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Evaluation of the South American gastropod *Heleobia parchappii* as test organism in cadmium toxicity bioassays

Cecilia L. Achiorno✉; Graciela Minardi; Marcela I. Schneider & Marilina Fogel

Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT La Plata-CONICET-UNLP. La Plata, Buenos Aires, Argentina.

Abstract. Gastropods are abundant key components in freshwater ecosystems, many of which are affected by anthropogenic activities. The genus *Heleobia* is widely distributed across Argentina, and the native *Heleobia parchappii* is very common in the Pampean Region. The identification of native candidate species for toxicity testing is gaining increasing interest. The objectives of the present study were to evaluate 1) the susceptibility of *H. parchappii* to a reference toxicant (Cl_2Cd), and 2) its suitability for toxicity testing by estimating LC_{50}. Also, we discussed the applicability of the proposed protocol. Snail sensitivity was assessed by exposure to concentrations between 0.5 and 12 mg Cd/L under acute static conditions during 72 h (exposure period). Then, snails were transferred to the control medium for 24 h (post-exposure period) and checked for recovery and those not responding to stimulation were considered to be dead. The endpoints were snail immobilization (recorded every 24 h during the exposure period) and mortality at the end of the post-exposure period. Snail sensitivity was analyzed with a generalized linear mixed model (GLMM) and LC_{50} (lethal concentration 50%) was calculated using probit analysis. Results indicated that the number of immobilized snails increased with increasing concentration. The toxic effect of Cd on snails persisted 24 h after exposure. The LC_{50} was 2.145 (1.675-2.641) mg Cd/L. The sensitivity of *H. parchappii* was similar to that reported for other freshwater standard species, supporting its suitability as test organism. The protocol was appropriate for use in toxicity testing as it is simple, inexpensive and reproducible.

[Keywords: mollusk, native species, bioassay, freshwater, acute toxicity, cadmium chloride]

Resumen. Evaluación del gasterópodo sudamericano *Heleobia parchappii* como organismo diagnóstico en bioensayos de toxicidad con cadmio. Los gasterópodos son componentes clave de los ecosistemas acuáticos, y muchos de ellos están afectados por la actividad antrópica. El género *Heleobia* está ampliamente distribuido en la Argentina, y la especie nativa *Heleobia parchappii* es muy común en la Región Pampeana. Actualmente, existe un interés creciente en identificar especies nativas para usarlas en evaluaciones de toxicidad. Los objetivos de este estudio fueron evaluar 1) la susceptibilidad de *H. parchappii* a un tóxico de referencia (Cl_2Cd), y 2) su idoneidad para ser utilizada en evaluaciones de toxicidad estimando la concentración letal 50% (CL_{50}). Además, discutimos la aplicabilidad del protocolo propuesto. La sensibilidad de los caracoles fue evaluada al exponerlos a concentraciones entre 0.5 y 12 mg Cd/L en un ensayo agudo bajo condiciones estáticas durante 72 h (tiempo de exposición). Los caracoles se transfirieron a un medio control durante 24 h (período de post-exposición) y se controló su estado para determinar si se recuperaban; aquellos que no respondían a los estímulos se consideraron muertos. Los puntos finales evaluados fueron la inmovilización de los caracoles (registrada cada 24 h durante el período de exposición) y la mortalidad al finalizar el período de post-exposición. La sensibilidad de los caracoles se analizó utilizando un modelo lineal generalizado y se calculó la CL_{50} usando el análisis probit. Los resultados indican que el número de caracoles inmovilizados aumentó con el incremento de la concentración. El efecto tóxico del Cd sobre los caracoles persistió 24 h tras la exposición. La CL_{50} tuvo un valor de 2.145 (1.675-2.641) mg Cd/L. La sensibilidad de *H. parchappii* fue similar a la informada para otros especies estándar dulceacuícolas, lo que respalda su idoneidad como organismo de evaluación. El protocolo fue apropiado para su uso en evaluaciones de toxicidad ya que es simple, económico y reproducible.

[Palabras clave: moluscos, especies nativas, bioensayos, dulceacuícola, toxicidad aguda, cloruro de cadmio]

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✉ achiorno@cepave.edu.ar

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Introducción

Freshwater gastropods are essential components of communities because they play an important role in trophic relationships by providing nutrients to both land and water ecosystems and may act as intermediate hosts for parasites (Martin and Cabrera 2018). The freshwater malacofauna of the Pampean Region in Buenos Aires province, Argentina, is represented by a small number of families and genera (Tietze 2011). Among these, the genus *Heleobia* (d'Orbigny 1835) belonging to the Cochliopidae, is widely distributed throughout the country. The most common species in this region is the benthic snail *Heleobia parchappii,* which lives associated with submerged vegetation, boulders and mud (Albariño et al. 2007; Tietze and De Francesco 2010) in all freshwater habitats, except under anoxic conditions. It shows a small body size, a relatively short life cycle and a high reproduction rate. In addition, it is widely distributed, and is abundant and ecologically relevant in natural habitats (Cazzaniga 2011a,b). In this regard, this snail plays an important role in the food web, as it is a detritivore/grazer, a prey for several fishes such as the commercially important *Odontesthes bonariensis* (Cazzaniga 2011b; Drago 2004; González Sagrario et al. 2018) and an intermediate host of different digenean species (Merlo et al. 2019).

The freshwater bodies of the Pampean Region are increasingly affected by anthropogenic activities related to cattle farming, agriculture, urbanization and industry (Giusto et al. 2014), leading to the presence of polluting substances in surface waters (Bollani et al. 2018) that are potentially hazardous for aquatic organisms. In particular, cadmium (Cd) is one of the main heavy metal pollutants due to its use in industrial activities and presence in phosphate fertilizers (Dhara et al. 2017). In freshwater environments, Cd is very_mobile, has a relatively long residence time and is highly toxic for aquatic organisms (Achiorno et al. 2010; Giusto et al. 2012). It is abundant in freshwater bodies of Argentina, reaching concentrations as high as 0.7-1.7 mg Cd/L (Giusto et al. 2012).

Toxicity bioassays are useful tools to evaluate the potential effects of contaminants on aquatic organisms (de Freitas Tallarico 2016). These involve exposing test organisms (bioindicators) to environmental samples or to a range of concentrations of at least one compound, using standardized protocols. The potential toxicological endpoints (biologically measurable responses) are evaluated at the organism level over a defined period of time (Oliveira-Filho et al. 2017; Schipper et al. 2010). According to Gopalakrishnan et al. (2008), bioindicators must fulfill a series of characteristics, such as being sensitive to potential contaminants, abundant in the field, easy to collect, handle and maintain in the laboratory, and showing a wide geographic distribution, among others. The suitability of candidate organisms for toxicity testing is determined by comparing endpoints to a range of concentrations of reference toxicants. These are useful for intra- and inter-laboratory calibration, and provide information for the interpretation of results (Achiorno et al. 2010; Gopalakrishnan et al. 2008). Among others, United States Environmental Protection Agency (USEPA) (1993) recommends cadmium (in the form of cadmium chloride, Cl_2Cd) as reference toxicant.

Bioindicators must be ecologically significant species, native to the region of interest (Charry et al. 2019; de Freitas Tallarico 2015) and sensitive to changes in their environment (Gómez et al. 2008). They have been selected from several taxa such as amphipods, oligochaetes, chironomids and ephemeropterans (Marchese et al. 2020) and from the main ecological or trophic positions (Piva et al. 2011). Mollusks, which are the second largest animal group, have gained increasing attention as test organisms for ecotoxicological studies since the second half of the 1990's (Oliveira-Filho et al. 2017). Among these, snails have shown to be efficient bioindicators of environmental pollution (see review of Baroudi et al. 2020). Moreover, freshwater snails have been used as test organisms in toxicity bioassays of cadmium (Coeurdassier et al. 2004; Dhara et al. 2017, among others). However, studies involving native South American species are scarce (Ansaldo et al. 2009; Salice and Miller 2003; Villar et al. 2015, among others).

The limited information on the interaction between pollutants and species native to freshwater bodies from the Pampean Region in Argentina highlights the importance of assessing the sensitivity of test organisms to different toxicants. In this regard, *H. parchappii* has many biological and ecological features making it a potential test organism. Moreover, the evaluation of its suitability as bioindicator requires the development of a standardized

and reproducible protocol, thereby allowing comparison of results across studies (Achiorno et al. 2010; Silva et al. 2007).

Based on the considerations mentioned above, the objectives of the present study were to evaluate 1) the sensitivity of *H. parchappii* to a reference toxicant (Cl_2Cd), and 2) the suitability of this species for toxicity testing through the estimation of LC_{50} values and further comparison with other bioindicator species. In addition, we discussed the applicability of the protocol proposed here for testing the potential effects of toxicants on snails and their persistence at 24 h following exposure.

Materials and Methods

Test organisms

Specimens of *H. parchapii* were collected from different sampling sites along the Martin stream (-34.860987, -58.065590) in the Pampean Region, Buenos Aires province, Argentina. Snails were randomly collected by hand and placed in plastic bags containing stream water. In the laboratory, they were transferred to 2-L glass containers filled with dechlorinated tap water at an ambient temperature of 23±1 °C and fed with flaked fish food. Snails of similar size that showed active movement were selected for the experiment. They were starved and kept in 1-L glass containers under the same conditions as in the bioassays for at least 24 h before the experiment.

Test chemicals

Cadmium ($Cl_2Cd.H_2O$) (PM=201.32, Biopack) was used as test substance. The stock solution (1000 mg Cd/L) was prepared with distilled water just before use. The test solutions were prepared by dilution of the stock solution. Dechlorinated tap water was used for dilution and as control medium. Preliminary tests were carried out to determine the final concentrations to be used in the bioassays. These were 0 (control), 0.5, 1, 2, 4, 8 and 12 mg Cd/L and are referred to as the nominal values. The analytical concentrations were determined by atomic absorption spectrophotometry at the Centro de Investigación y Desarrollo en Tecnología de Pinturas (CIDEPINT), and varied between 0.02 and 0.28 mg Cd/L from the nominal concentrations.

Bioassays

Figure 1 shows a schematic diagram of the experimental protocol for assessing cadmium toxicity to snails of *H. parchappii* in each set of bioassays. The experimental protocol consisted of two consecutive periods as follows: 1)

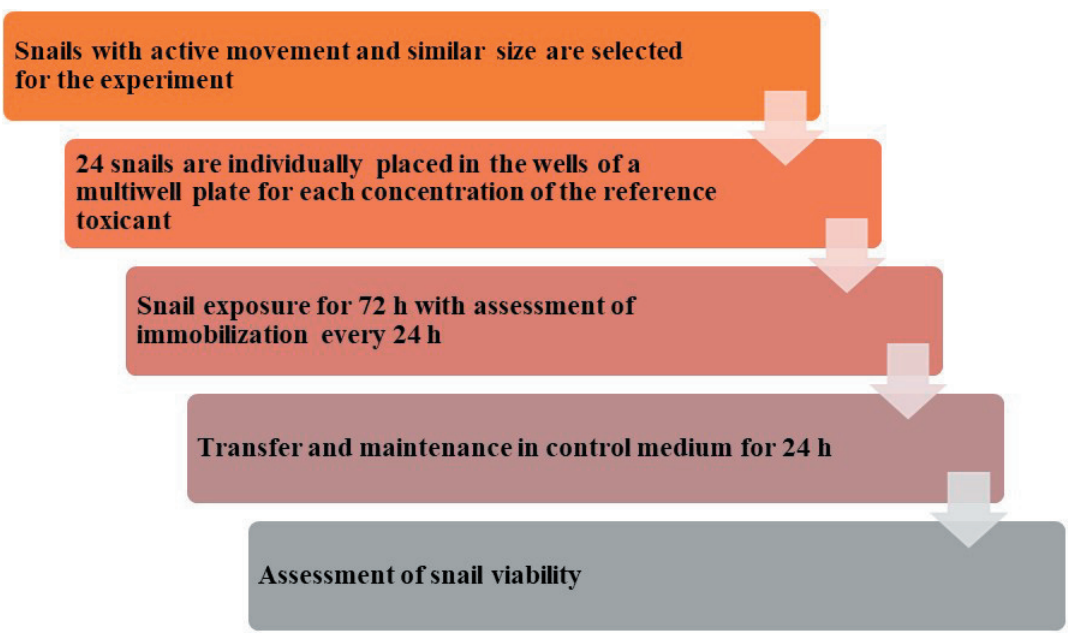

Figure 1. Flow diagram of the experimental protocol used in the present study for evaluating cadmium toxicity to snails of *Heleobia parchappi*.

Figura 1. Diagrama de flujo del protocolo experimental utilizado en este estudio para evaluar la toxicidad del cadmio en caracoles de *Heleobia parchappii*.

exposure: the specimens of *H. parchapii* were individually placed in a polystyrene 24-well plate with 2 mL per well of assay medium (Cd solution or control media) for 72 h; 2) post-exposure: after exposure, the media of all wells were totally replaced by fresh control medium, where snails were maintained for an additional 24 h. Each concentration including controls was tested in quadruplicate in a bioassay room at 25±1 °C, 70±5% relative humidity and 16:8 light: dark photoperiod, and under acute static conditions.

In the multiwell plate, each snail was placed with its shell pointing backward. Any change in body position was checked every 24 h and the number of immobile snails was recorded for statistical analysis. Snails that had moved were returned to the original position by gently holding the shell with dissection forceps, while the others were regarded as immobilized. Immobilization was the endpoint for sensitivity to the Cd solution. At the end of the experiment, immobile snails were subjected to mechanical stimuli and those failing to respond were considered dead. Mortality was the endpoint used for calculating LC_{50} (Lethal Concentration 50%).

Data analysis

In the bioassays, the endpoint variables were immobilization at 24, 48 and 72 h of exposure, and mortality at the end of the post-exposure period. We used a generalized linear mixed model (GLMM) for binomial responses with random effects. This model measures variations in the logit of the probability of immobilization, in function of Cd concentration and exposure time. The model provides estimates of these parameters, which can be positive or negative. If a parameter estimate is positive, the explanatory variable increases the probability of immobilization and if it is negative, this variable decreases its probability of occurrence. In addition, the model provides the 95% confidence intervals (CI) for the parameter estimates, which estimate the precision level of estimates. The random effects are given by *H. parchappi*i snails, since each individual was evaluated at four different times (24 h, 48 h, and 72 h during the exposure period and at the end of the post-exposure period) (Fox 2016; Gelman and Hill 2006). The GLMM was carried out using the glmer function from the lme4 package. The model was validated using a graphical residual analysis, and the marginal and conditional coefficients of determination were calculated to determine the amount of variance explained by the fixed and by the fixed and random effects together.

Considering that the response variable is snail immobilization, for each exposure time $Y_i=1$ if the i^{th} individual is immobile and $Y_i=0$ if the ith individual had moved actively; so, $Y_i \sim B(1,\pi)$ with $E(Yi)=\pi$ where π is the probability that one individual of *H. parchappii* is immobile. Then, $Logit(\pi)=\log[\pi/(1-\pi)]$ was modeled with Dosis and Exposure Time as fixed effects, and the effect given by the snail as random effect.

All analyses and visualizations were performed in the R statistical environment (R Core Team 2021). The LC_{50} value and their 95% confidence intervals (CI) were calculated using probit analysis (Chi 2008). Bioassays were considered valid when mortality in the control group was ≤10%.

Results

Our results showed that snail immobilization increased with increasing cadmium concentration for each exposure time and with increasing exposure time for each concentration (Figure 2). The model satisfactorily fit the data, as indicated by the graphical residual analysis and the proportion of variance explained by fixed and random effects (84% and 56.9%, respectively). Model estimation of the parameters evaluated is presented in Figure 3. The concentration estimate was positive, implying that an increase in concentration increased the probability of immobilization. Moreover, this estimate was found to be precise, as indicated by the length of the confidence interval. On the other hand, the estimations associated with exposure time were negative. The most negative estimate was observed at 24-h exposure, reflecting the lowest probability of immobilization. The estimates increased at 48 and 72 h, and the latter was almost identical to that obtained at the end of the post-exposure period.

The model also provided an estimate of the difference in the probability of immobilization between exposure times (Figure 4). These estimations were positive except for the contrast between 72-h exposure and post-exposure, indicating that they had a similar probability of immobilization. The highest differences were found between the 24-h exposure and the rest of the exposure times.

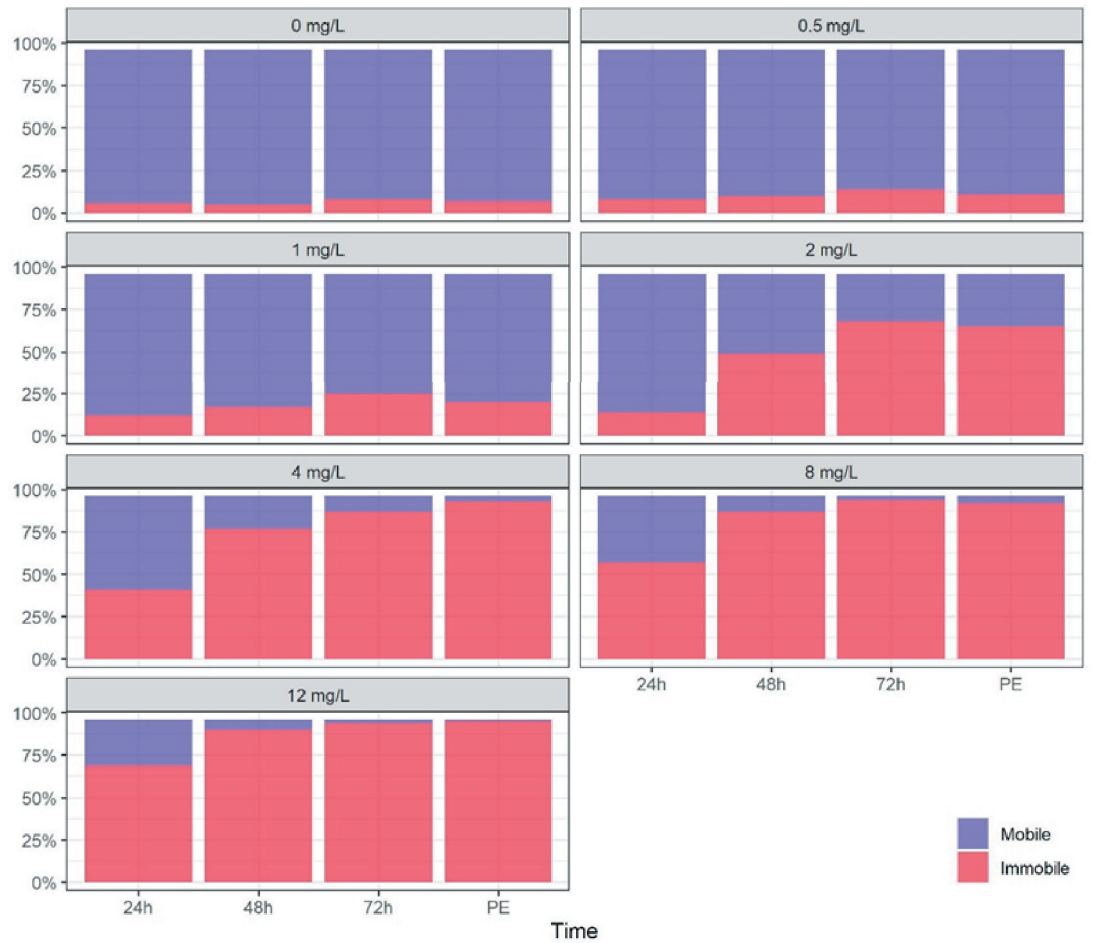

Figure 2. Percentage (Y-axis) of mobilized (violet) and immobilized (red) individuals of *Heleobia parchappii* exposed to different cadmium chloride concentrations (X-axis) for different exposure times (24, 48 and 72 h) and after post-exposure period (PE). N=96 for each concentration/time combination.

Figura 2. Porcentaje (eje Y) de individuos móviles (violeta) e inmóviles (rojos) de *Heleobia parchappii* expuestos a diferentes concentraciones de cloruro de cadmio (eje X) para el período de exposición (24, 48 y 72 h) y para el período de post-exposición (PE). N=96 para cada combinación de concentración/tiempo.

Model predictions of snail immobilization with respect to exposure and post-exposure times are shown in Figure 5. Experimental and predicted data were similar (Figure 2 and 5). The probability of immobilization increased with exposure time and especially with concentration. In average, the probability of immobilization increased up to 19% for each mg Cd/L increase in the concentrations of 2, 4 and 8 mg Cd/L. For 24, 48 and 72 h of exposure, the probabilities of immobilization predicted by the model at a concentration of 2 mg Cd/L were 2%, 20% and 39%, respectively; at 4 mg Cd/L, these probabilities were 10%, 54% and 75%, respectively, and at 8 mg Cd/L, were 70%, 96% and 98%, respectively. At all concentrations, the probability of immobilization increased with increasing time of exposure. Moreover, the differences between 24 and 48 h, 24 and 72 h and 48 and 72 h at 2 mg Cd/L were different from those obtained at 4 and 8 mg Cd/L. This may indicate that the effect of the exposure time on snails differed according to the concentration evaluated. In contrast, the probability of immobilization was similar at 72 h (final exposure time) and at the end of the post-exposure period at all concentrations. Moreover, the ranges of their confidence intervals overlap, implying that the post-exposure period did not have any effect on the probability of immobilization (Figure 5).

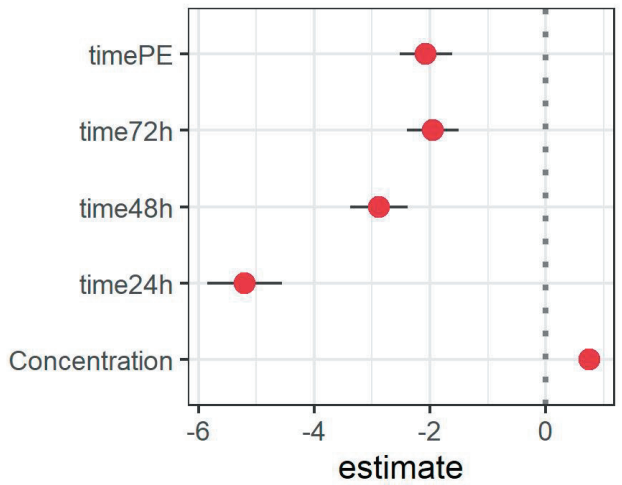

Figure 3. Estimates of model parameters. The X-axis corresponds to the model estimates and the Y-axis to the model parameters (concentration, exposure time at 24, 48 and 72 h, and post-exposure period). The 95% confidence intervals of each parameter are shown.

Figura 3. Parámetros del modelo estimados. El eje X corresponde a los estimados del modelo y el eje Y a los parámetros del modelo (concentración, tiempo de exposición a 24, 48 y 72 h, y el período de post-exposición). Se muestran los intervalos de confianza del 95% para cada parámetro.

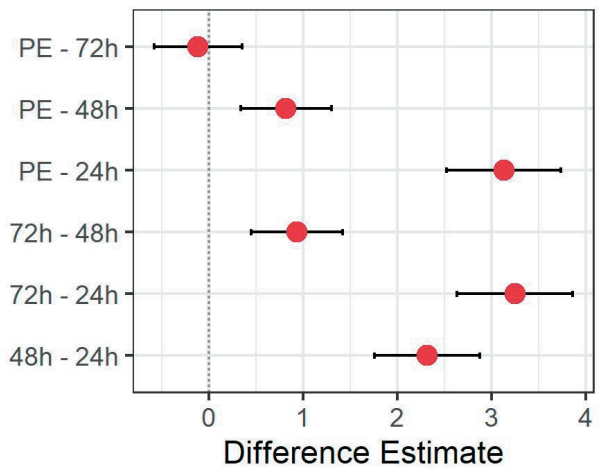

Figure 4. Estimated difference for the time effect. The X-axis corresponds to the model estimation of the difference between two probabilities of immobilization and the Y-axis to the contrast between exposure times. The 95% confidence intervals of each contrast are shown.

Figura 4. Diferencia estimada para el efecto tiempo. El eje X corresponde a el modelo de estimación de las diferencias entre las dos probabilidades de inmovilización y el eje Y a el contraste entre los tiempos de exposición. Se muestran los intervalos de confianza del 95% para cada contraste.

Finally, probit analysis of mortality data yielded LC_{50} value of 2.145 mg Cd/L, respectively, and Lower and the Upper 95% fiducial limits for LC_{50} of 1.675 and 2.641 mg Cd/L, respectively. These values are consistent with the results shown in Figure 5, which shows that 50% of the probability of immobilization at 72 h of exposure is within the concentration range of the LC_{50} estimated by probit analysis.

Discussion

In the present study we developed a simple, inexpensive and reproducible protocol to evaluate the effect of a reference toxicant on an aquatic snail species native to a wide geographic region, using immobilization and mortality as endpoints. Our results showed that 50% or more of the *H. parchappii* individuals would be immobilized after 72 h of exposure at concentrations of 2 and 4 mg Cd/L. In this species, concentration had a greater effect than exposure time on immobilization and the effect of cadmium was found to increase with exposure time at all concentrations assayed. Thus, the smallest effect of cadmium on snails was observed for the lowest concentrations at 24-exposure. The comparison between the results obtained at the end of the exposure period and the post-exposure period indicated that snails exposed to cadmium for 72 h could not recover from the toxic effect, thereby allowing us to consider them dead.

In assessing the suitability of *H. parchapii* as bioindicator for ecotoxicological evaluation,

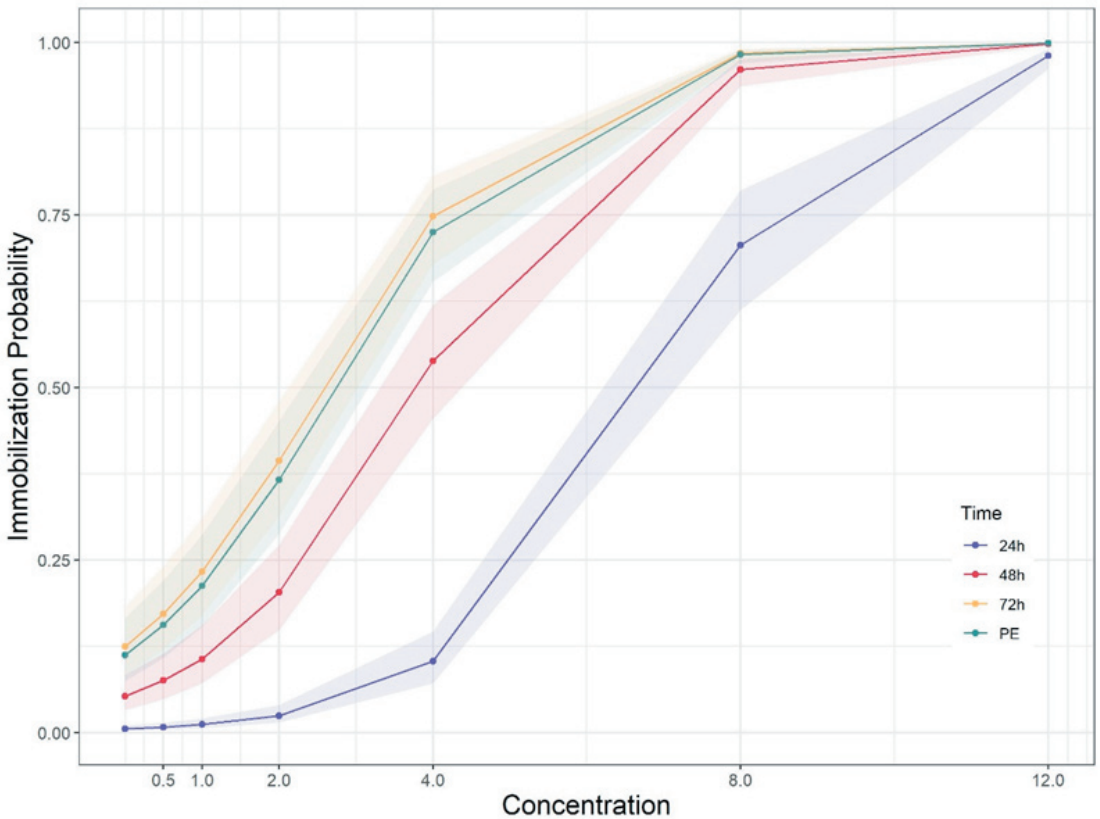

Figure 5. Predictions of the model to determine the probability of immobilization of *Heleobia parchappii* individuals (Y-axis) exposed to different cadmium concentrations (X-axis) for 24 h (violet), 48 h (red), 72 h (yellow) and during the post-exposure period, PE (green). The shaded areas around each line represent 95% confidence intervals.

Figura 5. Predicciones del modelo para determinar la probabilidad de inmovilización de individuos de *Heleobia parchappii* (eje Y) expuestos a diferentes concentraciones de cadmio (eje X) durante 24 h (violeta), 48 h (rojo), 72 h (amarillo) y durante el período post-exposición, PE (verde). Las áreas sombreadas alrededor de cada línea representan intervalos de confianza del 95%.

Table 1. Values of 96-h LC_{50} from mollusks exposed to Cl_2Cd under static acute test conditions. Source: database of US EPA ECOTOX (2022). *Heleobia parchapii* is included for comparison purposes.

Tabla 1. Valores de CL_{50} 96 h para moluscos expuestos a Cl_2Cd bajo condiciones estáticas en ensayos agudos. Fuente: base de datos de US EPA ECOTOX (2022). *Heleobia parchapii* se incluye para comparación.

Species	96-h LC_{50} (mg/L)	Distribution	References
Anodonta imbecilis (Mussel)	0.009	Cosmopolitan	Keller and Zam 1991
Anodonta imbecilis (Mussel)	0.107	Cosmopolitan	Keller and Zam 1991
Viviparus bengalensis (Gastropod)	1.55	Palearctic	Gadkari and Marathe 1983
Lymnaea stagnalis (Gastropod)	1.585	Holarctic	Coeurdassier et al. 2004
Heleobia parchapii	2.145 (1.675-2.641)	South America	This study
Brotia hainanensis (Gastropod)	15.21	South-East Asia	Lam 1996
Brotia hainanensis (Gastropod)	35.94	South-East Asia	Lam 1996

we compared its 72-h LC_{50} value (2.145 mg Cd/L, present study) with those of standard freshwater invertebrates exposed to cadmium chloride in freshwater static acute assays using the database from the ECOTOXicology Knowledgebase, US Environmental Protection Agency (epa.gov/ecotox). The LC_{50} value of *H. parchapii* was three orders of magnitude higher than that of the crustacean *Moina irrasa* (0.00748 mg Cd/L; lowest value of the range) (Zou and Bu 1994). It is important to highlight that the LC_{50} value of *H. parchapii* is comparable to that of the nematode *Caenorhabditis elegans* (1.5 mg Cd/L) (Williams and Dusenbery 1990). In turn, the sensitivity of *H. parchapii* was much higher than that of the nematode *Acrobeloides buetschlii* (96.68 mg Cd/L) (Kammenga et al. 1994). Then, we performed a search in the US EPA ECOTOX database (*loc. cit*) for 72-h and 96-h LC_{50} values from static acute tests using freshwater mollusks exposed to cadmium chloride. However, the search only yielded a limited amount of data that were easily available and could be used to compare with our results. The only 72-h LC_{50} value found corresponded to the great pond snail *Lymnaea stagnalis* (1.3 mg Cd/L) (Chouikhi 1979), which was similar to that of *H. parchappii*. The 96-h LC_{50} values of standard test mollusk species are listed in Table 1, which shows that they are of the same order of magnitude as *H. parchappii,* except for the mussel *Anodonta imbecilis.* It is worthwhile to note that, so far, the US EPA ECOTOX database (*loc. cit.*) includes no data from South American mollusks, highlighting the importance of the information provided in the present study.

In conclusion, *H. parchappii* appears to be suitable for toxicity testing because it showed a sensitivity similar to, and even higher than, those reported for standard test species, as well as a rapid response to the reference toxicant. This, along with its biological and ecological characteristics, makes it a promising candidate for toxicological bioassays. The next step is to determine if *H. parchappii* fulfils criteria related to laboratory rearing such as being amenable to routine maintenance and easily cultured in large numbers (Gopalakrishnan et al. 2008). Finally, the protocol presented here demonstrated to be appropriate for use in toxicity testing as it is easy to perform, provides reproducible results and allows comparison between laboratories.

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