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Acute Toxicity of Cd^{2+}, Cr^{6+}, and Ni^{2+} to the Golden Mussel *Limnoperna fortunei* (Dunker 1857)

Emiliano Bálsamo Crespo[1] · Patricio Javier Pereyra[2] · Anahí Silvestro[1,2] · Keila Hidalgo[1] · Gustavo Bulus Rossini[1,3]

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Abstract
In this study we test the sensitivity of three sizes of golden mussel (*Limnoperna fortunei*), an introduced species in Argentina, to a 96-h exposure to Cd^{2+}, Cr^{6+}, and Ni^{2+}. We also analysed the relative sensitivity of *L. fortunei* compared to other freshwater bivalve equivalent sensitivity data. The ANOVA results showed that both factors, heavy metal and size, had significant effects ($p = 0.0013$ and $p = 0.0091$, respectively) on the mortality of the golden mussel. Tukey's test showed significant differences for Cr^{6+} treatment and the smallest size class (7 mm ±1). The relative sensitivity analysis showed that LC_{50} values for the smallest size class of *L. fortunei* exposed to Ni^{2+} and Cd^{2+} were in the low range, with values of 11.40 mg/L and 12.65 mg/L, respectively. In the case of Cr^{6+} (1.66 mg/L), its LC_{50} was in the medium-low range of the freshwater bivalve sensitivity distribution.

Keywords Freshwater bivalves · Heavy metals · *Limnoperna fortunei* · Sensitivity

The pollution in aquatic ecosystems with heavy metals is a worldwide concern given the increase in their emissions (Vareda et al. 2019). Biomonitoring is a widely implemented technique that uses organisms in order to assess environmental pollution levels (Zhou et al. 2008). Due to satisfying many of the conditions for an ideal monitoring organism, bivalve molluscs have been extensively utilized for several decades in freshwater and marine environmental monitoring programs (Gupta et al. 2011). Elder and Collins (1991) pointed out the convenience of using introduced species as monitoring organisms because of their physiological tolerance range and their wide distribution. The freshwater golden mussel, *Limnoperna fortunei* (Dunker, 1857) (Bivalvia, Mytilidae), was introduced by ship ballast water, and reported for the first time in South America in 1991 (Pastorino et al. 1993). Byssate juvenile and adult forms live in dense groups attached to hard surfaces, where they feed on plankton by filtration (Darrigran and Damborenea 2006). The life cycle of *L. fortunei* presents a planktonic larvae which facilitates a rapid dispersion, assisted by commercial ship traffic, which has enabled the species to colonise five countries in South America (Oliveira et al. 2015). *L. fortunei* has been tested as a suitable organism for biomonitoring, with most studies focusing on bioaccumulation and biomarkers (do Amaral et al. 2019; Belaich et al. 2006; Villar et al. 1999), but very little has been reported regarding mortality of this species exposed to contaminants (Cataldo et al. 2003; Soares et al. 2009). The objectives of the present study were to (1) analyse the sensitivity (as mortality) of three sizes of *L. fortunei* to the acute exposure of three heavy metals of environmental relevance: Cd^{2+}, Cr^{6+}, and Ni^{2+}, and (2) establish the relative sensitivity range of *L. fortunei* among other freshwater bivalves used for toxicological assessment.

Materials and Methods

Individuals of *L. fortunei* were haphazardly collected during low tide at Palo Blanco Beach in Berisso (34°51′19.1″S, 57°50′17.3″W), Buenos Aires, Argentina. Individuals were acclimated in 100 L tanks with dechlorinated and oxygenated tap water (conductivity 1.0 mS/cm; hardness

✉ Emiliano Bálsamo Crespo
emilianobal@hotmail.com

[1] Centro de Investigaciones del Medio Ambiente (CIM), FCEx-UNLP-CONICET, La Plata, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[3] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC PBA), Buenos Aires, Argentina

215 mg/L $CaCO_3$; alkalinity 180 mg/L $CaCO_3$; pH range 7.6 ± 0.2; temperature 20 ± 2 °C; photoperiod 16:8 light:darkness) for at least 2 weeks before each assay. During acclimation, mussels were fed with a cultured Chlorophyceae algal solution. Food was supplied according to mussel's filtering activity in order to maintain a low green tinge in the water at the end of a 24 h period. Mussels were not fed during the assays. Individuals were measured along the maximum anterior-posterior axis (total length) with a digital calliper (precision 0.01 mm) and, based on the more abundant size intervals, were arranged in three size classes (SC): SC1(7 ± 1 mm), SC2 (13 ± 1 mm), and SC3 (19 ± 1 mm). Three heavy metals of environmental relevance were selected: Cd^{2+}, Cr^{6+}, Ni^{2+}. Dilutions were prepared from stock solutions using analytical grade (ACS) $3CdSO_4.8H_2O$ (Merck), $Cr_2O_7K_2$ (Analar), and Ni^0 granules (Biopack) previously dissolved in HNO_3. Concentrations were tested and run in triplicate in polyethylene containers. Sets of samples were randomly taken at different days of renewal. Actual metal concentrations of the samples were measured using a Varian Spectr AA 330 atomic absorption spectrophotometer with air-acetylene flame (APHA 1998 Method 3111 B). Quality assurance and control comprised the calibration of equipment using certified reference materials from Accustandard Inc. (Cd:AA08N-1, Cr:AA13N-1, Ni:AA37N-5), blanks and replicates of analytical samples, and bidistilled water. The detection limit for all three metals was 0.005 mg/L. All labware was previously cleaned in a 10% HNO_3 bath, and assay samples were all refrigerated and acidified with HNO_3 (Analar) analytical grade for storage. Since not every assay replicate and concentration was sampled at each medium renewal event, data of measured and nominal concentrations from each set of samples was used to calculate estimated concentrations by linear regression methods for each assay. Nominal concentrations in mg/L were: 5, 8, 14, 23, 39, 64 for Cd^{2+}, 1, 2, 4, 8, 16, 32, 64 for Cr^{6+}, and 7, 12, 20, 35, 60, 100, 165 for Ni^{2+}. Negative control contained the same water that was used to make dilutions. For each replicate, 15 mussels were allocated in 0.5 L of dechlorinated and mechanically aerated water and kept for 24 h. After this settlement period, only 10 mussels that had attached to the walls of each container were kept for the assays. Those individuals that had failed to attach were considered unhealthy and therefore discarded. Finally, containers were emptied and refilled with 0.5 L of the corresponding dilution; each of the assays were 96-h static-renewal tests. Test dilutions were renewed every 24 h. Mortality was the selected endpoint. Mussels were considered dead if they remained with opened valves when removed from the container or if they did not show signs of activity in response to physical stimuli with a plastic stick. The integrity of all individuals was assessed under stereo microscope. Statistical endpoints LC_{50} and LC_{10} were estimated by fitting measured concentration data to Finney's Probit model (Finney 1971) using the Probit software from USEPA (1993), or the Trimmed Spearmen-Karber method (TSK USEPA 1993) where needed. A two-way ANOVA without replication (Microsoft Excel) was performed to assess the effects of the two factors, size class and heavy metal, on the sensitivity (Log LC_{50}) of *L. fortunei*. Post-hoc Tukey HSD test for multiple comparisons was applied to evaluate the differences between the levels of the two factors. In order to calculate the relative sensitivity (RS) of *L. fortunei*, we conducted a bibliography search and consulted the USEPA ECOTOX database. Only mortality LC_{50} values from 96 h exposure to the same heavy metals, and from freshwater bivalves in juvenile and adult stage were considered. The toxicity of Cd^{2+} and Ni^{2+} are water hardness dependent, however, there is no evidence for this effect on the toxicity of Cr^{6+} (USEPA 1996). Neither the data from ECOTOX, nor the original publications provided consistently hardness-adjusted LC_{50} values. And in some cases, water hardness was not reported at all. Due to these limitations, no adjustments were made to data in our analysis. We calculated the $RS = Log(LC_{50Lf}/LC_{50i})$ (Santos-Medrano and Martinez 2019), where 'LC_{50Lf}' corresponds to the calculated lethal concentration for *L. fortunei*, and 'LC_{50i}' is any one species' LC_{50} value recorded from our search. In those cases where there was more than one record for a given 'LC_{50i}', the arithmetic mean was calculated. With respect to 'LC_{50Lf}', only those from the size class (SC) that had yielded significant differences in the ANOVA were included in the relative sensitivity analysis. Finally, we charted the frequency distribution of all Log LC_{50} values, and graphically showed where the sensitivity of the three size classes of *L. fortunei* lie for each heavy metal.

Results and Discussion

Details of the heavy metal estimated concentration adjusted by linear regression are shown in Table 1. Estimations averaged 58%, 103%, and 86% of the respective Cd^{2+}, Cr^{6+}, and Ni^{2+} nominal values. There was no mortality in the negative control for all replicates. The estimated LC_{50} and LC_{10} values with their respective 95% confidence limits for each heavy metal assay are shown in Table 2. In the case of Ni^{2+}, mortality data for SC1 did not show a monotonically increased response to the

Table 1 Estimated concentrations of heavy metals in mg/L. Values were adjusted by linear regression using the samples' measured concentration and their corresponding dilution coefficients

Estimated concentration										
Metal	Estimated value (mg/L)							Slope	R^2	Std Error
Cd^{2+}	2.9	4.64	8.12	13.34	22.61	37.11		0.5798*	0,9997*	0.5823*
Cr^{6+}	1.03	2.06	4.12	8.23	16.47	32.94	65.88	1.0293*	0,9957*	1.0878*
Ni^{2+}	5.99	10.27	17.11	29.95	51.34	85.57	141.19	0.8557	0,9976	1.5548

*Average value of data from three sets of samples taken at different times.

All results are statistically significant ($p < 0.05$, N=44)

Table 2 LC_{50}, LC_{10} values, and 95% confidence values for the three heavy metals and size classes tested. Values are in mg/L. SC1 LC_{10} values could not be calculated by TSK method

	Cd^{2+}			Cr^{6+}			Ni^{2+}		
	LC_{50}	Lower limit	Upper limit	LC_{50}	Lower limit	Upper limit	LC_{50}	Lower limit	Upper limit
SC1	12.65	10.74	14.96	1.66	1.31	2.01	11.40*	4.57*	28.46*
SC2	24.23	20.77	29.18	4.97	3.84	6.38	61.25	44.09	136.43
SC3	42.23	32.52	73.3	5.22	3.83	7.05	82.85	68.19	106.01
	LC_{10}	Lower limit	Upper limit	LC_{10}	Lower limit	Upper limit	LC_{10}	Lower limit	Upper limit
SC1	5.33	3.88	6.62	0.71	0.41	0.96	–**	–**	–**
SC2	11.78	8.42	14.37	1.19	0.68	1.72	18.34	10.03	24.35
SC3	16.32	10.8	20.5	0.85	0.39	1.37	29.31	19.14	37.88

* TSK method, **unable to be calculated with TSK method

treatments, and thus was fitted to a TSK method. Ni^{2+} LC_{10} values for SC1 could not be calculated by TSK method. The LC_{50} values for SC1 showed the toxicity trend was $Cr^{6+} > Ni^{2+} > Cd^{2+}$, whereas for SC2 and SC3 the trend was $Cr^{6+} > Cd^{2+} > Ni^{2+}$. LC_{10} values for SC2 and SC3 showed a consistent toxicity trend: $Cr^{6+} > Cd^{2+} > Ni^{2+}$. The two-way ANOVA results indicated significant effects ($p < 0.05$) on the mortality of *L. fortunei* for metals (p= 0.0013) and mussel size class (p= 0.0095). Tukey HSD test yielded significant differences between mussels from SC1 and those of SC2 and SC3 ($p = 0.0216$ and $p = 0.0091$, respectively). Heavy metal comparisons showed that sensitivity to Cr^{6+} was significantly different to that of Cd^{2+} ($p = 0.0036$), and Ni^{2+} ($p = 0.0016$). The combination of data from the USEPA ECOTOX database and bibliography searches yielded 29 Cd^{2+} LC_{50} entries corresponding to 13 species, 12 Cr^{6+} LC_{50} entries for 9 species, and 16 Ni^{2+} LC_{50} entries for 11 species. The calculations of the relative sensitivity of *L. fortunei* SC1 compared to that of other freshwater bivalves are shown in Table 3. In regard to Cd^{2+} and Ni^{2+}, it can be observed that *L. fortunei* SC1 is less sensitive than all freshwater bivalves. However, data for these two metals could not be normalised for water hardness. *Villorita cyprinoides cochinensis* is the organism with the highest RS scores of 3.67 and 2.27 for Cd^{2+} and Ni^{2+}, respectively. In the case of Cr^{6+}, *L. fortunei* SC1 showed to be more sensitive than *Diplodon chilensis* (RS -1.09) and *Hyriopsis cumingi* (RS -0.81), but scored a lower relative sensitivity than the rest of the test organisms included in the analysis. *V. c. cochinensis* (RS 2.16) was the most sensitive compared to *L. fortunei* SC1. A graphic representation of the RS values from Table 3 is depicted in Fig. 1. When compared to data from other freshwater bivalves, the sensitivity range of *L. fortunei* to Cd^{2+} (Fig. 2a) and Ni^{2+} (Fig. 2c) sits in the lower end of the distribution of sensitivities. Furthermore, the golden mussel's sensitivity to Cr^{6+} falls within the medium-low range (Fig. 2b). Small juveniles of *L. fortunei*, such as those of SC1 in this study, represent the most numerous size class in natural populations of the

Table 3 Results of the relative sensitivity (RS) quotient calculations for *L. fortunei* SC1. Values of LC_{50} were collated from the USEPA ECOTOX database and bibliography searches

Metal species	Test organism	96h LC_{50} † (mg/L)	Relative sensitivity**	References
Cd^{2+}				
	Actinonaias pectorosa	0.06*	2.34	Keller (2000)
	Corbicula fluminalis	0.52	1.39	Abdel Gawad (2006)
	Crassostrea rhizophorae	5.00	0.40	Chung (1980)
	Lamellidens marginalis	10.00	0.10	Raj and Hameed (1990)
	Lampsilis straminea claibornensis	0.04	2.52	Keller (2000)
	Lampsilis teres	0.02*	2.76	Keller (2000)
	Lasmigona subviridis	0.06*	2.34	Black (2003)
	Parreysia favidens	7.20	0.24	Bhamre et al. (1996)
	Pisidium casertanum	0.85*	1.17	Mackie (1989)
	Pisidium compressum	1.05*	1.08	Mackie (1989)
	Utterbackia imbecillis	0.07*	2.26	Keller and Zam (1991), Black (2003) and Keller (2000)
	Villorita cyprinoides cochinensis	0.003	3.67	Abraham et al. (1986)
	Villosa vibex	0.08	2.21	Keller (2000)
Cr^{6+}				
	Amblema plicata	0.23	0.85	Wang et al. (2016)
	Diplodon chilensis	20.40	− 1.09	Silva et al. (2007)
	Hyriopsis cumingii	10.60*	− 0.81	Chin and Chou (1978)
	Lampsilis siliquoidea	0.27	0.80	Wang et al. (2016)
	Margeritifera falcata	0.62	0.42	Wang et al. (2016)
	Megalonaias nervosa	0.14	1.08	Wang et al. (2016)
	Rangia cuneata	0.21	0.90	Olson and Harrel (1973)
	Utterbackia imbecillis	0.29*	0.76	Wang et al. (2016)
				Keller and Zam (1991)
	Villorita cyprinoides cochinensis	0.01	2.16	Abraham et al. (1986)
Ni^{2+}				
	Actinonaias pectorosa	0.52	1.34	Keller (2000)
	Amblema plicata	0.23	1.69	Wang et al. (2016)
	Hamiota perovalis	0.31	1.56	Gibson (2015)
	Lampsilis siliquoidea	0.43*	1.42	Wang et al. (2016)
	Lampsilis straminea claibornensis	0.13	1.94	Keller (2000)
	Margeritifera falcata	0.27	1.63	Wang et al. (2016)
	Megalonaias nervosa	0.17	1.82	Wang et al. (2016)
	Utterbackia imbecillis	0.41*	1.44	Keller (2000), Keller and Zam (1991) and Wang et al. (2016)
	Villorita cyprinoides cochinensis	0.06	2.27	Abraham et al. (1986)
	Villosa nebulosa	0.51	1.35	Gibson (2015)
	Villosa vibex	0.30	1.58	Keller (2000)

* Average of multiple LC_{50} data records. ** $RS=Log(LC_{50Lf} / LC_{50i})$. †Not adjusted to water hardness

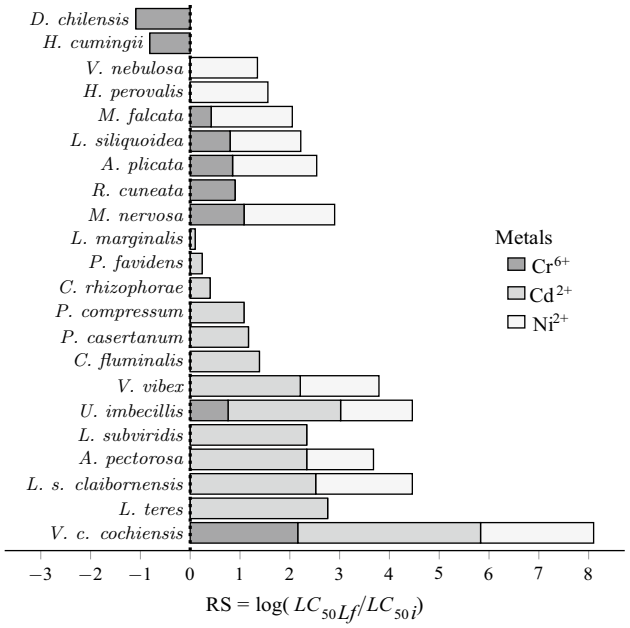

Fig. 1 Graphic representation of the relative sensitivity (RS) calculations from Table 3 for three heavy metals: Cd^{2+}, Cr^{6+}, Ni^{2+}. Positive values represent a greater RS, and negative values correspond to a lesser RS than *L. fortunei* SC1 (dotted line)

golden mussel (Bonel and Lorda 2015). They are easy to collect in the field, and given their encrusting nature they can establish colonies on artificial substrates that can be used as artificial units of habitat for manipulative experiments. *L. fortunei* presents a short life cycle (2/3 years) with a planktonic larval development, and a long actively reproductive period with external fecundation (Darrigran and Damborenea 2006). Because of the aforementioned characteristics, and the ability of *L. fortunei* to tolerate a wide range of environmental conditions (Ricciardi 1998), this species is a versatile test organism for different experimental scenarios and rearing in the laboratory. Conversely, most of the native species of freshwater bivalve families in Argentina live buried in sediments, with the exception of some epifaunal species such as *Byssanodonta paranensis* and *Eupera platensis* which have more specific distribution patterns (Darrigran and Lagreca 2005). Their life cycles include ectoparasitic larval stages (e.g. glochidia in Hyriidae, or lasidia in Mycetopodidae), or are ovoviviparous with less prolific reproductive periods (e.g. Euperinae) (Ezcurra de Drago et al. 2006; Ituarte 1988). Previous studies on *L. fortunei* tested for bioaccumulation and biomarkers in response to heavy metals such as mercury, copper, and cadmium (Belaich et al. 2006; do Amaral et al. 2019; Soares et al. 2009) and organic compounds (Iummato et al. 2018; Pereyra et al. 2011, 2012). This study provides new sensitivity data for *L. fortunei* under acute exposure to Cd^{2+}, Cr^{6+}, and Ni^{2+}. It also shows that the juveniles of the golden mussel present arange of sensitivity suitable for a sentinel species (e.g. studies with biomarkers). Its encrusting epifaunal nature that enables simple sampling methods, widespread distribution (which allows inter-regional comparisons), and easy maintenance in the laboratory, make *L. fortunei* a suitable candidate for biomonitoring programs.

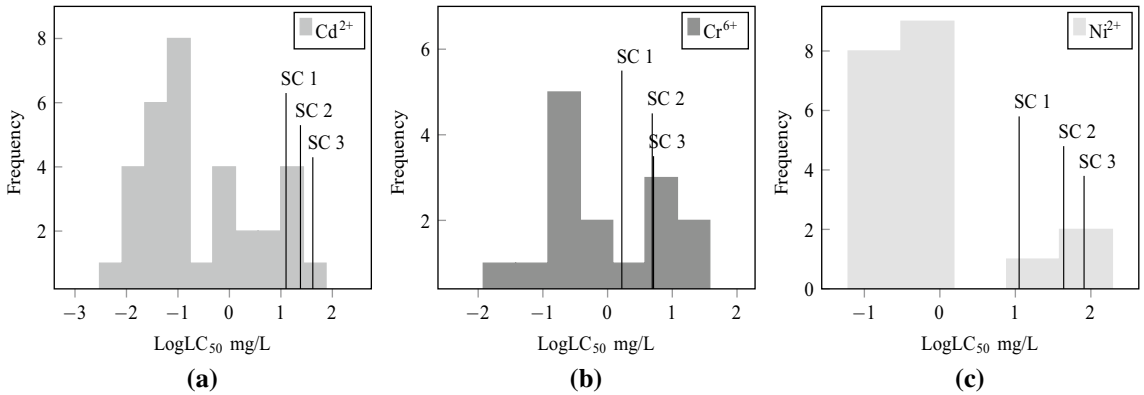

Fig. 2 Representation of the sensitivity range of *L. fortunei* for **a** Cd^{2+}, **b** Cr^{6+}, and **c** Ni^{2+}. Frequency distribution data was collated from the USEPA ECOTOX database and bibliography searches

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no confict of interest.

Ethical Approval The authors declare that all studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

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