

The use of the red swamp crayfish (*Procambarus clarkii*, Girard) as indicator of the bioavailability of heavy metals in environmental monitoring in the River Guadiamar (SW, Spain)

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Abstract

A translocation experiment of red swamp crayfish (*Procambarus clarkii*) to different sites located in the River Guadiamar was performed in order to assess the ability of this species as bioindicator of heavy metal and metalloid contamination. Crayfish were placed in cages and exposed to polluted environment during either 6 or 12 days in the three sites with different concentration of contaminants. Their tissues (exoskeleton + gills, hepatopancreas and abdominal muscle) were dissected and analysed by ICP–MS to assess for concentration of Cd, Cu, Zn, Pb and As. Both exposure times result in significant bioaccumulation of some metals in crayfish tissues as compared to their concentration in the environment. According to overall metal concentration, crayfish tissues rank as follows: hepatopancreas/viscera > exoskeleton/gills > abdominal muscle. Essential metals for crayfish metabolism (Cu and Zn) are always found in high concentrations independently of their quantities in the environment because of the ability of crayfish to manipulate their levels for their own metabolic profit. Metals not involved in crayfish metabolism (Cd, Pb, As) tend to increase with increasing concentration in the surrounding environment and with longer exposure times. Thus crayfish could be used as bioindicator of these pollutants because their dose- and time-dependent accumulation may be reflective of the levels of non-essential metals present in contaminated wetlands. Future guidelines in plans for monitoring contamination on polluted Mediterranean rivers and wetlands should take into account the implementation of the incubation of crayfish during 6 days and their subsequent analyses of metal contents, as a routine.

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1. Introduction

Spills of mining wastes are a relative frequent source of trace element pollution (Farag et al., 1998) and several authors have demonstrated the adverse effects of

heavy metals on aquatic at both the individual and the community level (Clements et al., 1988; Leslie et al., 1999). The Aznalcóllar mine accident in 1998 caused the discharge of 6 hm³ of sludge and acid water loaded with heavy metals into the Guadiamar River (Seville, SW Spain) directly affecting an area of approximately 4400 ha (Grimalt et al., 1999; Arenas et al., 2004). As a result of the spill, heavy metals accumulated in soils (Cabrera et al., 1999; Simón et al., 1999), sediments (Palanques et al., 1999), water (Van Geen et al., 1999),

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flora (Murillo et al., 1999), fauna (Benito et al., 1999; Blasco et al., 1999; Hernández et al., 1999) and therefore in food webs (Meharg et al., 1999) all through the Guadiamar catchment. The river belongs to the Lower Guadalquivir Basin and is a major contributor to the marshes of Doñana National Park, one of the most valuable wetland areas within the European Union (Montes et al., 1998).

Immediately after the mining accident an urgent procedure for cleaning up the soil was carried out (Grimalt et al., 1999). After finishing those critical activities—which were indeed very effective—a restoration plan focusing on socio-ecological systems and a pollution monitoring plan (the ‘Green Corridor’ of Guadiamar-Corredor Verde) were designed for the area. Activities related to that plan still continue nowadays (Montes et al., 2003). This paper reports on some field experiments aimed to contribute to the monitoring plan through the use of a widely distributed non-native species in the area as a bioindicator, the red swamp crayfish *Procambarus clarkii* Girard. In aquatic ecosystems, the use of indicator species seems to be a suitable way of monitoring environmental quality due to the ability of some aquatic animals to accumulate metallic ions either directly from the surrounding water or indirectly through food sources (Devi et al., 1996). Crayfish can be used to monitor the aquatic environments for heavy metal pollution because they are solitary bottom dwellers, which keep much of their bodies in contact with surrounding objects and tend to accumulate metals in their tissues (Khan et al., 1995; Schilderman et al., 1999). Previous studies have addressed contaminant accumulation into tissues of a variety of strains of crayfish under different concentrations and times of exposure in both, the field (Anderson and Brower, 1978: Pb in *Orconectes virilis*; Roldan and Shivers, 1987: Fe, Pb in *Orconectes propinquus*; Alikhan et al., 1990: Cu, Ni in *Cambarus bartoni*; Keenan and Alikhan, 1991: Pb and Cd in *Cambarus bartoni*; Khan et al., 1995: Pb, Cd, Cu, Zn, Co, Ni, Hg in *Orconectes virilis*; Currie et al., 1998: Cd in *Orconectes virilis*; Schilderman et al., 1999: PCBs, PAHs, Zn, Cu, Pb, Cd in *Orconectes limosus*) and the laboratory (Alikhan et al., 1990: Cu, Cd, Fe, Mn, Ni, Zn in *Cambarus bartoni*; Meyer et al., 1991: Pb, Cd in *Astacus astacus*; Allinson et al., 2000: Cu in *Cherax destructor*).

When looking for a species which might be used as such in the Guadiamar Basin attention was immediately directed towards *P. clarkii*. The Decapod crustacean *P. clarkii* was introduced in the Lower Guadalquivir Basin back in 1973. Once the crayfish established and

developed dense populations, local people started its commercial exploitation which, nowadays, comprises a significant fraction of their incomes (Gutiérrez-Yurrita et al., 1999). This crayfish species has been previously used as bio-indicator of organic contaminants in experiments done in laboratory conditions (Foster and Crosby, 1986; Blat et al., 1988; Barron et al., 1991) and metal pollution on behalf of its capacity to accumulate metals (e.g. lead and cadmium) in its tissues, thus reflecting environmental levels of those metals (Díaz-Mayans et al., 1986; Pastor et al., 1988; Devi et al., 1996; Anderson et al., 1997b; Bollinger et al., 1997). Field experiments, however, are scarce (Rincón-León et al., 1988; Madigosky et al., 1991; Anderson et al., 1997a). Other features also contribute to make out *P. clarkii* a model indicator species (e.g. abundant populations, long life cycle, widespread distribution, and relatively sedentary lifestyle) (Sánchez-López et al., 2004).

The most abundant heavy metals released to the river Guadiamar by the mine spill were Zn, Pb, As, Cu, Cd, Sb and Tl (Prat et al., 1999; Solà et al., 2003; Toja et al., 2003). All these metals can be accumulated by *P. clarkii* and their potential to seriously affect human health is widely documented (Czerczak and Fishbein, 2002; Antón and Lizaso, 2004; Oyarzun and Higuera, 2005). Indeed Cd, Pb and As have been registered in the database of the International Chemical Safety Cards of the CIS (International Occupational Safety and Health Information Centre) as carcinogenic to humans (CIS 2006).

This study describes a translocation experiment of red swamp crayfish (*Procambarus clarkii*) to several sites in the River Guadiamar that were differently affected by the toxic spill of the Aznalcóllar mine accident. The experiment was performed in order to assess the ability of this species as bioindicator of heavy metal and metalloid contamination.

2. Material and methods

2.1. Study area

The Guadiamar River runs through calcareous gypseous clayey substrates linking the northward highlands in Sierra de Aracena with the lowland marshes of Doñana National Park in the Lower Guadalquivir Basin. Although in former times it was one of the main tributaries that fed the marshes, the Guadiamar is nowadays channelled in its lower reach and flows directly into the Guadalquivir River; its waters no longer enter Doñana National Park except on high flooding.

From a hydrological point of view the Guadiamar is a typical Mediterranean stream (Giudicelli et al., 1985) with a pluvial hydrologic regime and an average annual flow ranging from 3.67 to 6.3 m³/s (minimum values: 0.15 to 0.4 m³/s in dry periods) (Prat et al., 1999). The mine where the toxic spill originated is placed near the Agrio, a small tributary of the Guadiamar (Fig. 1).

Although pollution diminished significantly after the remediation and restoration works done in 1998, there are still residual pollutants in the riverbed at several places along the basin. Three experimental sites were chosen to include the range of conditions concerning the spatial distribution of pollutants and were located at 4 (Site 1), 9 (Site 2) and 42 km (Site 3) downstream from the confluence with of the river Guadiamar with the Agrio stream (Fig. 1). Site 1 (Guijo, UTM coordinates: 29S 215608, 4148147) and Site 2 (Las Doblas; 29S 214340, 4143764) were fully covered by the mud discharged in the mine accident in April '98 whereas Site 3 (Puente Vaqueros; 29S

216632, 4117443) is located in the channelled part of the river Guadiamar, and although it was affected by acid waters from the spill, the polluted mud did not reach this point (Fig. 1).

2.2. Collection of baseline data

Before starting the experiment sediment and water samples were obtained from the experimental sites and analysed for heavy metal content. Sediment samples (4 replicates per site) were collected at random from the uppermost layer (3 cm) driving a 90 mm (Ø) methacrylate core into the sediment and plugging the upper end of the core with a rubber stopper so that the enclosed sediment and water column were withdrawn intact. Water was dropped away and the sediment core was extruded from the tube obtaining an undisturbed sample of the surface sediment up to a total of 250 ml per replicate. Water samples (3 replicates per site) were filtered (0.45 µm pore size cellulose nitrate filter), placed

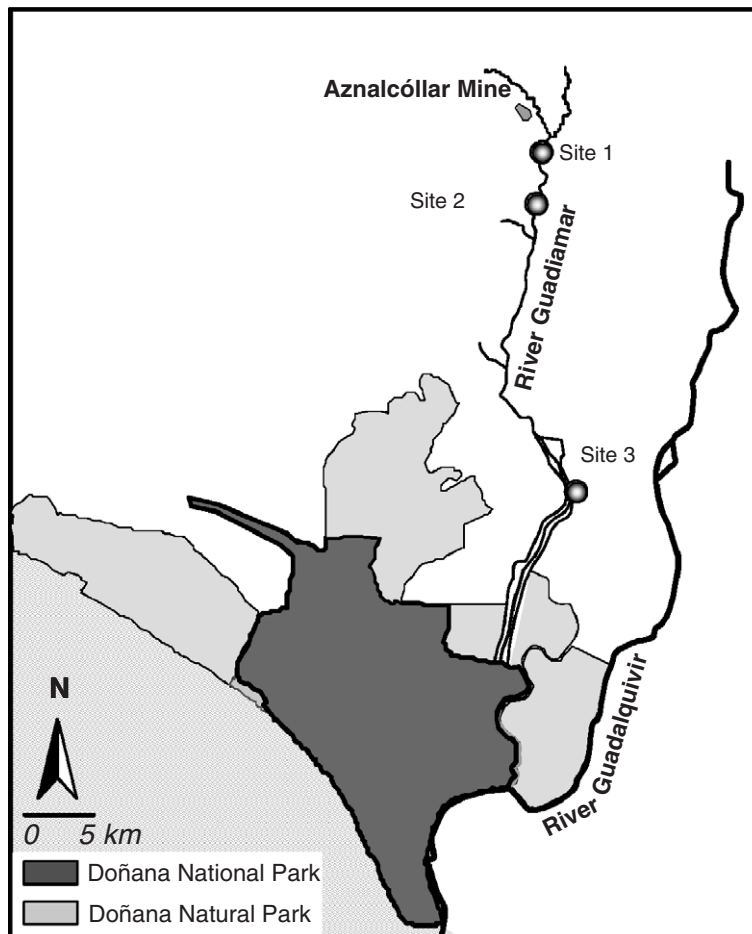


Fig. 1. Geographic location of the study.

in polyethylene bottles and for the analyses of metal content brought to a pH below 2 with high-quality concentrated nitric acid (Merck Suprapur®).

Physico-chemical variables (pH, conductivity and water temperature) were measured in situ using a WTW 330i-pHmeter and a WTW LF26 conductivimeter.

2.3. Experimental design

Experimental crayfish were purchased from a local seafood vendor and kept more than 24h in stocking pools in order to clean their digestive tracts. Fourteen control specimens (50% males and 50% females) were then removed from the set, rinsed with deionised water, measured (length and weight), sexed, introduced in polyethylene bags, frozen and stored at -20°C until analysed for heavy metals content (cadmium, copper, lead, zinc and arsenic) so to assess contamination initial conditions (Control). Remaining crayfish were translocated to the experimental sites.

A total of 32 adult crayfish (1:1 sex ratio; total length: 7.4 to 12.2cm) were placed in individual compartments (to avoid cannibalism) within special cages at each experimental site in May, 2002. The cages ($n=4$ per site with 8 compartments each) were built using plastic net fabrics (1 cm mesh size) (Fig. 2) and were totally submersed and anchored with sticks to the sediment, allowing the contact between crayfish, sediments and food sources (macrophytes, periphyton and benthic fauna).

After a 6-day period of exposure (t_6), two of the cages were collected at random and the crayfish processed as explained for the control individuals above. The same procedure was repeated with the other two cages after another 6-day period of exposure (t_{12}). Incubation periods were chosen based on existing experimental studies that demonstrated significant accumulation rates of heavy metals in crayfish tissues for 7- and 14-day periods of exposure in both lab (Devi et al., 1996) and field conditions (Anderson et al., 1997a).

2.4. Processing and analysis of samples

The different crayfish tissues were analysed separately because of their different roles with regard to heavy metals and metalloids. Thus, crayfish were dissected after thawing in abdominal muscle, hepatopancreas and exoskeleton (gills included). The hepatopancreas is a metabolically very active organ that sequesters metals and therefore may provide an integrated estimate of exposure to metal pollutants over time without the confounding effects of frequent moulting. Muscle is important to be monitored because it is the subject of human consumption. Finally, the exoskeleton is a tissue where heavy metals and other contaminants are frequently excreted to.

All laboratory dissecting tools and containers were plastic or Teflon-made, acid washed with high-quality concentrated nitric acid (Merck Suprapur®) and rinsed with deionised water (Milli-Q) to minimize the possibility of contamination. All crayfish sample tissues were rinsed with deionised water and the digestive tract eliminated. Exoskeleton and muscle were placed in Pyrex petri dishes and oven dried for 48h at 80°C to determine dry weight. Hepatopancreas were placed in ceramic crucibles and burnt at 550°C for 2h in a muffle furnace to determine ash-free weight. Samples were grounded with a pestle and mortar. The same procedure followed for muscle and exoskeleton tissues was followed with sediment samples.

2.5. Metal analysis

Analyses of heavy metals and metalloids content (hereafter named as “metals”) were carried out by inductively coupled plasma mass spectrometry (ICP–MS) at the Servicio Interdepartamental de Investigación (SIDI) lab of the Universidad Autónoma de Madrid. Certified ICP standards of Merck were used in the calibration and validation of the standard curves. The determination of total cadmium, copper, lead, zinc and arsenic content was performed with a Perkin-Elmer

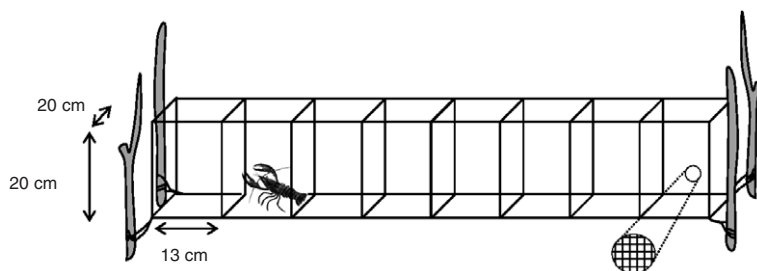


Fig. 2. Sketch of the cage designed for the experiment.

Sciex Elan 6000 ICP–MS equipped with an AS-91 autosampler.

High-purity deionised water purified with a Milli-Q analytical-reagent grade water-purification system (Millipore Ellix with Milli-Q Element) and high-quality concentrated nitric acid (Merck Suprapur®) were used for the preparation of reagents and standards.

Tissue (<20 mg of hepatopancreas, and 0.25 g of muscle, exoskeletons or whole homogenised crayfish) and sediment samples (0.25 g) were digested in 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 closed vessels, and pressure and temperature control following the protocol described by Anderson et al. (1997a).

For crayfish tissues, complete digestion was accomplished, whereas for sediments the analyses were performed on the lixiviate. After digestion, samples were driven to a final volume of 25 ml adding diluted nitric acid (1%). The following metal isotopes were selected for measurement: ^{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 samples analysed. All calibration straight lines had correlation coefficients >0.999.

2.6. Statistical analysis

Mean values of metal concentrations in sediments and water sampled before starting the experiment were compared through a Tukey's HSD equal sample size ($P < 0.05$). Multi-way analysis of variance (MANOVAs) were used to test for the effects of sites, exposure time and type of tissues, on the average metal content in *Procambarus clarkii*. Wilks' Lambda statistic was used to determine whether the test factors of interest affected the response variables (Cu, Zn, Cd, Pb, and As) in all MANOVAs. An initial four-way MANOVA compared metal levels by site, sex, exposure time and type of tissue. No significant differences in metal concentrations were found between the sexes within sites and exposure times; therefore, the data for both sexes were pooled prior to subsequent analysis.

Six MANOVAs were conducted afterwards. First, all data were combined in a three-way ANOVA to determine the overall effects of sites, tissues and exposure time on metal contents. Then, one-way ANOVAs was used to determine significant differences in average metal contents for tissues, sites or

exposure times. Specific contrasts between levels of each factor were performed. Finally, once significant differences between control and experimental crayfish were detected, two-way ANOVA was done to test the effects of tissues and exposure time on metal contents for each site. Further exploration of the effects of sites and exposure time over the metal content of the hepatopancreas was also carried out using two-way ANOVA. Significant levels were adjusted using Bonferroni's correction. Following all significant MANOVAs, Tukey's HSD multiple comparisons test was performed between groups for the main effects, or between interacting factors where interactions were significant.

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. Whenever heterogeneity of variances and/or skewness were detected the data were transformed ($X' = \arcsin((X+0.5)^{1/2})$) to achieve normality, a feature that was re-evaluated prior to analysis (Zar, 1999).

3. Results

Values physicochemical variables measured before starting the experiment in all sites are summarised in Table 1. As expected, water temperature and pH values increase downstream. Concentration of metals (Cd, Cu, Pb, Zn and As) in riverbed sediment and stream water measured at that time as the environmental baseline conditions are presented in Table 2. Average values for all metals were exceedingly higher in Site 2 than in the other two (Table 2).

Although a significant improvement with regard to abiotic (water and sediment quality) and biotic (species richness and community structure) conditions has been documented all along the riverbed (Toja et al., 2003; Martín et al., 2005), Site 2 is still at an early successional stage with low-density macrophyte beds and poorly developed macroinvertebrate communities (Solà et al., 2003).

The experimental results showed a significant overall trend towards an increase of metal concentration with increasing exposure time for all crayfish tissues at all

Table 1
Values of the physicochemical variables measured at each of the three sites

Site	Temperature (°C)	Conductivity (μScm^{-1})	pH
Site 1	20.1	1719	7.0
Site 2	21.72	1491.4	7.4
Site 3	22.14	1645.2	7.6

Table 2

Mean±S.E.M. values of metal concentration measured in sediments ($\mu\text{g g}^{-1}$ dry wt) and in water ($\mu\text{g L}^{-1}$) samples taken in the three sites at the beginning of the experiment

Sample	Site	n	As		Cd		Cu		Pb		Zn	
			Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Sediment ($\mu\text{g g}^{-1}$, dry wt)	1	3	82.984	2.887	5.812	0.140	177.930	5.869	132.882	7.199	1486.731	53.948
	2	3	227.035	3.937	7.626	0.341	415.290	10.842	769.328	14.358	2250.065	38.082
	3	3	27.382	0.434	4.001	0.090	91.906	2.236	53.920	0.658	1229.648	23.648
Water ($\mu\text{g L}^{-1}$)	1	2	2.537	0.099	0.365	0.167	4.698	1.480	0.742	0.103	142.034	29.673
	2	2	3.024	0.041	1.605	0.144	8.873	0.579	1.463	0.187	324.567	38.670
	3	2	5.933	0.035	0.568	0.147	6.916	1.233	1.964	0.892	92.432	18.812

sites (MANOVA 1 in Table 3; Fig. 3). There was a significant effect of both experimental exposure times on the bioaccumulation of metals in crayfish tissues at all sites as compared to Control (MANOVA 2, C vs. $t_6 + t_{12}$, $P < 0.0001$; Table 3). Average metal content

measured at each site was higher than the Control (MANOVA 3, C vs. $1+2+3$, $P < 0.0001$; Table 3). Among sites, Site 2 was the one with the highest metal contents during the experiment (MANOVA 3, 2 vs. $1+3$, $P < 0.0001$; Table 3).

Table 3

Effects of exposure time (C =control, t_6 =6days, t_{12} =12days), site (C =controls 1, 2 and 3) and tissues (H =hepatopancreas, E =exoskeleton, M =muscle), and contrasts on mean metal content of crayfish (Cu, Zn, Cd, Pb, As)

Type of analysis	MANOVA, source of variation	df effect	df error	Wilks' Lambda	F	P
MANOVA 1 (three-way ANOVA)	Tissue	10	538	0.0464	195.740	<0.0001
	Sites	10	538	0.4234	28.877	<0.0001
	Exposure time	5	269	0.9336	3.822	0.0023
	Tissues vs. Sites	20	893	0.2923	20.049	<0.0001
	Tissues vs. Exposure time	10	538	0.8164	5.742	<0.0001
	Tissues vs. Sites vs. Exposure time	10	538	0.8543	4.405	<0.0001
MANOVA 2 (one-way ANOVA)	Exposure time	10	574	0.8850	3.614	<0.0001
	Contrast C vs. t_6+t_{12}	5	287	0.9002	6.361	<0.0001
MANOVA 3 (one-way ANOVA)	Sites	15	789	0.6125	10.231	<0.0001
	Contrast C vs. $1+2+3$	5	286	0.8991	6.418	<0.0001
MANOVA 4 (one-way ANOVA)	Contrast 2 vs. $1+3$	5	286	0.7049	23.935	<0.0001
	Tissues	10	574	0.0999	124.139	<0.0001
MANOVA 5 (two-way ANOVA)	Contrast H vs. $E+M$	5	281	0.1823	252.032	<0.0001
	For Site 1 and without Control					
	Tissues	10	148	0.0081	148.98	<0.0001
	Exposure time	5	74	0.7604	4.66	<0.0001
	Tissues vs. Exposure time	10	148	0.6378	3.73	0.0001
Contrast	H vs. $E+M$	5	74	0.0209	690.014	<0.0001
Contrast	t_6 vs. t_{12}	5	74	0.7604	4.662	0.0009
For Site 2 and without Control						
	Tissues	10	148	0.0055	183.79	<0.0001
	Exposure time	5	74	0.3210	9.03	<0.0001
	Tissues vs. Exposure time	10	148	0.5871	4.51	<0.0001
Contrast	H vs. $E+M$	5	74	0.0148	983.626	<0.0001
Contrast	t_6 vs. t_{12}	5	74	0.6210	9.029	<0.0001
For Site 3 and without Control						
	Tissues	10	148	0.0110	126.15	<0.0001
	Exposure time	5	74	0.8792	2.03	n.s.
	Tissues vs. Exposure time	10	148	0.5959	4.37	<0.0001
Contrast	H vs. $E+M$	5	74	0.0311	460.964	<0.0001
Contrast	t_6 vs. t_{12}	5	74	0.8792	2.032	n.s.
MANOVA 6 (two-way ANOVA)						
For Hepatopancreas without Control						
	Sites	10	148	0.0759	38.888	<0.0001
	Exposure time	5	74	0.8008	3.681	0.0049
	Sites vs. Exposure time	10	148	0.6553	3.482	0.0003
Contrast	2 vs. $1+3$	5	74	0.1256	103.007	<0.0001
Contrast	t_6 vs. t_{12}	5	74	0.8008	3.681	0.0049

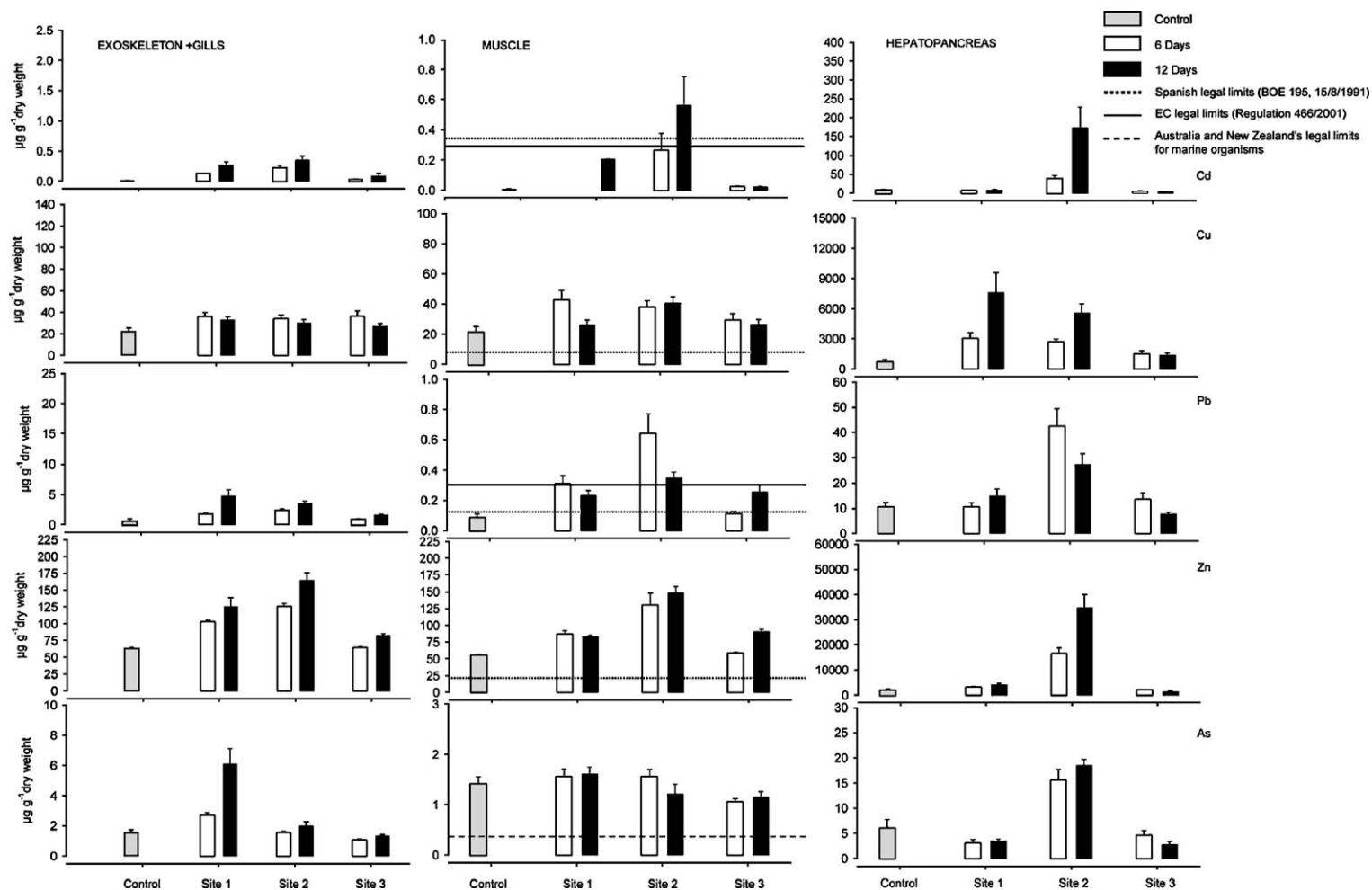


Fig. 3. Mean±S.E.M. values of metal concentration measured in different tissues of the crayfish of the translocation experiment ($\mu\text{g g}^{-1}$ fresh wt). All individuals (control and translocated) were purchased to the same seafood vendor. Horizontal dotted line corresponds to Spanish legal limits for Cu ($20 \mu\text{g g}^{-1}$ fresh wt) and for Pb and Cd ($1 \mu\text{g g}^{-1}$ fresh wt) (BOE 195, 15/8/1991). Straight line shows European legislation limits for Cd ($0.8 \mu\text{g g}^{-1}$ fresh wt) and Pb (0.5) (EC Regulation 466/2001). Short dashed line indicates maximum limits legislated for Zn ($70 \mu\text{g g}^{-1}$ fresh wt) and As ($1 \mu\text{g g}^{-1}$ fresh wt) in Australia and New Zealand for marine organisms, as reference values because there are no maximum values legislated in Europe. All these limits expressed in fresh weight have been converted to $\mu\text{g g}^{-1}$ dry weight, assuming a water content of 70% in crayfish (Rigler and Downing, 1984) thus, they were divided by a factor of 3.3.

The highest accumulations response for all sites and exposure time periods was found in the hepatopancreas followed by the exoskeleton and , the muscle (MANOVA 4, H vs. $E+M$, $P<0.0001$; Table 3).

When the effect of the exposure time on the average metal content and type of tissue was analysed site by site, no differences were found for Site 3 (MANOVA 5; Table 3). Post hoc comparison of the mean metal content of each element for all tissues pooled by exposure time was always significantly higher for t_{12} than for t_6 at Sites 1 and 2, but in Site 3, they showed the opposite pattern (except Zn which was bigger at t_{12} , $q=3.384$, $P=0.026$). This result in Site 3 is explained because crayfish were able to decrease their concentrations to about a 76% in Pb, 35% in Cd and 71% in As from the exposure time t_6 to t_{12} of the experiment in their hepatopancreas tissue, which is the one that accumulates more metals (Fig. 3).

When the hepatopancreas is the only tissue considered in the analyses a significant effect of the exposure time was found on the average metal content at all sites (MANOVA 6, t_6 vs. t_{12} , $P<0.004$; Table 3).

Post hoc comparisons revealed that the mean concentration of each metal in the hepatopancreas when they were compared in relation to sites, should be ranked in all of them as Site 2>Site 1>Site 3 (all $P<0.0001$), except for Cu, that presented the biggest value at Site 1 ($q=4.7661$, $P<0.0001$).

In general, the tissue accumulation of nonessential metals (Pb, As, Cd) reflected environmental concentrations (Fig. 3; Table 2). Other metals, like Zn and Cu, were by far the ones that showed the highest concentrations in the three tissues at all sites and independently of time exposure. They are two physiologically essential elements that are concentrated in the mainly in the hepatopancreas and regulated up to certain threshold levels, 5 to 200times higher than those of Pb and Cd.

The rapidity with which the changes in the concentrations of Pb, As, and Cd occurred in crayfish tissues indicates that there is a sensitive and dynamic equilibrium between the crayfish and their environment with regard to metals.

4. Discussion

Analyses of sediment samples revealed large amounts of metals still present at the study sites, suggesting that metal bioaccumulation in crayfish tissues might result from interactions of the organism with the substrate of the crayfish contact with the sediment rather than with the water, which contained lower levels of metals. Downstream the mine, metal

concentrations in water column tend to decrease exponentially due to adsorption to particles, precipitation, sedimentation and dilution (Solà et al., 2004). Metal concentrations in crayfish tissues from Sites 1 and 2 are higher than those from Site 3, a likely consequence of metals stored in the sediments which confirms the conclusions reached by Bradley and Morris (1986) and Alikhan et al. (1990) concerning the negative relationship between tissue metal concentration in bioindicator species and the distance of their habitat from the emission site. Anderson et al. (1997a) found a similar behaviour in the exposure experiment of crayfish to a contaminated bayou with petroleum wastes and coolant discharges from a manufacturing plant in Louisiana (USA).

According to our results, a period of exposure time to metal contamination from 6 to 12 days, was enough to produce a significant and bioaccumulation of metals in several tissues of *Procambarus clarkii*, mainly in hepatopancreas. Bioaccumulation level is related to the metabolic role of each of the metals studied and to their abundance in the surrounding environment.

Hepatopancreas and the exoskeleton (plus gills) were the tissues with the highest amount of metals. The gills are in direct contact with the environment; they have high permeability and are involved in the exchange of gases through respiration and the regulation of ion fluxes, which contributes to osmotic, excretory, and acid–base homeostasis (Vogt, 2002). Thus, they are likely one of the first logically tissues to show metal accumulation (Bollinger et al., 1997). The hepatopancreas is involved in a variety of physiological processes that include the secretion of digestive juices, the absorption and storage of digested food, and the detoxification and storage of heavy metals (Icely and Nott, 1992). It has the capability to concentrate metals from the haemolymph and the digestive tract and to store them in intracellular vacuoles F and R (Roldan and Shivers, 1987), and it is the major organ of detoxification aside from the antennal gland (Vogt, 2002). Furthermore, it is usual to find high levels of metals, specially Pb, in the exoskeleton tissue, but it has been postulated that this fact is mainly related with adsorption rather than with bioaccumulation (Knowlton et al., 1983).

The abdominal muscle has consistently been found in the literature to be the tissue containing the lowest concentration of metals (Rincón-León et al., 1988; Devi et al., 1996; Anderson et al., 1997a; Bollinger et al., 1997), and our results also agree with this, which is of special relevance for human health in this case since this is the edible part.

Each metal displays a different concentration pattern depending on their role in crayfish metabolism. Copper and zinc, which are essential micronutrients, do not accumulate in decapod crustaceans, but are regulated up to certain threshold levels. Uptake at the cell membrane level is governed by specific carrier-mediated transport, transport through protein channels, passive diffusion of lipid soluble metals and endocytosis (Rainbow, 1997). Zinc is used as an active centre for metalloenzymes and activators of other enzyme systems (carbonic anhydrase), while copper is an integral part of the respiratory pigment haemocyanin, accounting for the high copper levels observed at the hepatopancreas. Due to the fact that these metals are essential, they are also subject to strong regulation, being detoxified by metallothioneins (Canli et al., 1997), eliminated by excretion through faeces or urine, and via haemolymph through excretory organs or gills (Arumugtan and Ravindranath, 1987).

Zinc level in hepatopancreas tissue was found to be approximately six times higher than the concentration in the sediment, whereas in muscle and exoskeleton with gills, are fourteen to sixteen times lower than in the sediment. The copper content of the hepatopancreas tissue was found to be approximately sixteen times higher than in the sediment levels, and in muscle and exoskeletons seven times more than that of the sediment copper levels, consequently both elements were mainly regulated regardless of environmental levels.

Conversely, concentration of lead in the hepatopancreas was an order of magnitude lower than in the surroundings, and related to the concentration of contaminants in the environment and to changes in time exposure. Lead is not essential and tends to be detoxified by metallothioneins or phosphoric granules and stored permanently in tissues (Rubio et al., 1991; Rainbow, 1997). Thus, in our results, crayfish were found capable of doing a clearance of lead in their different tissues after diverse times of exposure to contaminants. Differences in concentration of lead of up to one order of magnitude were found among tissues (in decreasing order exoskeletons > abdominal muscle). Similar results were found in other field studies (Rincón-León et al., 1988; Madigosky et al., 1991; Khan et al., 1995) and laboratory experiments (Knowlton et al., 1983; Pastor et al., 1988; Meyer et al., 1991; Anderson et al., 1997b), which conclude that lead accumulation in crayfish is dose and time dependent (Anderson et al., 1997b). After 30 years of its arrival, *P. clarkii* has changed the structure and functioning of the invaded ecosystems where it readily occupied a central position in the food webs (Geiger et al., 2005). Thus from an ecosystem perspective, attention should also be

paid to the crayfish role as vector of pollutants as it can carry heavy metals and other harmful substances to higher trophic levels, human populations included.

Pollution level at each sampling site is related to remaining patches of contaminated soil and diffuse inputs of additional sources of pollutants independent from the mine spill occurred in 1998 (e.g. like urban sewage outputs, oil mill spills, and pesticides used in olive-tree plantations) (Solà et al., 2004).

Crayfish captured in the two more contaminated sites (Sites 2 and 1) had high levels of heavy metals that, in some cases, exceed the thresholds of Spanish regulations for trade and consumption of crustaceans (Fig. 3). According to Spanish legislation, maximum limits for heavy metals in edible wet mass are $20 \mu\text{g g}^{-1}$ for Cu and $1 \mu\text{g g}^{-1}$ for Pb and Cd (BOE 195, 15/8/1991), whereas European legislation limits are $0.8 \mu\text{g g}^{-1}$ for Cd, and 0.5 for Pb (EC Regulation 466/2001). No maximum legal limits exist in Spain for Zn and As, but adequate reference values are those provided by qualified agencies (FAD, EPA: $70 \mu\text{g g}^{-1}$ for Zn, and $1 \mu\text{g g}^{-1}$ for As (Australia and New Zealand legal limits for marine organisms). In Fig. 3, these limits have been converted to dry weight to make easier the comparison with metal concentrations measured in the different tissues and sites. These results might be relevant, for instance, for future management plans of the fishing activities in the area and for restoration plans focused on improving water quality, and therefore, the ecosystem's health.

5. Conclusions

This study shows the fast (6 days are enough) bioaccumulation response of crayfish when exposed to a polluted environment. Metals that show less concentration variability in crayfish tissues are the more toxic, metabolically non-essential, elements: Pb, Cd and As. The amount of metals that accumulates in crayfish is related to their abundance in the sediments around and can be used to identify sites under pollution stress. Copper and Zinc, are always found in high concentrations independently of their quantities in the environment because of the ability of crayfish to manipulate their levels for their own metabolic profit.

Considering that the whole area has been subjected to intensive cleaning activities after the mine spill took place, current contamination is likely to have another origin (pesticides of olive tree plantations, waste water from villages and other industrial activities).

River sediments, rather than water seem to be the source of metals that crayfish bioaccumulate.

As a corollary, we proposed to implement the use of *Procambarus clarkii* as a sentinel species to monitor environmental health of this and other Mediterranean rivers and wetlands. The protocol here applied (the measure of metal content in the hepatopancreas of originally reference individuals after exposure to study site conditions for no longer than 6 days) provides a fast, efficient and accurate indicator of contamination, if existing.

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