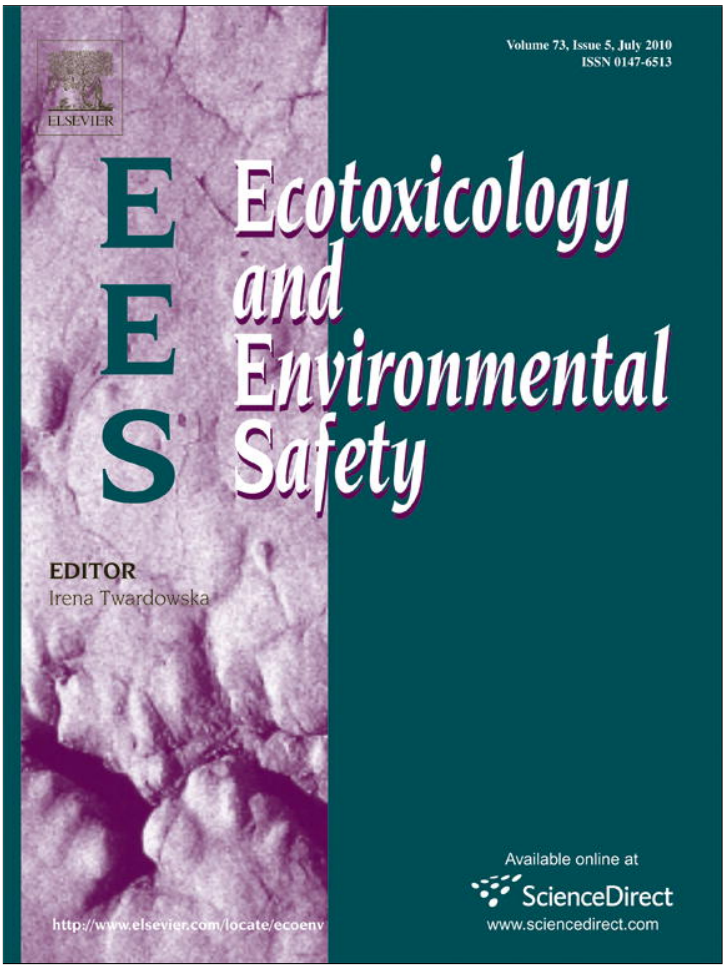

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Age-related differential sensitivity to cadmium in *Hyalella curvispina* (Amphipoda) and implications in ecotoxicity studies

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ABSTRACT

The standardization of toxicity tests requires the selection of the most suitable test species and their developmental stages, as well as the selection of the appropriate assay matrix and the evaluation of the sensitivity of the test species to the reference toxicants. International protocols recommend the use of the amphipod *Hyalella azteca* from the Northern Hemisphere for sediment toxicity tests. We selected the widely distributed amphipod *Hyalella curvispina*, representative of pleustonic, epiphitic and zoobenthic assemblages in austral South America, as test species to be used in regional studies. Our goals were to evaluate the sensitivity of three developmental stages of *H. curvispina* to cadmium as a reference toxicant and to select the most suitable age and exposure time for aquatic ecotoxicity assessment. The three ages were highly susceptible to cadmium, with sensitivities: neonates > adults > juveniles. Our results validate the use of the native *H. curvispina* as a standard species for ecotoxicological assessment studies.

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1. Introduction

There are many and varied consequences of chemical contamination on the biota, ranging from lethality to sublethal and chronic effects such as alterations in reproduction, growth and development (Cooney, 1995; Anguas-Cabrera et al., 2004; García-Medina et al., 2004).

Changes in population structure and dynamics may also become evident (Iannacone Oliver and Alvariño Flores (2003); García-García et al., 2004). The responses of organisms to contaminants depend on the toxicant concentration, the environment they live in and their vulnerability according to life-history traits. Hence, the effects of a given toxicant on a population, and ultimately on the whole community, are expected to be particularly harmful during the reproduction and breeding periods (Pascoe et al., 1989; Lagadic et al., 1994; Roex et al., 2000; Pietrock et al., 2008).

The bioassays are useful tools for evaluating the toxicity because test organisms show an integrated response to the adverse effects of chemical substances to which they are exposed, providing information complementary to physicochemical analyses.

Bioassay standardization requires not only a methodological protocol but also the selection of the most suitable test species and developmental stages, the assay matrix and the reference substance among others. Toxicity tests using reference substances are one way of monitoring the environment and of understanding the effects of xenobiotics on aquatic organisms (Jorge and Moreira 2005).

Most standardized bioassay protocols use Northern Hemisphere test species, but regional studies should use native species to obtain reliable results. The use of native species, which are better adapted to the environmental conditions of the region, can provide more useful information than exotic species do.

The amphipod *Hyalella azteca* (usually found in lentic habitats, ponds and occasionally in lotic habitats such as streams) is a common test organism for aquatic toxicity evaluation (Ingersoll, 1995; Borgmann and Munawar, 1989; González Ortíz and Martínez-Tabche, 2004; Ramírez-Romero et al., 2004; Wang et al., 2004, Bartlett et al., 2005, Borgmann et al., 2005).

The species used in this work, *Hyalella curvispina*, was first reported in Argentina by Shoemaker (1942). It is representative of zoobenthic and epiphitic communities in austral South America, from Punta Arenas, Chile (Cunningham, 1871) and Islas Malvinas, Argentina (Stock and Platvoet, 1991) in the south, to Cangallo and

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Río de Janeiro, Brazil (Oliveira, 1953) in the north. This species has also been cited as *H. knickerbuckeri* (1931), *H. simplex* (1943) and *H. pernix* (1985) (Grosso and Peralta, 1999). *H. curvispina* (being mainly found in ponds and streams) is suitable as test organism for regional studies of toxicity due to its wide distribution, easy breeding under laboratory conditions, and because it is part of the native fauna. In Argentina it has been increasingly used in ecotoxicity testing both in the field and under laboratory conditions (Rodrigues Capítulo, 1984; Di Marzio et al., 1999; Graça et al., 2002; Argemi et al., 2003; Galassi et al., 2004; Jergentz et al., 2005; Anguiano et al., 2005; Doyle, 2007, Giusto et al., 2008, Giusto and Ferrari, 2008). All these considerations underline the need for establishing a protocol using *H. curvispina*, and in this context, to determine its sensitivity to reference toxicants.

Reference substances are used to make comparisons among tests and among methods that use different organisms, and for interlaboratory calibration (Hunt and Anderson, 1989). Furthermore, they can reveal differences in the sensitivity of different batches of test organisms in periods of acclimation, disease, density or handling stress and may also be used to evaluate reproducibility and validate tests (Ong and Din, 2001).

The ideal reference substance must be toxic in low concentrations, quickly lethal, stable, non selective, detectable through known analytical techniques and further, able to furnish consistent laboratory results (Ong and Din, 2001). Cadmium is recommended as a reference toxicant in toxicity studies by international protocols (EPS 1/RM/33, 1997; DFO, 1992; ASTM, 1991, 1993, 1995; Borgmann et al., 1989; USEPA, 1991, 1993, 1994; NWRI, 1992). It is a non-essential element of unknown biological function, with a global ratio of approximately 7:1 between release from anthropogenic sources and release from natural sources (Abel, 1996).

Most of the cadmium discharged into freshwater ecosystems accumulates in sediments and can be resuspended into the water column under certain conditions (Wright and Welbourn, 1994). The bioavailable form of cadmium is the free ion Cd^{+2}. According to water quality criteria for the protection of aquatic life, allowed cadmium concentrations range between 0.2 and 4 μg Cd^{+2}/L, for hardness between 0 and 60 and up to 180 mg $CaCO_3$/L, respectively (Law Nº 24051, 1991; USEPA, 2002). Several aquatic organisms such as crustaceans, insects and fishes show high mortalities after exposure to 0.8–9.9 μg Cd^{+2}/L for 4–33 days, while concentrations exceeding 1 μg/L cause negative effects on the reproduction and growth of *H. azteca* (Borgmann et al., 1989). The response of test organisms to a reference toxicant must be quick and easy to distinguish, and these requirements are met when *H. azteca* is exposed to cadmium (McNulty et al., 1998). In Argentina, cadmium is used as a reference toxicant in ecotoxicological bioassays with different aquatic organisms (Ferrari et al., 1997; 2005; García et al., 1998; Demichelis et al., 2001).

Taking into account the need to standardize the use of *H. curvispina* as a test organism in ecotoxicological assays, the purposes of this study are to evaluate the sensitivity of *H. curvispina* to cadmium as a reference toxicant in function of age and exposure time, and to select the most suitable age for standardized ecotoxicological tests and their duration.

2. Materials and methods

The specimens of *H. curvispina* were collected in two relatively pristine freshwater bodies in the surroundings of La Plata City, located in Punta Lara: 34°4749.11S; 58°0144.92′ W and in Berazategui: 34°45664S; 58°10824W Buenos Aires, Argentina (Gómez and Toresani, 1999). In the laboratory they were acclimated in plastic trays with water and aquatic plants (*Egeria densa, Lemna gibba*) from the collection site. The water was gradually replaced with non-chlorinated tap water over a 48 h period and the plants were left to serve as substrate and shelter for the amphipods. Animal density varied between 60 and 100 individuals/L. The maintenance conditions were as follows: temperature 19–25 °C, natural photoperiod, dissolved oxygen 8.2–9.1 mg/l, conductivity 962–1054 μS/cm, 25 mg/l of commercial fish food 2–3 times a week.

The following life-cycle stages were isolated from the population maintained under laboratory conditions and acclimated for 7days before use: 1) neonates/breeding (7–20 d of age, between 0.88 and 2.3 mm length), obtained from isolated ovigerous females; 2) juveniles (between 3.2 and −5.0 mm length); and 3) adult males (between 5.1 and 10 mm length), see Fig. 1. The size ranges established here for the ages of *H. curvispina* are comparable to those for another native amphipod, *Hyalella pampeana*, with neonates/amphipodites between 1.28 and 2.87 mm, juveniles/prereproductive stages between 3.37 and 4.06 and adults >4.06 mm (Lopretto, 1983). The sizes of male adults of both regional species are in accordance with those reported in the taxonomic descriptions by González Balbontín Exequiel (2001).

All bioassays were conducted under static conditions, at controlled temperature of 21 ± 2 °C, and a photoperiod of 16 h light/8 h darkness. The dilution medium was moderately hard reconstituted water (MHW) with the following chemical composition (mg/L): 96 $NaHCO_3$; 60 Ca $SO_4 2H_2O$; 60 $MgSO_4$; 4 KCl; 7.4–7.8 pH; hardness 80–100 mg/L $CaCO_3$; alkalinity, 60–70 mg/L $CaCO_3$, (APHA, 1995). The exposure time of the assay was 14 days for juveniles and adults and 4 days for neonates.

The different cadmium concentrations assayed were prepared using dilution series of a stock solution of 1 mg Cd^{2+}/mL (as $CdCl_2$) in bidestilled water. Nominal concentrations were 2.5, 5, 8, 10, 15, 25, 50 and 100 μgCd^{2+}/L.

In each assay, all treatments were performed in quadruplicate; each beaker contained 5 animals, 150 ml of water and a piece of nylon as substrate. Table 1 shows a detailed description of the assays, in terms of age and total number of individuals, number of assays and concentrations used.

The following variables were measured in MHW at initial exposure times: dissolved oxygen and pH with sensors (oxymeter Hanna 600-ESD [± 0,1 mg/L], pH meter Hanna HI 8633 [± 0,01 mg/L], respectively); water hardness, estimated by the volumetric method, with the Aquamerck test kit (Merck, sensitivity of 1 mg/L $CaCO_3$); and the analytical Cd concentration was determined using inductively coupled plasma atomic emission spectrometry (ICPES, Perkin-Elmer Optima-3100 Icp-XL) with a method detection limit <1.5 μg Cd2+/L (APHA, 1995).

The assays were performed in translucid containers of polypropylene with a capacity of 250 ml, covered with transparent plastic lids throughout the experiment. Animals were fed 20 mg/l of ground fish food (Tetra Min) three times a week.

The number of live and dead animals in each replicate was recorded on days 2, 4, 7, 10 and 14 of exposure for juveniles and adults and on days 1, 2, 3 and 4 for neonates. The water in the replicates was oxygenated during animal count.

The lethal concentrations (LC_{50}) were calculated by the Probit method (Finney, 1972) with the TOXSTAT-EPA program (1996, software package version 3.5), using measured concentration values and the total number of repetitions for each age.

The significance of the differences among treatments and the survival kinetics as a function of time for each age was analyzed using a non-parametric test (Kruskal Wallis test) followed by pairwise comparisons (Sparks, 2000; Zar, 1999). Statistical analyses were conducted with the InfoStat software package (InfoStat, 2004). The significance level was set at $p < 0.05$.

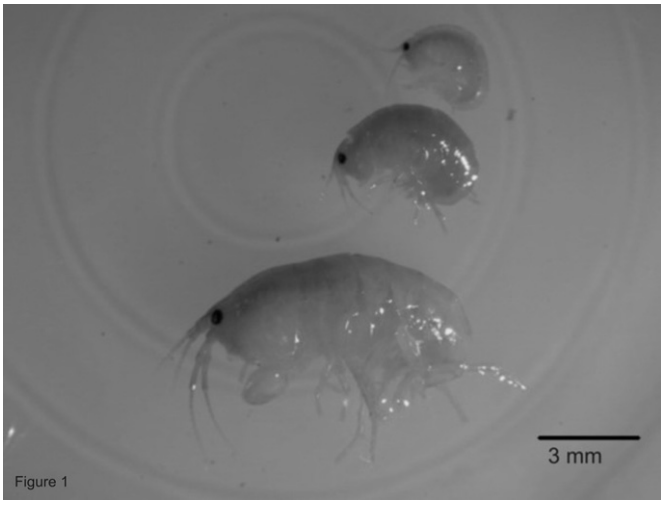

Fig. 1. Neonates, juveniles and male adults of *Hyalella curvispina*. Note differences in external morphology and size.

Table 1
Detail of the assays performed with neonates, juveniles and adults of *H. curvispina*: number of individuals (N), number of bioassays (no) and mean analytical and nominal (·) cadmium concentrations (μg Cd/L) assayed (per quatriplicate) in each bioassay.

age	N	no	Cadmium concentrations ($\mu g\ Cd^2 \pm /L$) nominals (·)and effectives (means values)										
			0	0.625	1.25	2.5	5	8	10	15	25	50	100
Neonates	260	2	0	0.625	1.25	2.9	4.8		10.2		22.7	45.1	88.5
Juveniles	280	2	0			2.9	4.8	8.1	10.2	14.8	22.7	45.1	
Adults	542	5	0			2.9	4.8		10.2		22.7	45.1	

Table 2
Mean lethal concentrations of cadmium (LC 50 μg $Cd^2 \pm /L$) in moderately hard water (MHW) obtained for neonates of *H. curvispina* at different exposure times. (N=260; Chi^2 critical value=12.592). "ns" denotes a not statistically significant difference ($P < 0.05$).

Exposure time (days)	LC50 (μg $Cd^2 \pm /L$)	Confidence limits	slope	intercept	Chi^2
1	8.02	2.71–30.42	1.071	4.031	18.314 ns
2	4.47	2.64–6.89	1.215	4.210	7.919
3	2.26	1.23–3.47	1.228	4.565	5.802
4	1.71	0.78–2.82	1.191	4.721	6.622

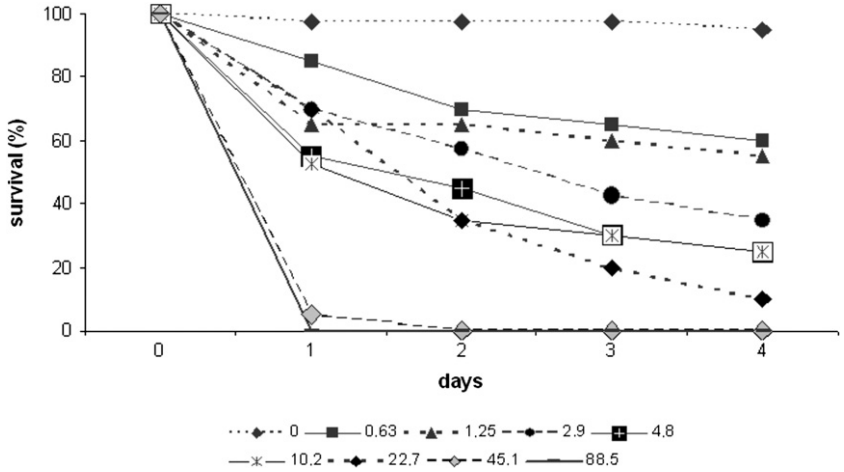

Fig. 2. Survival (%) of *H. curvispina* neonates exposed to different cadmium concentrations (μg $Cd^2 \pm /L$) in moderately hard water (MHW).

3. Results

The values of dissolved oxygen, pH and hardness in MHW at time zero ranged between: 8.2 and 9.1 mg/L, 7.6–7.8 and 94.3–101.7 mg $CaCO_3$/L, respectively. For all the assays, the analytical values of Cd in solution were between 86–96% of the nominal values for the concentration range between 2.5 and 100 μg Cd^{2+}/L. The effective concentrations of cadmium (mean±SD, n=5) in 8 of the concentrations assayed (2.5, 5, 8, 10, 15, 25, 50 and 100) were 2.9±0.2, 4.8±0.5, 8.1±0.8, 10.2 ± 0.7, 14.8 ± 0.6, 22.7 ± 2.2, 45.1 ± 5.6 and 88.5 ± 1.4 μg $Cd^2 \pm /L$, respectively.

The two lowest concentrations (0.625 and 1.25 μg $Cd^2 \pm /L$) were not included in the analysis because they were below the detection limits of the technique.

3.1. Assays with neonates

Table 2 shows the obtained values of LC_{50} (μg $Cd^2 \pm /L$) for the different times of exposure. Although the LC_{50} on day 1 was not significantly different (ns), there was an evident time-dependent effect with increasing toxicity.

Fig. 2 shows the survival as a function of time for the different concentrations of cadmium. The kinetics analysis indicated four homogeneous groups at the conclusion of the exposure period: (a) concentrations of 45.1 and 88.5 μg $Cd^2 \pm /L$ with survival rates less than 10%, (b) concentration of 22.7 μg $Cd^2 \pm /L$ with a survival rate of around 10%, (c) intermediate concentrations between 2.9–22.7 μg $Cd^2 \pm /L$ with survival rates between 10% and 55%, (d) intermediate concentrations between 0.63–1.25 μg $Cd^2 \pm /L$ (nominal values) with survival rates between 35% and 60%, and the control with 95% of survival on exposure day 4 (Table 3 Part A).

The comparison of survival between times of exposure (days 1, 2, 3 and 4) revealed a different response between the two highest concentrations and the remaining ones in the first 24 h of the assay (Table 3 Part B), and this result was consistent with the mean lethal concentrations (Table 2).

3.2. Assays with juveniles

Table 4 shows the values of LC_{50} (μg $Cd^2 \pm /L$) obtained for the different times of exposure. A time-dependent increase in toxicity can clearly be observed.

The survival curves as a function of time for each tested concentration are presented in Fig. 3. The kinetics analysis identified three homogeneous groups at the conclusion of the exposure period: (a) concentrations of 45.1, 22.7 and 14.8 μg $Cd^2 \pm /L$ with survival rates less than 25%, (b) concentrations

between 22.7 and 10.2 $Cd^2 \pm$ /L with survival rates between 10% and 40%, and c) low concentrations ($\leq$8.1 μg $Cd^2 \pm$ /L), controls included, with survival rates exceeding 80% (Table 5 Part A).

The analysis between times of exposure indicated the presence of three homogeneous groups, with no significant differences in survival between concentrations from exposure day 7 onward (Table 5, Part B).

3.3. Assays with adults

Table 6 shows the values of LC_{50} obtained for the different times of exposure. There was a gradual decrease in LC_{50} with time of exposure, with confidence intervals overlapping at all times of exposure.

Survival curves as a function of time for each tested concentration are shown in Fig. 4. The kinetics analysis identified three homogeneous groups at the conclusion of the exposure period, excluding the control: a) concentrations of 88.5, 45.1 and 22.7 μg $Cd^2 \pm$ /L with survival rates less than 10% on exposure day 14, (b) concentrations of 10.2 and 4.8 μg$Cd^2 \pm$ /L with survival rates around 50%, and (c) concentration of 2.9 μg $Cd^2 \pm$ /L with a survival rate of 70% (Table 7 Part A).

The comparison between times of exposure revealed no significant differences between concentrations from exposure day 4 onward (Table 7 Part B), which is concordant with the considerable overlap of the confidence intervals for the LC_{50} values between successive exposure times (Table 6).

3.4. Comparison of sensitivity to cadmium among developmental stages

The toxic effects of cadmium differed according to age, with neonates showing the highest sensitivity followed by adults and then juveniles. Fig. 5 shows the LC_{50} values obtained for the three developmental stages and their respective confidence intervals. On day 4 of exposure, the LC_{50} value for neonates was 5 times lower than that for adults and 20 times lower than that for

Table 3
Survival of *H. curvispina* neonates. Matrix of pairwise comparisons (Kruskal Wallis) between cadmium concentrations and exposure times. Statistically homogeneous groups ($p > 0.05$) are identified by the same letter. Part A, $Chi^2_{0.05;8}$=15.507, Part B, $Chi^2_{0.05;4}$=9.488.

Concentration H: 83.2 Part A					Exposure time H: 93.17 Part B				
Cd (μg/L)	Homogeneous groups				Time (days)	Homogeneous groups			
88.5	A				4	A			
45.1	A				3	A			
22.7	A	B			2	A	B		
10.2		B			1		B		
4.8		B			0				C
2.9		B	C						
1.25		B	C						
0.63			C						
0				D					

Table 5
Survival of *H. curvispina* juveniles. Matrix of pair wise comparisons (Kruskal-Wallis) between cadmium concentrations and exposure times. Statistically homogeneous groups ($p > 0.05$) are identified by the same letter. Part A, $Chi^2_{0.05;7}$=14.067, Part B, $Chi^2_{0.05;5}$=11.070.

Concentration H: 87.61 Part A				Exposure time H: 83.47 Part B			
Cd (μg/L)	Homogeneous groups			Time (days)	Homogeneous groups		
45.1	A			14	A		
22.7	A	B		10	A		
14.8	A	B		7	A		
10.2		B		4		B	
8.1			C	2			C
4.8			C	0			C
2.9			C				
0			C				

Table 4
Mean lethal concentrations of cadmium (LC 50 μg $Cd^2 \pm$ /L) in moderately hard water (MHW) obtained for juveniles of *H. curvispina* at different exposure times (N=280; Chi^2 critical value=11.070).

Exposure time (day)	LC50 (μg $Cd^2 \pm$ /L)	Confidence limits	Slope	Intercept	Chi^2
4	29.99	24.35–39.07	2.819	0.837	3.451
7	17.76	14.19–21.63	3.075	1.157	5.703
10	14.52	11.40–18.36	3.079	1.422	3.290
14	10.20	8.19–12.08	3.817	1.150	5.479

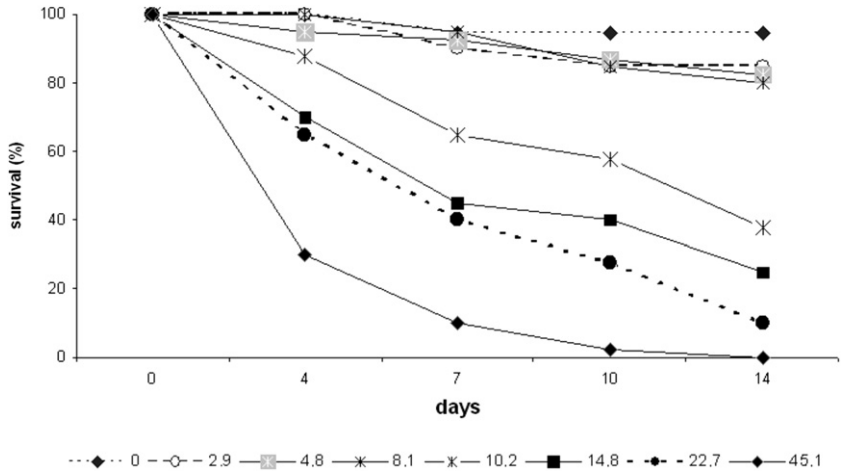

Fig. 3. Survival (%) of *H. curvispina* juveniles exposed to different cadmium concentrations (μg $Cd^2 \pm$ /L) in moderately hard water (MHW).

Table 6
Mean lethal concentrations of cadmium (LC 50, μg $Cd^2 \pm$ /L) in moderately hard water (MHW) obtained for adult males of *H. curvispina* at different exposure times (N=542; Chi^2 critical value=7.815).

Exposure time (days)	LC50 (μg $Cd^2 \pm$ /L)	Confidence limits	Slope	Intercept	Chi^2
4	8.68	7.18–10.67	2.033	3.092	6.062
7	7.22	5.96–8.76	2.205	3.106	6.028
10	6.41	5.28–7.73	2.205	3.220	4.981
14	6.12	4.87–7.53	2.105	3.344	6.928

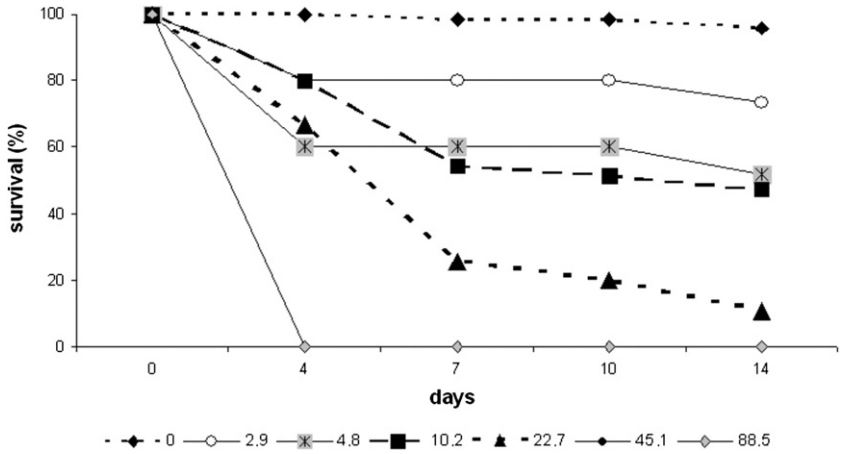

Fig. 4. Survival (%) of *H. curvispina* adult males exposed to different cadmium concentrations (μg $Cd^2 \pm$ /L) in moderately hard water (MHW).

Table 7
Survival of *H. curvispina* adult males. Matrix of pairwise comparisons (Kruskal-Wallis) between cadmium concentrations and exposure times. Statistically homogeneous groups ($p > 0.05$) are identified by the same letter. Part A, $Chi^2_{0.05;6}$=15.592, Part B, $Chi^2_{0.05;5}$=11.070.

Concentration H: 209 .67 Part A				Exposure time H: 98.77 Part B			
Cd (μg/L)	Homogeneous groups			Time (days)	Homogeneous groups		
88.5	A			14	A		
45.1	A			10	A		
22.7	A			7	A		
10.2		B		4	A	B	
4.8		B		2		B	
2.9			C	0			C
0			D				

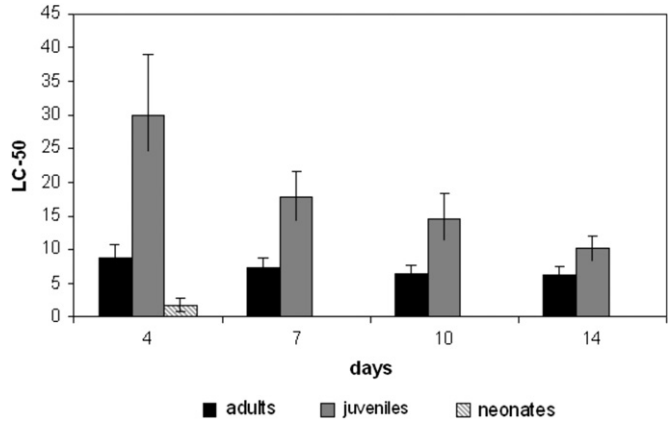

Fig. 5. Mean lethal concentrations of cadmium (CL50, μg $Cd^2 \pm$ /L) and confidence intervals at different exposure times for neonates, juveniles and adults of *H. curvispina*.

juveniles. At successive exposure times, the LC_{50s} for juveniles were approximately twice those for adults.

4. Discussion

The available data suggest that the sensitivity of invertebrate species to toxicants is developmental stage-dependent, with juvenile stages being more vulnerable than mature ones. On this basis, the use of toxicity data derived with these juveniles will eventually result in the most protective water quality criteria (Muysen and Janssen, 2007). Based on a database of comparative toxicity of chemicals substances to different life stages of aquatic invertebrates, Hutchinson et al.(1998) showed that in most cases, larvae were more sensitive than juveniles (66% of the substances) which, in turn, were more sensitive than adults (54% of the substances). However, particularly for cadmium, these authors found that juveniles were more sensitive than either larvae or adults. Our results indicate that the *H. curvispina* juveniles are less sensitive than the other two ages. Collyard et al. (1994) in evaluating the toxicity of five substances with different action modes (including cadmium) found 96 h LC_{50s} values typically varied by 50% or less among the various age classes of *H. azteca*.

The use of the LC_{50} value, which is the most common measure of toxicity, assumes that survival depends on the concentration of the toxic substance and that, in general, the LC_{50} value decreases with time of exposure. Despite its limitations as a toxicity indicator, it is considered to play an important role in ecological risk assessment (Kooijman, 1998).

The metal accumulation in aquatic invertebrates and the resulting acute and chronic effects have been evaluated in numerous species, particularly from the Northern Hemisphere (Amiard-Triquet et al., 1987; Borgmann et al., 1993a, 1993b, 2005; Toussaint, Shedd et al., 1995; Shaw and Chadwhich,1998; Gillis et al., 2004), and, to a lesser extent, in local species

(Rodrígues Capítulo, 1984; Achiorno et al., 2005; 2008; Vásquez et al., 2005; Gagneten, 2006).

Among the 18 taxonomic groups of invertebrates tested for the evaluation of toxic effects, cladocerans are the most commonly used, and amphipods are regarded as highly vulnerable (Janssen, 1998). A study concerning chronic exposure of *Daphnia magna* and *H. azteca* to heavy metals showed that amphipods proved to be the most sensitive in almost all cases (Borgmann and Munawar, 1989). The sensitivity of 21 genera of aquatic organisms to chronic exposure to cadmium was determined in order to establish guide levels for the protection of aquatic life; *Hyalella* ranked within the four most sensitive genera (Mebane, 2006).

The recommended age and/or size for *H. azteca* in toxicity tests is 2 ± 1 mm between 1 and 3 days old (USEPA, 1991), between 0 and 7 days old (DFO, 1989, 1992; , EPS 1/RM/33, 1997), between 7 and 14 days old (EPS 1/RM/33, 1997; USEPA, 1994) or between 2 and 3 mm (EPS 1/RM/33, 1997, ASTM, 1991). The current USEPA (2000) sediment toxicity method for *H. azteca* calls for starting long-term tests with a duration of 42 days and 7 or 8 days old animals.

In Argentina (Law Nº 24051), the maximum permissible level of cadmium for the protection of freshwater aquatic life in surface waters is 2 μg $Cd^2 \pm$/L for water hardness between 60 – 120 mg/L $CaCO_3$ and 0.2 μg $Cd^2 \pm$/L) for water hardness between 0–60 mg/L $CaCO_3$. Our results indicate that the neonates of *H. curvispina* (0–20 days old and 0.88–2.3 mm in length), are very sensitive to cadmium with LC_{50} values between 2.26 and 1.71 μg $Cd^2 \pm$/L on the 3th and 4th of exposure days; these concentrations are slightly below the maximum permissible level for aquatic life protection (Table 2). A LC50 of 1.6 μg $Cd^2 \pm$/L with confidence levels between 0.9 and 2.8 μg $Cd^2 \pm$/L were reported for *H. azteca* exposed for 6 weeks (Borgmann et al., 1993a, 1993b).

The juveniles showed to be much less sensitive to cadmium than neonates under the same experimental conditions and exposure times, with LC_{50s} values between 29.99 and 10.20 μg $Cd^2 \pm$/L for days 4 and 14, respectively (Table 4). These concentrations are detectable by the currently available techniques, which is necessary for reliable, comparable and reproducible results. The survival response tended to stabilize with the increasing of the exposure time and increasing cadmium concentration from exposure day 7 onward. According to our experience, the juveniles of *H. curvispina* can be handled and identified easily (Fig. 1) and show high survival rates under laboratory maintenance conditions.

Adults were remarkably more sensitive to cadmium than juveniles (with an adult: juvenile LC_{50} ratio of 4 on exposure day 4) and showed a stable response as a function of time and concentration from the first day of survival evaluation. Boxal, et al. (2001) pointed out that to interpret the age-dependent sensitivity to a particular toxicant it is required to determine its route of entrance and the physiology of the organism.

In ecotoxicity tests, the time of exposure depends on the objectives of the analysis. For acute toxicity tests with *H. azteca* neonates, the recommended time of exposure to a reference toxicant varies from 48 to 96 h (NWRI, 1992, USEPA, 1991, 1994). For spiked sediment tests with *H. azteca* neonates, recommendations vary from 7 days, (USEPA, 1991), 10 days (USEPA, 1994), 14 days (EPS, 1985; EC, 1994; EPS, 1997) to 28 days (EPS 1/RM/33 (1997)), from 10 to 30 days (ASTM, 1991) or 28 days (DFO, 1989, 1992, NWRI, 1992). In both cases, no recommendations have been made for the assessment of toxic effects during exposure; for example, mortality is only recorded at the conclusion of the exposure period. The chronic toxicity tests usually last from 4 to 8 weeks, and Borgmann and Munawar (1989) have proposed not to exceed this short period to avoid response variability. Nevertheless, it is important to assess the response of potential test species to the selected endpoints in function of time and in function of the concentration of the reference toxicant.

In brief, the neonates of this species are extremely sensitive to cadmium at concentrations from 22.7 to 0.6 μg $Cd^2 \pm$/L and exposure times up to 72 h. The juveniles are the least sensitive of the three ages, with a time and concentration-dependent response between 22.7–8.1 μg $Cd^2 \pm$/L and an optimum final exposure time of 7 days. Adult males have an intermediate sensitivity, and show a stable response between 22.7–2.5 μg $Cd^2 \pm$/L and 4–14 days of exposure. Notwithstanding these considerations, Cd can be regarded as highly toxic to *H. curvispina* at the three ages studied and could be used as reference toxicant in assays with this species. In addition, juveniles are proposed as the preferential age for ecotoxicological assessment at a regional level.

5. Conclusions

The life stages of *H. curvispina* had a different sensitivity to cadmium, in particular, neonates, which showed a LC50 96 h value similar to the guide level for the protection of aquatic life.

H. curvispina resulted an adequate test species and the Cd a suitable reference substance for assessment of ecotoxicity in regional water bodies.

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