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Research article

Heavy metals effects on life traits of juveniles of *Procambarus clarkii*

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Abstract: An incubation experiment of juvenile crayfish (*Procambarus clarkii*) following a three-level treatment design approach was performed to assess the effect of different heavy metal concentrations on their life history traits (lifespan, growth, moult and feeding activity). The aims were to: (1) address the response of the life traits; (2) check for the correlation between heavy metal concentrations in crayfish whole bodies with the ones of the experimental solutions; (3) analyse the variation of crayfish carbon and nitrogen stable isotopes signatures grown under these treatments. Treatments were: control or absence of pollutants (C); low level contamination (L) similar to those found in the water of the Guadiamar River (SW, Spain) one year after the Aznalcóllar mine accident, and high level contamination (H) maximum concentrations of metals measured in the water of the river after the spill. The study concludes that the H treatment produced lethal effects on juveniles of crayfish, whereas those undergoing the L treatment showed less marked effects. Crayfish's juveniles grown in L treatment seemed able to regulate and manage this range of pollution while maintaining their biological traits. Juvenile's capacity to bioaccumulate toxic substances also changes with the nature of the particular metals. The reduction in lifespan was mainly influenced by Cu, Zn and As. ^{13}C of C and juveniles from L treatment had similar values but different from those individuals of H treatment, reflecting the isotopic signature of the food source used (liver), and were also influenced by the concentration of Cu and As.

Keywords: crayfish; feeding activity; Guadiamar River; metal accumulation; moulting; stable isotope analysis

1. Introduction

River systems are continuously subjected to anthropogenic disturbances in most parts of the world. Typical examples of these alterations are pollution and waste disposal, bank alterations, riparian simplification, simplification of the river network, water abstraction and the introduction of alien species. These combined factors contribute to a loss of river function and associated

hydrological services [1]. The Aznalcólllar mine accident held in 1998 caused the discharge of 6 Hm^3 of sludge and acidic waters, containing high concentrations of heavy metals in solution, into the Agrio stream, a tributary of the Guadiamar River (SW Spain) [2]. The toxic mine spill contaminated 62 km of the beds and banks of the Guadiamar River affecting an area of approximately 4400 ha. The ecological functions of the river were consequently heavily affected [3,4]. Following this catastrophic event, urgent action was undertaken to clean the soil and, a research-based management project was launched by the regional authorities aimed at the restoration of the Guadiamar Basin as a whole (*The Green Corridor Protected Area*). This project actively monitored the contamination of water, sediments and different bioindicators from 1999 until 2008. One of the sentinel species used to analyse the effect of the spill was the red swamp crayfish, *Procambarus clarkii*. This exotic species was introduced into the Lower Guadalquivir Basin (SW Spain) for commercial purposes, from Louisiana (US), in 1974 [5] and then later became widespread throughout Spain and the other European countries [6,7].

P. clarkii can ingest and eventually store in its tissues large amounts of heavy metals [8] as it is able to adjust the physiological mechanisms involved in metal metabolism in order to survive in polluted environments [9]. Therefore, *P. clarkii* has been used bioindicator in several studies carried out in the Guadiamar River, so that the relocation of heavy metals from non-biological (mainly sediments) to biological compartments (food webs) in the system could be efficiently traced [10–16]. It also occupies a keystone and central position in the aquatic food webs and serves as a vector of contaminants to higher trophic positions [17]. Further, the estimation of the amount of pollutants that the crayfish mobilises and stores as biomass is of great relevance because *P. clarkii* has recently become a main prey for a number of protected vertebrate species, some of them endangered, in the Lower Guadalquivir Basin [18]. This means that pollution effects can potentially travel far from the source area through trophic relations linking crayfish with large range organisms (e.g. herons, spoonbills, storks, otters, etc.).

Although *P. clarkii* is highly resistant to toxic substances it is not fully protected against the harmful effects of heavy metal pollution and can suffer serious drawbacks in growth and reproductive functions when exposed to toxic waste [19]. Whereas lethal effects imply that crayfish biomass is lost as a resource for predators and that pollutants are sequestered back in the sediments, sub-lethal effects mean that contaminants accumulate in the organisms and are readily passed onto higher levels in the food web. Unfortunately, it is difficult to predict the magnitude and kind of the response that different contamination exposure scenarios might induce in crayfish populations because of the complex interactions that occur between heavy metals and the idiosyncratic composition of actual toxic spills [20].

In recent years, the biological interpretation of changes in the relative abundance of naturally occurring stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) has provided an alternative method to characterize the food web structure and dynamics [21–27].This approach is based on the fact that stable-isotope ratios of nitrogen and carbon in the consumer tissues reflect those in their prey in a predictable manner [28,29]. In this context, the development of studies that combine both, the use of stable isotopes and the analysis of bioaccumulated contaminants, on bioindicator species in polluted environments could provide new insights and tools for a better understanding of the flow of contaminants through food webs.

This study was carried out within the framework of the research activities done for the monitoring and assessment of heavy metal contamination of the *Green Corridor of the Guadiamar Restauration Project*. The study aims to deepen understanding of two aspects of the already demonstrated capability of *P. clarkii* to withstand heavy metal pollution and intends to: (1) address

the response, in terms of lifespan, growth and feeding activity, of the red swamp crayfish to different concentration levels of a mixture of pollutants similar to those released in the Aznalcóllar mine accident; (2) check for the correlation between heavy metal concentrations in crayfish whole bodies as compared to those in the surrounding environment; and (3) analyse the variation of crayfish carbon and nitrogen stable isotopes signatures grown under different concentration of pollutants.

2. Material and methods

2.1. Collection of baseline data

Juveniles of *P clarkii* were preferred over adult crayfish, not only because they can be manipulated with greater ease under experimental conditions, but mainly because the features under scrutiny are far more variable during early, metabolically very active development stages. 10 gravid females were caught using a hand-net from a rice field in Isla Mayor (Seville, Spain) in November 2002. Crayfish were isolated individually in plastic boxes filled with water from the rice field to avoid the frequently detected aggressions among them [30], and then, transported to the lab in travel-fridge. Once at laboratory, crayfish were placed in individual aquariums (20 L capacity) filled with standard dechlorinated tap water (Table 1) and fed daily with chicken liver pieces. Also, artificial aeration and plastic tubes were provided as shelters to avoid cannibalism in juveniles [31]. After a week, juvenile crayfish hatched and were cultured in the same conditions until they reached a total length close to 2 cm, after the acclimation process a total of 105 equally sized individuals were selected for the experiment.

2.2. Experimental design

An incubation experiment of crayfish following a three-level treatment design w**as** used to test the effect of different heavy metal concentrations in the different life history traits of crayfish was carried out in a laboratory environment. Crayfish size (5.8 ± 0.9 mm mean ± S.E. cephalothorax length) was similar to avoid for bias in initial size distribution. Each animal was randomly assigned to one of the treatments (each containing 35 individuals) and incubated during 20 weeks in experimental solutions whose composition was based on the nature of the toxic sludge released during the Aznalcóllar mine accident. Treatments were: *Control* (C), solution made up of standard dechlorinated tap water considered here for the aim of the experiment as free of toxic substances (Table 1); *Low level contamination* (L) solution containing heavy metal concentrations similar to those found in the water of the Guadiamar River one year after the mine spill, 2001, and considered sublethal for crayfish; and finally, *High level contamination* (H) solution with the maximum heavy metal concentrations measured in the water of the river after the mine accident (Table 2). These two levels of contamination had been used in other ecotoxicological experiments using other organisms of the Guadiamar River [32,33].

Table 1. Mean and range (in brackets) values of the heavy metal concentrations for tap water during the period of the study (MSC, 2004).

Element	Concentration of tap water	Legal limits (R. D. 140/2003)
As	0.5 (0.4–0.6) μg L^{-1}	10 μg L^{-1}
Cd	0.06 (0.05–0.18) μg L^{-1}	5 μg L^{-1}
Cu	20 (10–20) μg L^{-1}	2000 μg L^{-1}
Pb	0.9 (0.8–1.0) μg L^{-1}	10 μg L^{-1}
Zn	No data	5000 μg L^{-1}

Throughout the experiment each juvenile crayfish remained isolated in a closed transparent plastic box (Ø 11.5 cm; 4.5 cm height) with a perforated lid to allow for gas exchange. Each box contained 125 mL of the corresponding experimental solution (C, L or H) that was renewed daily. All the boxes remained stored inside large containers where heated water was pumped in to maintain a 2 cm deep water layer in permanent circulation and with a constant temperature (aprox. 22 ºC, Table 3). Such temperature is well within the optimal range for the development of crayfish in experimental conditions (20–25 ºC) [34,35] and not considered high enough to enhance metal toxicity [36].

Crayfish were fed daily with chicken liver in pieces. Faeces and non-consumed liver were removed daily. When an animal died it was frozen and stored for further analyses. The experimental setup continued until all specimens in treatments L and H (low and high pollution level, respectively) were dead. At that point, all the remaining crayfish were frozen and stored (Figure 1).

Table 2. Above, summary of the heavy metal concentrations of the experimental solutions. Below, summary of the concentrations of salts (reactive) used to build the mother solution for each metal. To obtain the experimental solutions[a], specific volumes of the mother solution of each metal were diluted into 22 L of tap water. The solution of As was added first to avoid interferences with Pb. The mother volumes used were 5 mL for Cu, Zn, Pb for L and H treatments, and for Cd of H-treatment; and 1 mL for Cd of L-treatment and for As of both treatments. [b]To build the mother solutions of each metal a particular weight of each reactive[c] was diluted to 1 L of distilled water.

Experimental solutions	As (μg L^{-1})	Cd (μg L^{-1})	Cu (μg L^{-1})	Pb (μg L^{-1})	Zn (μg L^{-1})
[C-Control]	0.5	0.06	20	0.9	-
[L-Low Metals][a]	15	15	50	30	600
[H- High Metals][a]	45	150	180	275	2000
Mother solutions and reactive used	As_2O_3	$3CdSO_4\ 8H_2O$	$CuSO_4\ 5H_2O$	$Pb(NO)_3$	$ZnSO_4 7H_2O$
[L-Mother] (μg L^{-1}) [b]	330	330	220	132	2640
L-reactive (g) [c]	0.436	0.753	0.864	0.211	11.611
[H-Mother] (μg L^{-1}) [b]	990	660	792	1210	8800
H-reactive (g) [c]	1.307	1.506	3.112	1.934	11.611

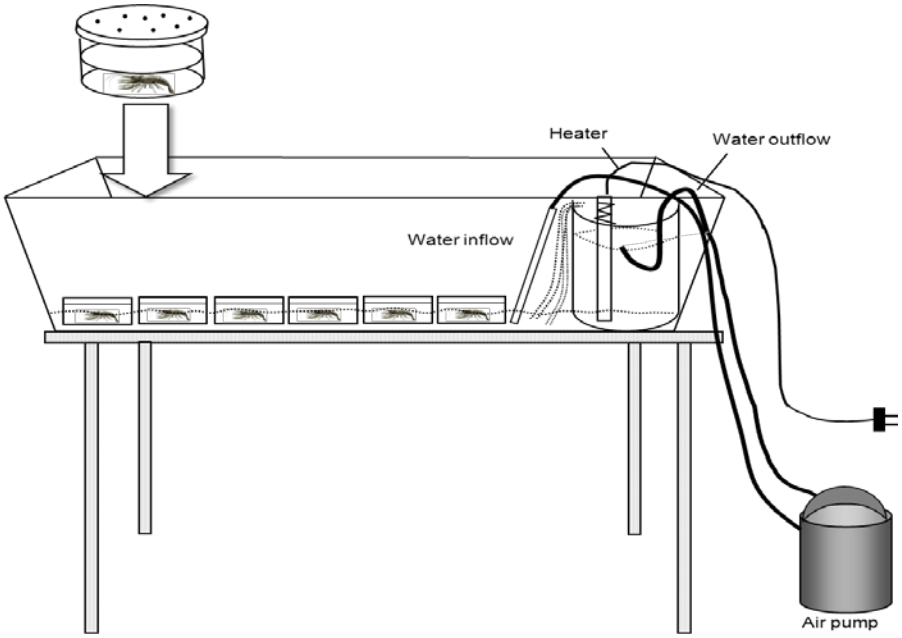

Figure1. Diagram of the experimental containers of each treatment. Each crayfish was individually placed in a plastic box with the lid punctured.

New solution stocks (22 litres) for L and H treatments were prepared fortnightly diluting 1 or 5 mL respectively of specific mother solutions made for each metal (i.e. Cu, Zn, Cd, Pb, As, see Table 2 for further details). In order to avoid the precipitation of some of the metals, the pH in the experimental solutions was kept at a value of 6 by adding drops of high-quality sulfuric acid (H_2SO_4). Tap water used in treatment C had a pH of around 7. Physico-chemical variables (pH, dissolved oxygen and temperature) were weekly measured using a WTW 3310-oxymeter and a WTW 330i-pHmeter (Table 3).

Table 3. Average and range values of the physicochemical parameters monitored at each treatment throughout the study.

Treatment	T^a (^oC)	O_2 (mg/L)	O_2 (%)	pH
C-Control	21.7 (20.5–22.2)	8.7 (7.8–9.78)	89.2 (80–108)	6.92 (6.52–6.93)
L-Low Metals	22.1 (20.8–22.5)	7.9 (7.1–9.94)	80.6 (101–109)	6.53 (6.5–6.81)
H-High Metal	21.9 (20.9–22.3)	8.9 (8.1–10.1)	90.8 (101–110)	6.46 (6.4–6.54)

The variables used as descriptors of life history traits and recorded throughout the experiment were:

- *Lifespan (days),* the total number of days lived by each crayfish from the beginning of the experiment to crayfish death (or to the end of the experiment).
- *Growth (mm),* increase in size (cephalothorax length in mm) from the *beginning of* the experiment to crayfish death (or to the end of the experiment). Growth was measured under a dissecting microscope (Olympus SZ 30) and total growth rate was calculated as "final size-Initial size/time". In addition, growth was measured once a week and then used to estimate the weekly growth rate as describe below.
- *Growth rate (mm/week),* cephalothorax size increase (mm) per observation unit time (weeks).

- *Feeding activity (%),* total number of days in which crayfish ingested food *relative* to the total number of days they lived.
- *Moult*, number of moults standardised to crayfish lifespan.
- *Intermoult period*, mean number of days between successive moults in each single crayfish calculated as "number of moults/longevity".

Heavy metal content accumulated in crayfish whole bodies and their stable isotopic signature were also measured when the experiment finished.

- *Bioaccumulation* (μg g^{-1} dried weight), content of Cu, Zn, Cd, Pb and As after full exposure in crayfish whole bodies.
- *Isotopic signature* (‰) of carbon and nitrogen in crayfish whole bodies at the end of the experiment.

In summary, lifespan, moult and feeding activity were collected on a daily basis (except for weekends). Crayfish size (cephalothorax length) however was measured weekly, whereas intermoult period and growth rate were estimated once all data were collected and the experiment was terminated.

2.3. Processing samples for metal analysis

All laboratory dissecting tools and containers were plastic or teflon-made, acid washed with high-quality (65%w/v) nitric acid (Merck) and rinsed with deionized water (MilliQ). Animal bodies were placed in pyrex petri dishes and oven dried for 48 hours at 80 ºC to determine dry weight. Samples were then ground with a pestle and mortar.

Twenty five animals per treatments were thawed and processed for metal analysis that were carried out by ICP-MS (Inductively Coupled Plasma Mass Spectrometry) at the SIdI (Servicio Interdepartamental de Investigación) laboratory of the Autónoma University of Madrid.

Certified ICP standards of Merck were used in the calibration and validation of the standard curves. Determination of total Cd, Cu, Pb, Zn and As was performed with a Perkin-Elmer Sciex Elan 6000 ICP mass spectrometer (MS) equipped with an AS-91 autosampler.

Whole homogenised crayfish tissues were digested by a solution of 5 mL of concentrated (65%) nitric acid (Merck Suprapur®) with 5 mL deionised water (Milli- Q) using a high pressure microwave digestion system (Milestone ETHOS SEL) with Teflon used in Alcorlo et al. [11]. After digestion, samples were driven to a final volume of 25 mL adding diluted nitric acid (1%w/v). The metal isotopes selected for measurement were ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{68}Zn, ^{75}As, ^{114}Cd and ^{208}Pb. The ICP-MS system was calibrated using ^{72}Ge and ^{103}Rh. In order to check for contamination during the digestion procedure and sample manipulation, a blank solution was prepared and carried through each ten sample analysed. All calibration straight lines had correlation coefficients >0.999.

2.4. Processing samples for stable isotopes analysis

The remaining 10 animals per treatment were processed for stable carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope analysis. They were thawed and placed in 250 mL Erlenmeyer flasks and suspended in 100 mL of 0.2 N HCl for 24 h at room temperature to remove carbonates. Crayfish were then rinsed thoroughly with deionised water and dried at 80 ºC (48 hours). Samples were then homogenised with a mortar and pestle and stored dry in 15 mL vials until analysed.

Stable isotope analysis were performed on 1 mg subsamples of homogenized materials by loading into tin cups and combusting at 1800 ºC in a Carbo Erba 1108-CHNS elemental analyser. The resultant CO_2 and N_2 gases were then analysed using a Micromass Isochrom continuous-flow

isotope ratio mass spectrometer (CFIRMS) with every 9 unknowns separated by two or three laboratory standards (NBS22, sucrose, atropine, benzoic) at the SIdI.

Stable isotope abundances were expressed in δ notation as the deviation from standards in parts per thousand (‰) according to the following equation:

$$\delta X = \left[(R_{sample} / R_{standard}) - 1 \right] \times 1000$$

where X is the isotope ^{15}N or ^{13}C, and R is the corresponding ratio $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The $R_{standard}$ for ^{15}N is that for atmospheric N_2 (air) and for ^{13}C is that for Pee Bee Belemmnite (PDB) limestone formation. Based on numerous measurements of organic and inorganic standards done by the lab performing the analysis, the precision of these measurements is estimated to be ±0.1 and ±0.2 ‰ for carbon and nitrogen respectively.

2.5. Statistical analysis

To test the effect of the experiment on the recorded life traits and isotope signatures of crayfish from the different treatments, one-way ANOVA was chosen because the different number of observations per trait and treatment reached at the end of the experiment. It was followed by Tukey's HSD test for multiple comparisons when the former rendered significant results [37]. To meet the assumptions of ANOVA, all data were tested for heterogeneity of variance by Levene's test, and for deviations from normality by the Kolmogorov–Smirnov test. Normality of the variables involved was checked and transformations (logarithmic) applied when required.

A Kaplan-Meier analysis based on χ^2 survival curves comparison was used to test the effect of the treatments on the survival of crayfish. In addition, multiple linear regression analysis was performed to analyse the relationship between heavy metals concentrations (i.e. independent variables) measured in crayfish whole bodies and their lifespan (i.e. dependent variable) with Pearson´s correlation coefficient.

The same analysis was used to analyse the relationship between heavy metals concentrations (i.e. independent variables) of crayfish and their isotopic signatures (i.e. dependent variables).

Statistical analysis was carried out by means of the STATISTICA (v. 8.0) statistical package. The level of significance under which the null hypothesis was rejected was $\alpha = 0.05$.

3. Results

The experiment lasted for nineteen weeks (133 days) and was concluded when all crayfish in the 'polluted' treatments (L and H) were dead. At that point, ten juveniles from the control treatment were still alive. Most individuals of L treatment died before the week 12^{th} and from that time until the end of the experiment there was only one crayfish that survived, while in treatment H all the animals died at the 11^{th} week.

3.1. Lifespan

Control crayfish lived significantly longer than those juveniles developed on heavy metals solutions ($F_{2,91} = 70.58$, $P < 0.0001$). Post hoc comparison showed that the mean lifespan duration of individuals should be ranked as C>L>H (all $P < 0.0001$).

This results shows that the juvenile's survival is reduced when the heavy metal doses in experimental solutions increase. A Kaplan-Meier analysis based on χ^2 survival curves comparison

was completed and significant differences among juvenile survival across the three treatments were assessed ($\chi^2 = 57.985$, $P < 0.0001$, n = 105) (Figure 2A).

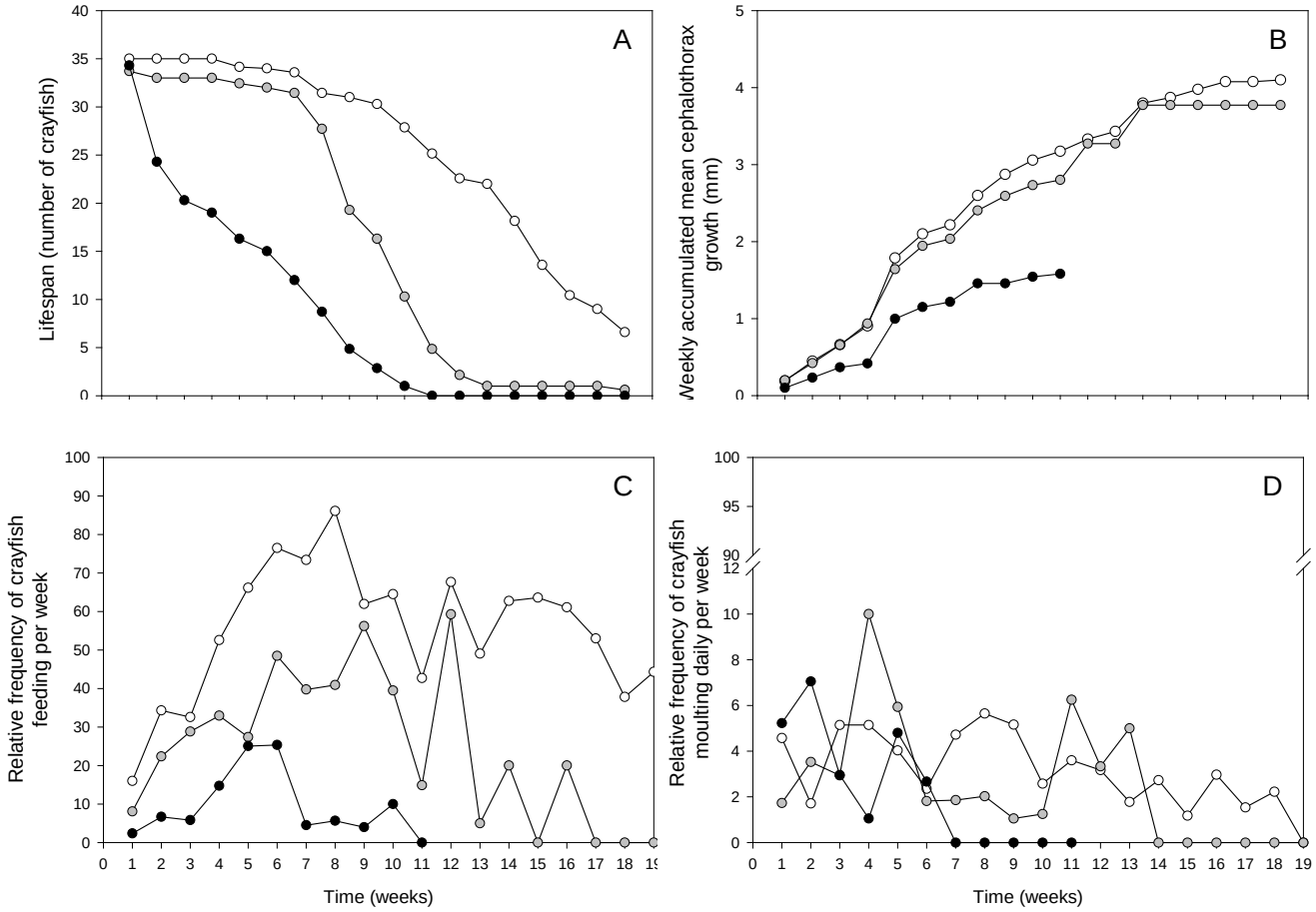

Figure 2. Summary of the trends of the recorded life traits of juvenile crayfish grown under experimental conditions. A) Lifespan (number of days lived by each crayfish), B) Weekly accumulated mean cephalotorax growth (mm), C) Relative frequency of crayfish feeding per week, D) Relative frequency of crayfish moulting daily per week. Treatments, white dots: C; gray dots: L; black dots: H.

3.2. Growth

Mean accumulated cephalothorax growth estimated until the 11th week showed significant differences among treatments ($F_{2,91} = 3.23$, $P = 0.04$). Control individuals reached bigger sizes, followed by the individuals of L and then H treatments (post hoc comparisons showed H individuals as the smallest, $P < 0.04$; Table 4). The estimated growth rates for C and L treatments were similar (0.22 and 0.28 mm/11th weeks) and bigger than the rate seen in the H treatment (0.16 mm/11th weeks) (Figure 2B, Table 4).

Table 4. Means and Standard Deviation for the variables studied. ANOVA significant results are expressed with (*) for α = 0.05.

Treatment	Longevity (days)	Cephalotorax growth rate (mm)	Cephalotorax growth (11^{th} wk, mm)	Feeding activity (days)	Moult (total no.)	Intermoult (days)	Biaccumulation (μg g^{-1}) Cu	Zn	Cd	Pb	As
C	101.13 ± 25.7*	0.22 ± 0.07	3.1 ± 0.77	0.39 ± 0.14*	0.03 ± 0.01	30.25 ± 16.15	905.84 ± 321.98	209.11 ± 138.71*	0.34 ± 0.20*	0.73 ± 0.60*	0.35 ± 0.27*
L	65.06 ± 17.55	0.28 ± 0.12	2.95 ± 0.61	0.19 ± 0.10	0.02 ± 0.01	24.58 ± 13.12	1080.25 ± 347.38	592.09 ± 404.43	56.77 ± 22.64*	71.90 ± 62.15*	0.94 ± 0.53*
H	33.23 ± 23.9	0.16 ± 0.20	1.65 ± 0.21	0.05 ± 0.05	0.06 ± 0.08	11.75 ± 7.85	595.36 ± 273.37*	669.04 ± 401.71	132.28 ± 96.05*	36.65 ± 28.08*	1.46 ± 0.77*

Table 5. Results of the multiple regression Lifespan = $a + b$ logCu + c logZn + d logAs, where each metal is expressed as μg g^{-1} whole experimental animals analyzed.

Variables	Adj. R^2	F-value	Standard deviation error of estimate	Intercept	Regression coefficients log Cu b Mean±SD	log Zn c Mean±SD	log As d Mean±SD	log Cd e Mean±SD	log Pb f Mean±SD
n = 64 log Cu log Zn log As log Cd log Pb	0.73	36.791	Est ± 15.694	−116.04 ($P < 0.05$)	42.596 ± 4.01 ($P < 0.05$)	−15.06 ± 3.24 ($P < 0.05$)	−0.69 ± 6.78 ($P < 0.05$)	14.46 ± 15.58 ($P = 0.35$)	−21.12 ± 11.51 ($P = 0.071$)

3.3. Feeding activity

The specimens from the C treatment fed more times than the others ($F_{2,91}$ = 88.33, P < 0.0001) (Table 4, Figure 2C). All crayfish fed more often during the first weeks of the experiment and then, this activity slows down after the 6th (H) and 8th (C) week. Individuals of L-treatment showed a more erratic pattern, they showed a drastic decrease in the number of times they fed after the 9th week, a recovery in the feeding activity during the 12th week and then another decrease on it.

3.4. Moult

The average number of moults per crayfish standardised to their lifespan was different across treatments ($F_{2,78}$ = 7.50, P < 0.0001; Table 4). Control animals exhibited a higher value with a more uniform pattern through time, while individuals of the L and H treatments showed a strong marked pattern with contrasted peaks of moults during the first two weeks (H) and during the 4th and 11th weeks (L) (Figure 2D).

The intermoult period showed longer values in treatment C and shorter in H, as could be expected in relation with the higher number of moults concentrated during the first weeks of the experiment, however these differences were not found to be significant ($F_{2,45}$ = 3.01, P = 0.059).

3.5. Bioaccumulation

The whole body concentration for each metal (μg g^{-1}) measured in experimental animals is different in each treatment through time (Figure 3). In general, the lifespan of crayfish is related to the contamination level, as it became shorter as the heavy metal concentration increased as observed in the results of the multiple regression analysis of lifespan *vs* metals ($F_{5,59}$ = 36.79, P < 0.001, r^2 = 0.75, Table 5). Cu (t_{59} = 10.61, P < 0.05, r = 0.68), Zn (t_{59} = −4.63, P < 0.05, r = −0.36) and As (t_{59} = −3.05, P < 0.05, r = −0.23) are considered to be the elements that contribute towards this global pattern. However, the metal content accumulated by crayfish through time in each treatment did not follow the same variation trend. The As and Cd average internal levels found in the crayfishes followed a similar increasing trend as H>L>C. These differences were significant for As, taking into account an α level = 0.05 ($F_{2,62}$ = 28.0754, P < 0.0001), but not for Cd that was marginally significant when an α level = 0.1 was used ($F_{2,62}$ = 3.0176, P < 0.0562) (Table 5).

Conversely, average Cu and Pb concentrations found in the crayfish from L treatment were higher than in other experimental solutions (Figure 3, Table 4) following the trend L>C>H significantly for Cu ($F_{2,62}$ = 14.4164, P < 0.0001) and L>H>C for Pb if an α level = 0.1 is used ($F_{2,62}$ = 2.7682, P < 0.0705).

Finally, the differences in Zn content were ranked as H≥L>C ($F_{(2,62)}$ = 30.21, p < 0.0001).This metal also exhibits the same pattern as that seen for Cd and As (Figure 3, Table 4).

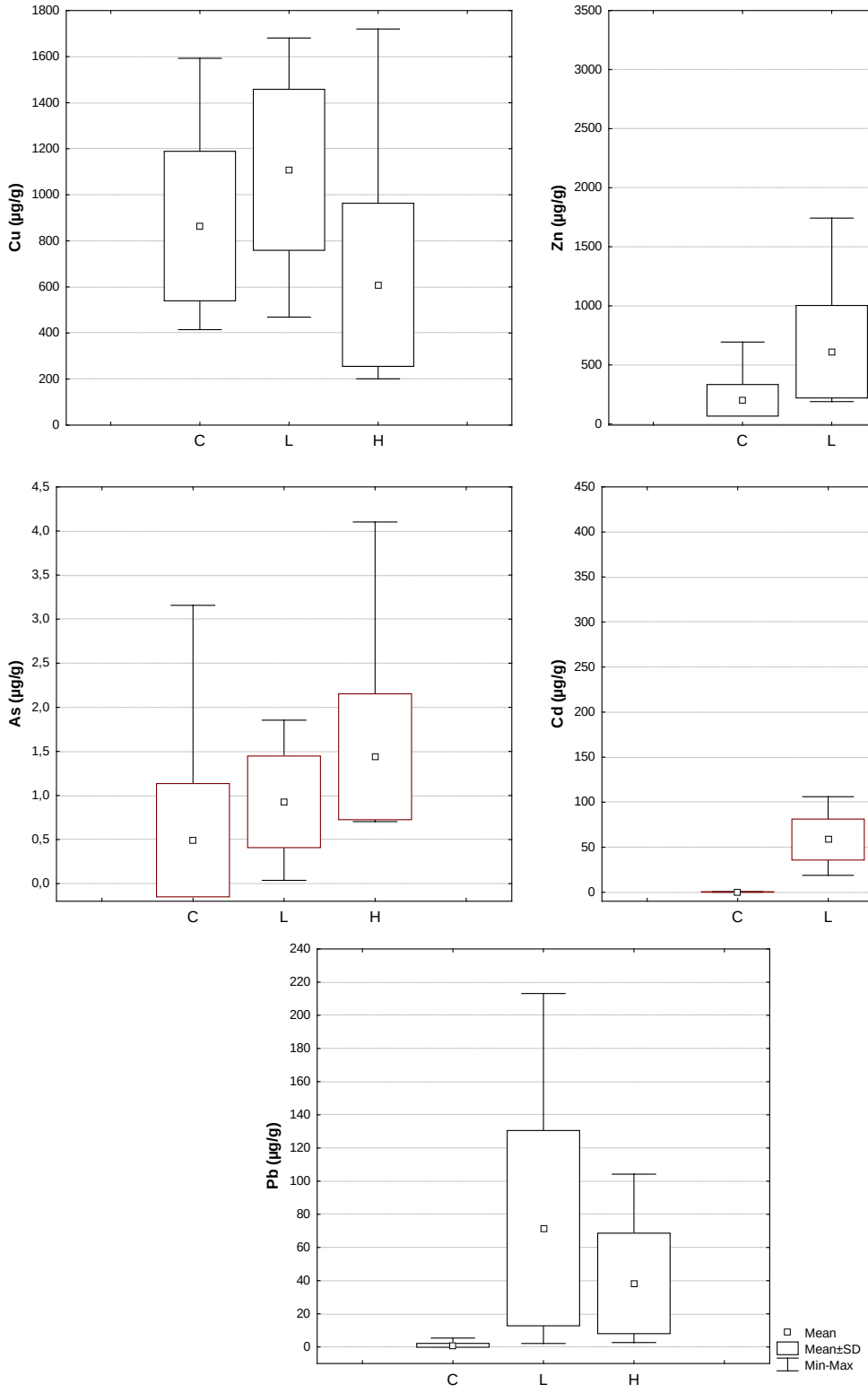

Figure 3. Metal bioaccumulation in juvenile crayfish per treatment. The box represents the mean ± standard deviation values of the heavy metal concentrations measured at crayfish whole bodies and the whisklers their range values. All concentrations are expressed as µg g^{-1} of dry weight.

3.6. Stable isotopes

Stable isotopes signatures of $\delta^{13}C$ were lighter in crayfish grown under contaminated solutions H<L≤C ($F_{2, 23}$ = 18.34; P < 0.0001) while no differences were found for $\delta^{15}N$ among treatments ($F_{2,23}$ = 0.52; P = 0.55) (Figure 4). In addition, relationships among metal concentrations and isotopic signals of crayfishes when they were analysed through multiple regression were only significant for $\delta^{13}C$ ($F_{5,23}$ = 6.76, P < 0.0005, r^2 = 0.59) and explained mainly by Cu (t_{23} = 2.37, P = 0.026, r = 0.33) and As (t_{23} = −2.09, P = 0.047, r = −0.39).

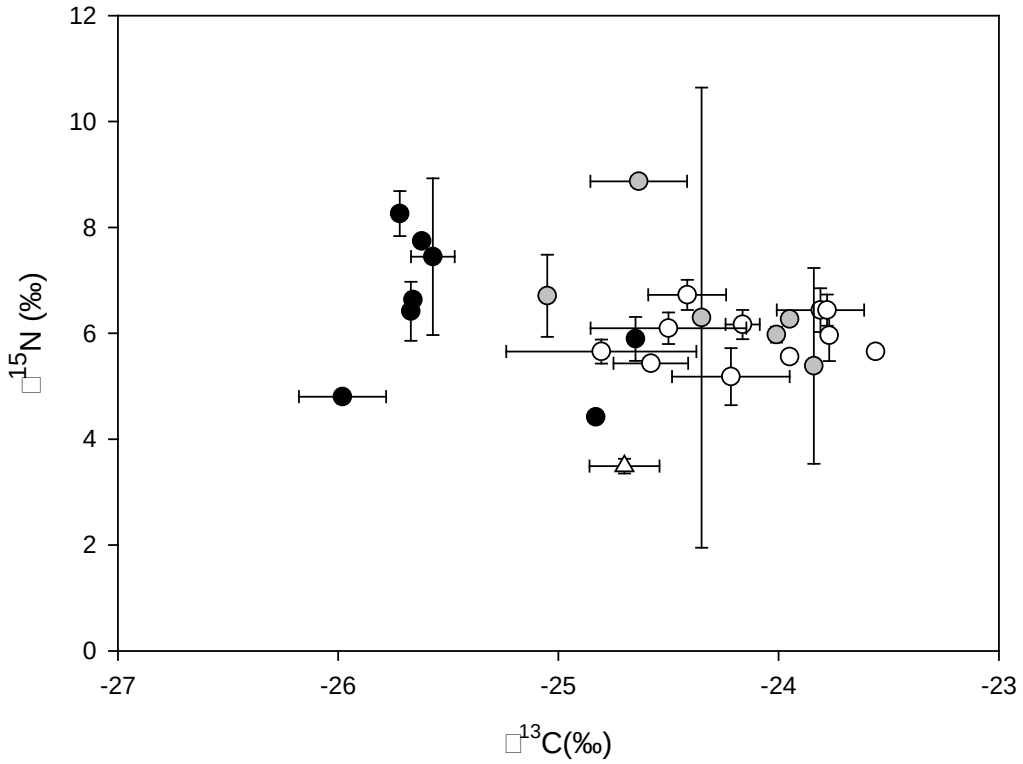

Figure 4. Treatment-specific $\delta^{13}C$ and $\delta^{15}N$ signatures of juvenile crayfish. Circles: individual mean isotopic values of each crayfish, lines: standard deviation. Treatments, white: C; gray: L; black: H. Triangles represent the isotopic signature of the liver used to feed them

4. Discussion

4.1. Influence of heavy metals in life history traits

The presence of heavy metal polluted environments has been shown to reduce the presence and abundance of crayfish populations [31]. However, in general, the concentration of metals in the environment is not sufficient to be a direct cause of death [20]. *Procambarus clarkii* has the capability to rapidly reach a balance with contaminated environments, and as soon as there is an increase in contaminants, crayfish bioaccumulate them in its tissues [16,38,39]. This accumulation of metals has been found to be dose-and time-dependent in other studies [11] and explains why the responses of the different life history traits measured in this experiment showed such a heterogeneous pattern.

In this study, the life history traits (i.e. lifespan, feeding activity, growth and number of moults) were significantly reduced as the concentrations of toxic substances increased. The exposure to two levels of concentrations of the mixture of metals used (i.e, Cd, Cu, Pb, Zn and As) caused complex responses in the biological variables examined and these are reviewed individually below. The heavy metal concentration levels used in this experiment were lower than the ones utilized in other studies for lethality or sublethality [36,40–43] although the duration of the experiment was similar to former studies (i.e. 15–20 weeks). Nevertheless, in this study two solutions with a mixture of five heavy metals have been employed, as opposed to specific solutions for analysing the effects of a single metal element as done in the formerly cited studies. This methodology therefore contributes considerably to the knowledge about the effects of toxic spills on crayfish and the final effect achieved on the life history traits was a consequence of both the different concentration levels of L and H treatments and the composition of the mixture of heavy metal elements. Due to this experimental design the subsequent and expected dose-response effect for each single metal alone was not evident in some cases. It is known that, some metals promote an antagonist influence on other toxic substances assimilation by crustaceans [44], such as competition between Ni and Cu in assimilation routes [45] or Pb inhibitory effect on Cd, or Cr on Pb [46] with different effect on their life history traits.

The mean *lifespan* decreased as the metal concentration increased (33, 65 and 101 days in H<L<C treatments respectively). However, it cannot be assessed as the final cause of mortality. Some studies comment that crayfish develop compensatory adaptations to survive in contaminated environments, such as a reduction in gill efficiency [47,48], thus, the lower oxygen consumption done by the organisms under these circumstances could produce the high mortality rate found in these environments. In our experiment dissolved oxygen in the solutions had always concentrations bigger than 7 mg/L and the could be considered well oxygenated

The weekly *growth rate* of the cephalothorax in this study was seen to be generally low in all treatments, even in C (0.22 mm/week, Table 4). H treatment crayfishes showed the smallest growth rate (0.16 ± 0.2 mm/week) and final size (1.65 ± 0.21 mm). This is seen in other crayfish that grow at a reduced rate and show smaller sizes when they grow in contaminated environments and are compared to those living in unpolluted systems [31]. A slower growth rate in crayfish exposed to metals affects the final size reached by the animals, due to a change in energy investments caused by the overall energy cost involved in detoxifying the metals from tissues as found by Rowe et al. [49]. In addition, it has been shown that prolonged exposure to heavy metals inhibits the lipolytic activity in the hepatopancreas which contributes towards biological processes of crayfish such as moult and growth [42,50].

The results of this experiment confirmed that sublethal metal concentrations of the L treatment influenced the decrease of the observed *feeding activity* of crayfish juveniles. Similar assays performed with other crayfish species, *Cambarus bartonii*, where animals were exposed to sublethal Cu concentrations, ranging from 0.02mg/L to 0.2mg/L, caused the loss of the food detection capacity after 7 days [51], and the concentrations of Cu used in our study (0.05 mg L^{-1}) could also cause such an effect in *Procambarus clarkii* juveniles. Despite L metals level used could not cause a quick death in crayfish, this concentration of contaminants in other studies become seriously harmful for crayfish tissues, especially kidneys and antennal glands [51]. H metal concentrations level had even a stronger lethal effect and decreased the feeding activity of juveniles until almost zero values (0.05 days/lifespan).

The *number of moults* in treatment H was larger than those seen in C and L (H>C>L)**,** however it did not show a homogeneous response as would be expected in contaminated environments where

adult crayfish exhibited an increase in the moult number and an extension of the intermoult period [31,52,53]. In this study the *intermoult period* duration seemed to decrease with the decline of metal levels in solutions, although the differences across treatments were not significant (H<L<C, Table 4). Thus *P. clarkii* juveniles in H treatment had a higher moult frequency. Toxic solutions seemed to stimulate the moulting process in juvenile crayfish, as it occurs with other stress situations like the existence of high temperatures that promote metabolic activation [54].

The growth, number of moults and the duration of the intermoult period are related life history traits because crustaceans grow according to the number of moults. Thus, the time spent in the intermoult is important to calculate the ecdysis frequency. The moulting process of crayfish involves several steps, each controlled by physiological changes instigated by hormones from different organs [30]. The process is similar in juvenile and adult stages, although juveniles moult much more often. The crayfish functions normally found during the intermoult period are to feed, and to build up reserves. During the moulting period crayfish are highly vulnerable to adverse environmental factors and to predation. The crayfish ceases to feed and becomes less active, often taking shelter. The delicate process of moulting and its interactions with external factors could explain why a rise in mortality was observed in the specimens of H treatment. It is well known that during the post-moult stage, crustaceans need elevated amounts of Ca to create the new exoskeleton [55], but in an environment with a high abundance of heavy metals there is competition between Ca and the other metal elements with the same molecular proprieties (i.e. Cd, Cu and Zn). These metals are incorporated into the Ca proteins, so the crayfish assimilates massive concentrations of metals after moulting that cannot be metabolically regulated. Consequently, most part of the juveniles from the H treatment died immediately after moulting. Also the cephalothorax growth reached by crayfish at the 11^{th} week in the H treatment was the smallest, so, in this case to have a shorter intermoult duration period did not mean a higher cephalothorax growth.

4.2. Bioaccumulation of metals

Cu and Zn concentration were the more abundant elements in crayfish tissues in all treatments. Both metal levels are regulated and controlled in crustaceans inside certain limits [56,57] as both metals are essential elements for crayfish [58]. They are part of active enzyme centres and respiratory pigments; they regulate protein synthesis, their transport, cellular reproduction, respiration, etc [59,60]. The results of the experiment showed a higher variability in the measured internal concentrations of Cu than in Zn. Indeed, accumulation of Cu (L>C>H) is mediated by a time-dependent factor more than a dose-dependent factor. The high levels of Cu in C juveniles (905.8 ± 321 μg g^{-1}) could not be explained by the natural concentration found in wild populations of the same place of origin of crayfishes which is approximately on average 607.7 μg g^{-1} [61]. Moreover, as the composition of the solution of this treatment is free heavy metal tap water, the elevated gain of this compound would be through the supplied food, as chicken liver is rich in Cu and other essential minerals and metals [62]. The bioaccumulation of Zn (H>L>C) was more dose-dependent than Cu, reflecting the levels of the experimental concentrations used (L and H).

Pb, Cd and As are non-essential elements for crayfish that tend to be detoxified by metallothioneins or can be stored in vacuoles in the organisms tissues [35,36,57,59], thus the accumulation of these metals is related with the environmental concentration (60). In our experiment, *P.clarkii* juveniles accumulated Pb following a time dependent factor, like Cu, and agreed with the results of other experiments [42]. Despite Pb toxicity, *P.clarkii* is able to accumulate great amounts of Pb through time without any appreciable lethal effect [63]. On the other hand, moulting in

freshwater crayfishes seems to be a good method for Pb detoxification [64], as it is usually incorporated in the exoskeleton by adsorption [35,36]. The low Pb concentration obtained in crayfish from the H treatment could be explained by the short intermoult period that these crayfishes exhibited allowing them to continually eliminate Pb.

The more effective elements for indicating the external toxicity of the treatments were Cd and As, as the juveniles' internal concentrations of these metals are in proportion with the different experimental solutions. They are the most lethal or toxic elements for crayfish: Cd, for example, is 700 fold more toxic than Pb [41] and its accumulation is also higher (56.8 and 132.3 μgCd g^{-1} *vs* 71.9 and 36.7 μgPb g^{-1} in L and H treatments respectively). This trend of Cd to be concentrated in crayfish tissues is related to the existence of specific Cd blind metallothionein proteins situated in the digestive glands of *P. clarkii* [65]. In addition, Cd is an element that due to its chemical prop**er**ties is able to compete with Ca for the same metabolic routes and can easily enter tissues through the canal proteins of the cellular membranes of the cuticle and gills [35].

The accumulated As levels in crayfish juveniles reflected concentrations found in the external heavy metal solutions. Thus, there was also a dominant dose-dependent factor in its assimilation. The origin of the As measured in C treatment (0.35±0.2 μg g^{-1}) could come from the tap water used (Table 1) because there is evidence that shows how small As concentrations from the tap water used throughout time produced concentrations of As 0.1–0.3 ppm in crayfish tissues [66].

4.3. Isotopes

$\delta^{13}C$ has been widely used in food web ecology as a tracer of the food sources used by each organism while $\delta^{15}N$ has been used as a tracer of the trophic position [22,67]. Also, recently, $\delta^{15}N$ had been used to explore the biomagnification or biodilution of heavy metal concentrations in macroinvertebrates [68], crayfish [69,70] and fishes [71].

In our experiment, juvenile crayfish from the H treatment showed a lighter $\delta^{13}C$ signature but neither of the analysed metals was correlated with $\delta^{15}N$. Similar absence of correlation between isotopic signatures and heavy metal concentration has been found in the crayfish *Pacifastacus leniusculus* from lakes of different trophic status in southern Sweden [69]. These authors found that the metal concentrations in biota were strongly correlated with the descriptor variables of the trophic status, while the stable isotopes reflected a baseline signature of each of the lakes in this omnivorous crayfish species. Nevertheless, Wanatabe et al. [72] found a biodilution of Pb and Ag in the macroinvertebrate food web of a stream that receives drainage from an abandoned mine and found a negative correlation between the $\delta^{15}N$ and the Pb and Ag tissue concentration of macroinvertebrates, although this was not seen in the other metals (Zn, Cu, As). However, there was no correlation between $\delta^{13}C$ and tissue metal concentrations, indicating that the relative contributions of autochthonous and allochthonous carbon sources to macroinvertebrate biomass did not influence the tissue metal concentrations. These results were related with the different assimilation metal routes used by macroinvertebrates, direct uptake from water at low levels of metal contamination sites or indirect uptake from food at high levels of contamination sites. In our study, the food source is the liver and there was a consistent isotopic signature of all the pieces of liver used, explaining why there was a significant effect of the treatments in the overall $\delta^{13}C$. No isotopic differences were found in specimens grown in C and L treatments (i.e. the ones that fed regularly and acquired the liver carbon isotope signal) even though H specimens became isotopically lighter (i.e. the ones that almost no fed and for instance did not acquired the liver carbon isotope signal). Indeed, the effect of Cu and As on the overall $\delta^{13}C$ measured through multiple regression analysis points in this direction. It seems that

the $\delta^{13}C$ of C crayfish could be associated with the ingested food source rich in Cu (liver) and by the As of the environment (water).

4. Conclusions

In summary, we can conclude that the level of toxic pollutants used in the H treatment produced lethal effects on juveniles of *P. clarkii*, whereas the levels used in the L treatment, resulted in less marked effects. The concentration used in the L treatment corresponded to a pollution level similar to that found in the River Guadiamar three years after the mining disaster, and the crayfish's juveniles seemed to be able to regulate and manage within this range of pollution to maintain their biological traits. Longevity, feeding activity and growth rate, are negatively affected by the experimental solutions used in the L treatment and more strikingly by H treatment. Juveniles capacity to bioaccumulate and depurate toxic substances changes with the nature of the heavy metal element. Cd, Zn and As showed a dose-dependent assimilation, while Cu and Pb exhibit a time-dependent accumulation. The Zn element seems to be regulated with efficiency although its concentration is higher in juveniles from the H treatment. These results assess the high resistance quality of *P. clarkii* against high heavy metal concentrations and enhance its interesting role as bioindicator. Also, further field studies that combine the employment of stable isotopes with heavy metal concentration measurements as this, will aid in improving the understanding of metal transfer through food webs.

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Conflict of interest

The authors declare no conflict of interest.

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