-1} of Cu(II). The IC_{25} and IC_{50} TCC values and regression plots are very similar for all the studied clones (no significant differences; $p \leq 0.05$). The effect of Cu(II) on the Chla/Chlb ratio shows a similar trend for all clones. A general view of the results of the Cu(II) phytoxicity on *Lemna* clones indicates a similar sensitivity on all the studied endpoints, except for clone LgJ, which is more affected on the frond area.

Results from tests with Cr(VI) show that the growth rate sharply declines at low concentrations and the shape of plots (Fig. 1d) for the three clones is very similar (no significant differences; $p \leq 0.05$). Sensitivity assessed by means of the growth rate IC_{50} estimate indicates that the response of the three clones is also similar (Table 1). This behavior is slightly different at low concentrations of Cr(VI) at which *L. minor* (Clon LmJ) shows a significant effect at 0.1 mg L^{-1} (Fig. 1d insert) and a lower IC_{25} respect to *L. gibba* clones (Table 1). The assessment of the effect of Cr(VI) on the reduction of the frond area shows differences between clones. In particular, *L. minor* appears to be the most affected at low concentrations (Fig. 1e and insert) but at higher concentration exposure the inhibition response remains constant. *L. gibba* (clone LgJ) is the most sensitive clone according to the IC_{50} values (Table 1). Differences between clones of *L. gibba* are observed for the FA at low and high concentrations of this toxicant (Table 1 and Fig. 1e insert). As regards the response on growth rate, all the clones show a similar trend when assessing the effect of Cr(VI) on the total chlorophyll content (Fig. 1f). No significant interaction between clones and the Cr(VI) effect was detected ($p \leq 0.05$). According to the IC_{25} and IC_{50} values (Table 1) *L. gibba* (clon LgJ) is the most sensitive species, also showing significant inhibition in total chlorophyll content at 0.25 mg L^{-1} (Fig. 1f insert). The highest effect on the Chla/Chlb ratio was also observed in clon LgJ (Table 1). When comparing the response to Cr(VI) on the different endpoints in each clone it could be observed (Table 1) that in general chlorophyll content was the most affected.

The analysis of the results of the phytotoxicity tests to Cd(II) indicates that *L. minor* shows a fitted growth rate plot (Fig. 1g) significantly different from that of *L gibba* ($p \leq 0.05$), the clone of *L. minor* being the least sensitive (Table 1). Regarding the effect on the frond area (Fig.1h) this endpoint indicates a slightly higher effect than that on the growth within the same concentration interval for the three clones (Table 1). On the other hand, exposure to Cd(II) shows significant

differences on the response of total chlorophyll content ($p \leq 0.05$). Clone LgP is the most sensitive (Fig. 1i insert) with higher differences at lower concentrations. The Chla/Chlb ratio follows the same trend as the total chlorophyll content (Table 1). The comparison of the response to Cd(II) indicates that LgP clone is most sensitive and exhibits a more uniform response than the observed for the other clones on all endpoints.

The general tendency of the results from toxicity, expressed as the mean IC_{50} values of all endpoints for the three clones and the maximum and minimum obtained values between brackets for the studied metals –Cd(II) 0.24 [0.13-0.35] mg L^{-1}, Cu(II) 1.03 [0.68-1.25] mg L^{-1} and Cr(VI) 3.12 [0.9-5] mg L^{-1}–, indicates that cadmium is one order of magnitude more toxic than chromium, while copper is five times less toxic than cadmium. When comparing these mean values with reported sensitivity ranges for different *Lemna* species –Cd(II): 0.07-6.13 mg L^{-1}, Cu(II): 0.12-1.3 mg L^{-1} and Cr(VI): 3.3 to >10 mg L^{-1}– (Huebert and Shay 1991; Mohan and Hosetti 1999), it can be observed that mean sensitivity is within the reported intervals and that the response of some of the measured endpoints is similar to the most sensitive species (Table 1). The comparative response of clones indicates that variability between those from the same species is low for all endpoints and metals, except for Cr(VI) effect on chlorophyll content and on the frond area, where a "3-fold" difference can be observed, *L.gibba* LgJ being the most sensitive. The response of the local clone is also very similar to the collection clone of *L. minor*, the latter being slightly more sensitive to Cr(VI) and Cu(II). The maximum assessed difference was observed for chlorophyll content, where the IC_{50} ratio between clones reached a value of 2.4 for LgP:LmJ with Cr(VI) and LmJ:LgP with Cd(II). Significant effect on the root elongation for the three clones was observed at concentration levels of 0.1, 0.25 and 0.025 mg L^{-1} for Cu(II), Cr(VI) and Cd(II), respectively. Different reports also describe metal damage on root system (Hendry et al. 1992; Sobrero et al. 1996; Samantary 2002).

Results found in literature on intra-species variability with respect to metal phytotoxicity indicate a wide interval of metal concentrations leading to an effect, ranging from the same order to one order of magnitude. This pattern has been reported for algae where one order of magnitude in the sensitivity to Cd(II) was observed for two cellular lines of *Chlorella* sp (Kaplan et al. 1995). For vascular plants such as grasses, Hendry et al. (1992) found differences in sensitivity to Cd within the same order of magnitude for *Holcus lanatus* clones. Wu and Zhang (2002) also found them for different barley genotypes. For the case of leguminous plants, Samantary (2002) showed variations of sensitivity to Cr within one order of magnitude for two cultivars of *Vigna radiate*. Sensitivity to Zn within one order of magnitude was also reported on three *L. minor* clones by Van Steveninck et al. (1992). On the other hand, although the results reported in the present study reveal significant differences in sensitivity among *Lemna* clones, even from different species, such differences are smaller than those observed for algae and other vascular plants. Low variability in the sensitivity was also detected among 14 clones of *L.gibba* to the herbicide simazine (Mazzeo et al. 1998).

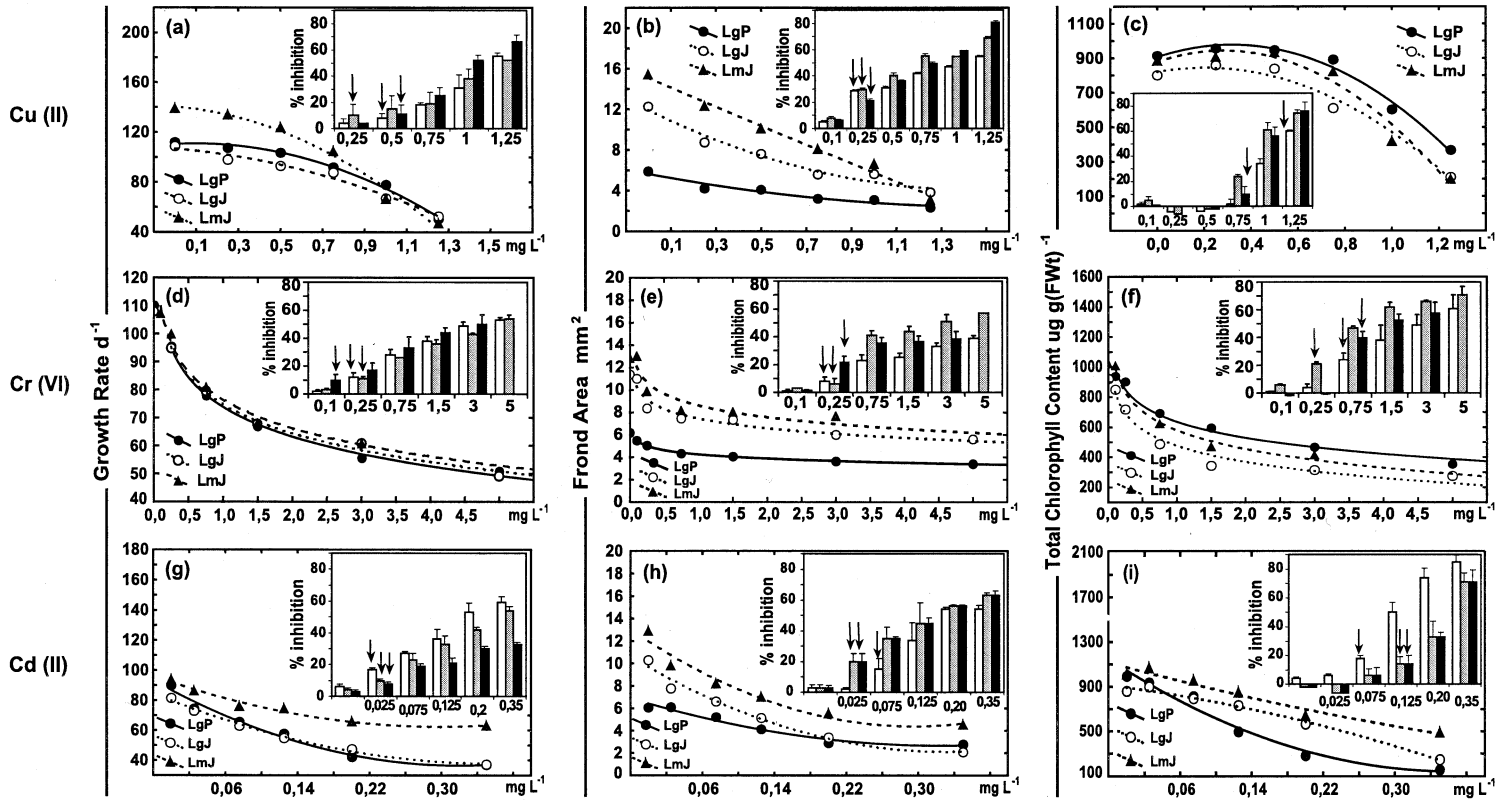

Figure 1. Fitted regression lines showing the response of *Lemna* clones to Cu(II), Cr(VI) and Cd(II). Effect on growth rate (a, d, g), frond area (b, e, h) and total chlorophyll content (c, f, i). Inserts indicate mean inhibition respect to control on clones LgP (white), LgJ (grey) and LmJ (black columns). Bars indicate standard error (n=2) of mean values from replicates. Arrows show the lowest statistically significant inhibition ($p \leq 0.05$).

Table 1. Sensitivity of duckweed clones to copper, chromium and cadmium for different endpoints.

	Endpoint IC_{25}-IC_{50} ($mg.L^{-1}$)							
	Growth Rate		Frond Area		Total Chlorophyll Content		Chlorophyll a/Chlorophyll b Ratio	
	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}
Cu(II)								
***L.gibba* (LgP)**	**0.87** (0.73-0.99)	**1.19** (1.15-1.23)	**0.10>CI_{25}< 0.25***	**1.05** (0.91-1.32)	**0.93** (0.90-0.96)	**1.15** (1.12-1.18)	**1.08** (1.04-1.11)	**1.21** (1.16-1.25)
***L.gibba* (LgJ)**	**0.82** (0.64-0.91)	**1.23** (1.18-1.25)	**0.10>CI_{25}< 0.25***	**0.68** (0.66-0.72)	**0.74** (0.71-0.77)	**0.93** (0.89-0.97)	**0.90** (0.87-0.95)	**> 1.25 ***
***L.minor* (LmJ)**	**0.67** (0.40-0.83)	**0.97** (0.89-1.04)	**0.33** (0.29-0.37)	**0.81** (0.68-0.93)	**0.85** (0.79-0.91)	**0.99** (0.92-1.08)	**0.81** (0.74-0.89)	**1.10** (1.06-1.13)
Cr(VI)								
***L.gibba* (LgP)**	**0.64** (0.52-0.78)	**3.49** (2.72-4.62)	**1.00** (0.57-1.76)	**> 5 ***	**0.80** (0.61-1.29)	**3.05** (2.52-3.73)	**1.67** (0.61-3.22)	**4.43** (3.75-4.96)
***L.gibba* (LgJ)**	**0.70** (0.51-0.98)	**4.17** (3.63-4.77)	**0.53** (0.49-0.60)	**2.55** (2.05-3.24)	**0.32** (0.15-0.49)	**0.90** (0.75-1.04)	**0.89** (0.61-1.12)	**3.54** (1.50-4.84)
***L.minor* (LmJ)**	**0.49** (0.25-0.69)	**3.00** (2.55-3.60)	**0.40** (0.25-0.69)	**> 3 ***	**0.55** (0.44-0.64)	**1.28** (1.06-1.46)	**2.12** (1.45-2.55)	**> 3 ***
Cd(II)								
***L.gibba* (LgP)**	**0.07** (0.05-0.08)	**0.19** (0.16-0.25)	**0.11** (0.08-0.13)	**0.19** (0.17-0.20)	**0.09** (0.08-0.10)	**0.13** (0.11-0.15)	**0.13** (0.11-0.15)	**0.21** (0.18-0.24)
***L.gibba* (LgJ)**	**0.09** (0.06-0.11)	**0.27** (0.23-0.31)	**0.04** (0.03-0.06)	**0.13** (0.09-0.16)	**0.16** (0.13-0.19)	**0.26** (0.23-0.29)	**0.24** (0.18-0.32)	**> 0. 35***
***L.minor* (LmJ)**	**0.16** (0.10-0.21)	**> 0. 35***	**0.04** (0.03-0.07)	**0.15** (0.10-0.21)	**0.15** (0.12-0.17)	**0.32** (0.28-0.35)	**> 0.35***	**> 0. 35***

Values in parentheses correspond to confidence intervals (95%). *IC_{50} or IC_{25} estimation was not possible within tested concentration interval. Data refers to the nominal concentration of each metal.

The comparison of the relative endpoint response measured by means of the IC_{50} (Table 1) indicates that TCC is most affected in all clones and for all the studied metals with respect to GR. On the contrary, when analyzing the response at lower tested concentrations, GR seems to be the most affected endpoint. Although inhibition FA is the most variable endpoint, it is very sensitive to some of the metals, indicating a higher specificity of the response. Although the battery of measured endpoints is complementary, the significance of the GR inhibition of a population has a higher ecological relevance. Besides, the persistence of the damage after exposure assessed by means of plant recovery is higher for growth parameters than in chlorophyll content (Sobrero et al. 1996).

The results show that the local clone is within the order of sensitivity to copper, chromium and cadmium as the two collection clones. It will also show a toxicant effect at levels of metal concentration admitted for discharges in surface waters for the local regulation (Ley 5965/58, Dec. Regl. 2009/60, Res. 287/90, Provincia de Buenos Aires). Consequently, when selecting a reference organism to be used in the assessment of the impact of metals in water bodies with a vascular plant, L. gibba LgP may be considered a potential surrogate organism for the Pampa's region.

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