Ecotoxicology and Environmental Safety 185 (2019) 109682

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Food safety risk assessment of metal pollution in crayfish from two historical mining areas: Accounting for bioavailability and cooking extractability

Jaime Rodríguez-Estival*, Carlos Morales-Machuca, Jennifer Pareja-Carrera, Manuel E. Ortiz-Santaliestra, Rafael Mateo

Instituto de Investigación en Recursos Cinegéticos (IREC – CSIC, UCLM, JCCM), Ronda de Toledo 12, 13005 Ciudad Real, Spain

ARTICLE INFO

Keywords:
Bioaccessibility
Red swamp crayfish
Procambarus clarkii
Lead
Mercury
Abandoned mines

ABSTRACT

Here we characterize the bioaccumulation of mercury (Hg) and lead (Pb) in red swamp crayfish (*Procambarus clarkii*) from two river courses in Central Spain that are impacted by historical Hg and Pb mining activities, respectively. We estimate the absolute oral bioavailability of metals in crayfish tissues by means of *in vitro* bioaccessibility simulations, and assess whether their consumption may imply a health risk for humans by estimating target hazard quotients and safe consumption rates. We also study the effect of cooking crayfish on the mobilization of the metal body burden in the context of the traditional Spanish cuisine. The results showed that crayfish from the mining districts accumulated a high level of Hg and Pb pollution in both the tail muscle and the carcass. The *in vitro* bioaccessibility of Hg and Pb in the edible part was 27.86 ± 4.05 and 33.73 ± 5.91%, respectively. Absolute bioavailability was estimated to be 38.31 for Hg, and 20.21 (adults) and 67.35% (children) for Pb. Risk indices indicated that, even after adjusting for bioavailability, it is not safe to consume crayfish from the mining-impacted rivers because of their high levels of Hg and Pb. Using the carcass as a condiment for flavouring should also be avoided. The cooking procedure extracted relatively small amounts of the total Hg (8.92 ± 2.13%) and Pb (1.68 ± 0.29%) body burden. Further research that will support human and ecological risk assessment, along with the implementation of advisory measures for the local population as regards crayfish consumption, are recommended.

1. Introduction

The global production and consumption of freshwater crayfish have undergone a sharp increase over the last two decades (FAO, 2018). This growth is exclusively owing to the boom in the production of the red swamp crayfish (*Procambarus clarkii*), a crustacean species that has been introduced into and/or cultivated in many parts over the world outside its native range since the early 20th century (Gherardi and Panov, 2006; Huner and Bonham, 2011; Nagy et al., 2015). More than 90 % of the current total freshwater crayfish production in Europe is derived from *P. clarkii*, which was first legally introduced into the marshes of the Guadalquivir River (southern Spain) for aquaculture production in the 1970s, and quickly established itself in the wild (Ackefors, 1999). It is currently the most widespread and abundant crayfish species in the fluvial ecosystems of the Iberian Peninsula (Vedia and Miranda, 2013).

As a highly invasive alien species, it has triggered a series of negative socioeconomic, health and environmental impacts on the colonized ecosystems (Gherardi, 2006; Loureiro et al., 2015). However, the *P. clarkii* fishery and its associated food industry is a thriving socioeconomic sector in some parts of Spain (Conde and Domínguez, 2015; Fernández Díaz et al., 2016). Although far behind China and USA, Spain, and specifically Andalusia, is the third largest producer of *P. clarkii* in the world and the first in Europe, and is responsible for 8–10 % of the commerce of this species worldwide (Fernández Díaz et al., 2016). Moreover, outside the area in which it is caught for legal commercialization, recreational crayfish fishing is deeply rooted in the local culture throughout Spain, especially in rural areas, where *P. clarkii* constitutes an integral part of the local communities' diet for at least part of the year (Conde and Domínguez, 2015).

As a food resource, *P. clarkii* is considered a healthy dish that provides a variety of nutritional benefits (Cremades et al., 2003; El-Sherif and El-Ghafour, 2015). Nevertheless, this crayfish tends to

* Corresponding author.. .
E-mail addresses: jaime.rodriguez.estival@gmail.com, Jaime.Rodriguez@uclm.es, jaime.rodriguez@uclm.es (J. Rodríguez-Estival), Carlos.Morales2@alu.uclm.es (C. Morales-Machuca), Jennifer.Pareja@uclm.es (J. Pareja-Carrera), ManuelE.Ortiz@uclm.es (M.E. Ortiz-Santaliestra), Rafael.Mateo@uclm.es (R. Mateo).
URL: http://www.irec.es (J. Rodríguez-Estival).

https://doi.org/10.1016/j.ecoenv.2019.109682
Received 24 June 2019; Received in revised form 12 September 2019; Accepted 15 September 2019
Available online 23 September 2019

bioaccumulate large amounts of metals in its tissues (Kouba et al., 2010; Alcorlo and Baltanás, 2013), as a consequence of which it is a major vector in the effective transfer of potentially toxic metals and other pollutants to higher trophic levels, including humans, through biomagnification processes (Henriques et al., 2014; Johnson et al., 2014; El-Kady and Abdel-Wahhab, 2018). This is of particular significance for crayfish populations in freshwater environments within historical metalliferous mining districts (see, e.g., Higueras et al., 2006; Schmitt et al., 2011; Allert et al., 2013), since acid mine drainages from non-remediated mining residues make a significant contribution to the pollution of aquatic ecosystems for decades or even centuries after mines have closed (Strzebońska et al., 2017).

Monitoring residues of toxic metals in *P. clarkii* populations established in highly polluted ecosystems is of critical importance if food safety hazards are to be prevented (Peng et al., 2016a). In the context of assessing risks to human health, the risk of suffering from the toxic effects of pollutants in foodstuffs is not governed by their total concentration, but rather by their oral bioavailability, which refers to the fraction of the administered chemical that reaches the systemic circulation from the gastrointestinal (GI) tract (bioavailable fraction) (Moreda-Piñeiro et al., 2011). When *in vitro* assessment models are used, a first step in the assessment of bioavailability includes the study of bioaccessibility, a concept based on the fact that chemical dissolution in GI fluids is a necessary precursor to intestinal absorption (Ruby et al., 1999). Bioaccessibility, therefore, represents the fraction of the administered chemical that is theoretically released (solubilized) from its matrix (e.g., food, soil) into the GI fluids (bioaccessible fraction), and thus becomes potentially available for absorption (Ng et al., 2015). Failure to adequately adjust exposure estimates for the bioaccessibility or bioavailability of chemicals in specific matrices may lead to a significant misdiagnosis of health risks (Safruk et al., 2015). Furthermore, cooking can modify the levels of pollutants in food products or change their physicochemical form and bioavailability, which may also contribute to defining the risk as regards food safety (Mateo et al., 2011; Maulvault et al., 2012).

In the present study, our objective was to characterize Hg and Pb bioaccumulation in selected body parts of *P. clarkii* from naturalized populations living in two river courses impacted by historical mining districts in Central Spain, one of which is impacted by Hg pollution and the other by Pb pollution. The results may help to assess, with previous and future monitoring studies, the effect of the cessation of mining activities and the restoration measures adopted. We also assessed whether consuming crayfish from these rivers would imply a health risk for humans as the result of Hg and Pb exposure by estimating target hazard quotients (THQ) and safe monthly consumption rates (CR_{mm}) as risk indices. Moreover, we determined the absolute *in vitro* bioaccessibility of metals in crayfish tissues as a surrogate method by which to estimate their absolute *in vivo* bioavailability in order to provide a more realistic risk assessment outcome. Finally, we examined the effect of cooking crayfish on the mobilization of metal burdens, especially those in the exoskeleton and viscera, by following a traditional Spanish recipe.

2. Material and methods

2.1. Study area

This study was carried out in the main river courses flowing through two historical mining areas located in the natural region of Sierra Madrona and the Alcudia Valley, in the central part of the Iberian Peninsula (province of Ciudad Real, Spain; Fig. 1): the Valdeazogues River, which flows within the largest Hg mining area in the world – the former Almadén Mining District (Hg-AREA) (Hernández et al., 1999); and the Montoro River, which flows in what was the major Pb-producing area in Spain during the second half of the 19th century – the former Alcudia Valley Mining District (Pb-AREA) (Palero-Fernández and Martín-Izard, 2005). In both districts, mining started in Roman times and continued until the end of the 20th century (Pb-AREA, in 1979 in the Montoro area) or at the beginning of the 21st century (Hg-AREA, in 2003). Although some of the main waste dumps in the Hg-AREA were effectively restored between 2004 and 2008 (Higueras et al., 2013), most mining operations in both districts ceased in the absence of specific regulations concerning the restoration of land on mining sites after closure. As a result, nearly 52 (Hg-AREA) and 484 (Pb-AREA) decommissioned, abandoned mines and their related waste (spoil heaps, tailing ponds and other mining residues) are currently scattered throughout areas spanning about 500 and 2500 km^2, respectively (Fig. 1). After centuries of extensive exploitation and subsequent abandonment, this has led to the widespread dispersion of toxic metals into the local environment (see, e.g., Reglero et al., 2008; Higueras et al., 2012, 2016; Campos et al., 2018), has been associated with environmental and health risks for terrestrial and aquatic wildlife and livestock, and has posed a threat to food safety (see, e.g., Taggart et al., 2011; Pareja-Carrera et al., 2014; Ropero et al., 2016).

The Montoro and Valdeazogues Rivers receive significant inputs of metals owing to leachates flowing into the fluvial ecosystems from upstream and the surrounding mines scattered in these areas. The water and sediments in these rivers and/or in fluvial courses within their drainage networks have, therefore, been found to contain high levels of Hg (Valdeazogues catchment) and Pb (Montoro catchment) (Higueras et al., 2006, 2012; García-Ordiales et al., 2017). In the Hg-AREA, the average values of Hg in water courses, sediments and soils are 0.736 μg/L, 74 μg/g dry weight (d.w.) and 477.03 μg/g d.w., respectively; in the Pb-AREA, the average environmental concentrations of Pb are as high as 26.6 μg/L in waters, 8897 μg/g d.w. in soils and 52.6 μg/g d.w. in plants (Ortiz-Santaliestra et al., 2019, and references therein). A third river course in the northwest of the province of Ciudad Real, the Bullaque River, was considered as a reference (REF-AREA) (Fig. 1). This river is located in the Ciudad Real Mountains area, which is the same natural macro-region and has similar climatic and biogeographic characteristics (typical vegetation formations of the Mediterranean forest combined with pasture and agricultural fields) to the mining districts. However, it is not recognised as being rich in metallic deposits, has never been mined and is known to have background metal concentrations in soils, plants and/or stream sediments (Reglero et al., 2008).

One sampling site was set in each river course (Fig. 1). Since *P. clarkii* is an alien invasive species, its recreational fishing is authorized with no size limit in order to carry out population control in these three river courses, and the peak harvesting season runs from June through September. In the Montoro (Pb-AREA) and the Bullaque River (REF-AREA), crayfish are unrestrictedly caught for consumption by the local population during this period. However, one exception applies to the Valdeazogues River (Hg-AREA), where recreational crayfish fishing is not authorized by the regional regulation on fishing in a stretch of about 30 km, including the sampling site selected in this river course in the present study, owing to the presence of high levels of metals in crayfish (Lacal et al., 2013). This regulation also establishes the recommendation of not consuming fish and crayfish within part (about 20 km) of this stretch, but advisory signs regarding this restriction are not yet shown in the fishing areas, so that part of the local population still use it to catch crayfish for self-consumption. On the other hand, the technical report supporting this regulation highlights the need for further research to obtain conclusive results and a reliable overview of the situation (Lacal et al., 2013). Furthermore, sampling this stretch may be relevant for areas few kilometers downstream, since the Valdeazogues River joins the Guadalmez River in the Region of Extremadura, where the consumption of crayfish is not restricted.

2.2. Crayfish trapping and sample preparation

The trapping on the three sampling sites was carried out in July

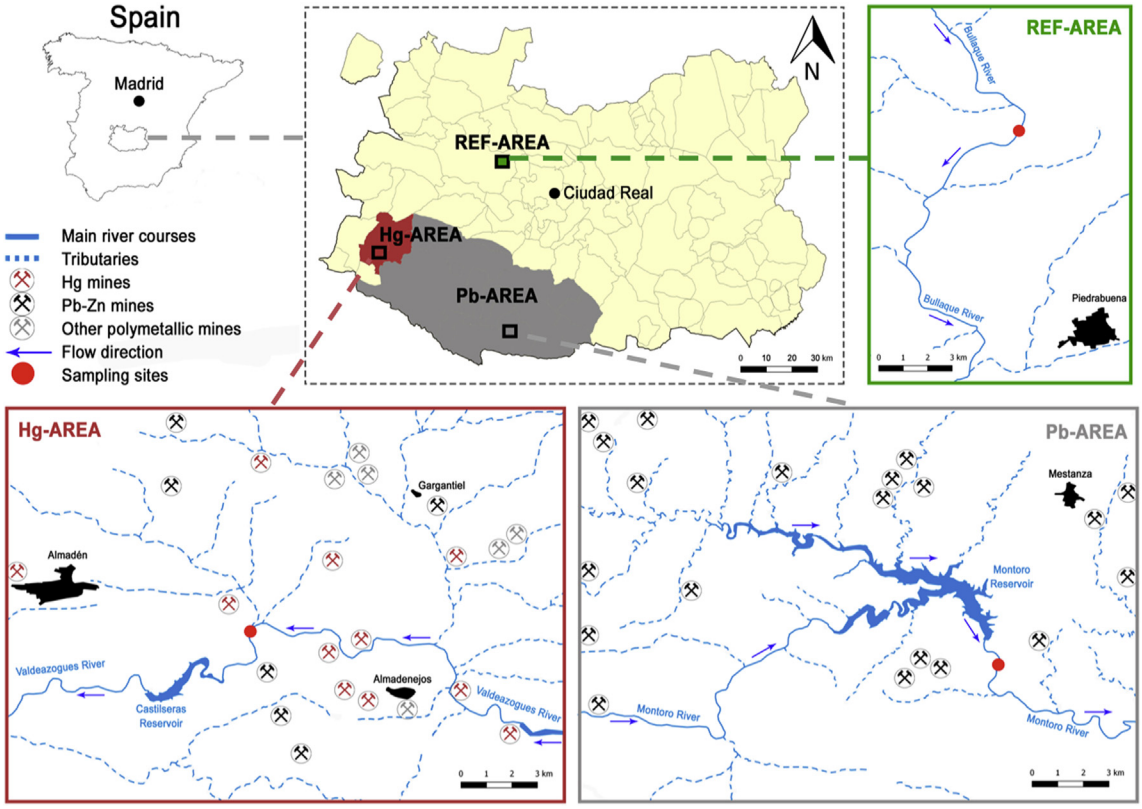

Fig. 1. Study areas within the province of Ciudad Real (Central Spain). The REF-AREA (marked with green) was located in the municipality of Piedrabuena. The Almadén Mining District (Hg-AREA, marked in dark red) comprises the municipalities of Almadén, Almadenejos, and part of the municipalities of Chillón and Almodóvar del Campo, covering about 500 km^2. The Alcudia Valley Mining District (Pb-AREA, marked in grey) comprises, totally or partially, the municipalities of Abenójar, Almodóvar del Campo, Brazatortas, Cabezarrubias del Puerto, Calzada de Calatrava, Fuencaliente, Hinojosas de Calatrava, Mestanza, Puertollano, San Lorenzo de Calatrava, Solana del Pino, Villamayor de Calatrava, and Villanueva de San Carlos, covering about 2500 km^2.

2016 using long double-funnelled traps baited with fish trimmings. Traps were set before sunrise at a depth of about 1 m and at a distance of 2–3 m from the shoreline, and were checked early in the morning. All individuals captured in the three river courses belonged to the species *P. clarkii*, and a total of 57 crayfish of a similar size (19 from each study site) were selected for this study. After collection, the crayfish were placed in independent ziplock polyethylene bags and kept on wet ice in a cooler. They were transported to the laboratory within 2 h after capture and stored at −80 °C until processing. The handling and trapping protocol followed the standards for the ethical and humane treatment of animals established by the University of Castilla-La Mancha according to national regulations. Permits for animal collection and manipulation were provided by the Department of Agriculture, Environment and Rural Development of the Junta de Comunidades de Castilla-La Mancha (references DGPFEN/SEN/avp_16_024 and 178/17).

After thawing, each specimen was cleaned with distilled water and patted dry using lint-free absorbent paper towels, and its total length (TL, mean ± SD = 101 ± 14 mm), cephalothorax length (CTL, mean ± SD = 43.77 ± 6.50 mm), body weight (BW, mean ± SD = 16.87 ± 8.22 g) and gender (sex ratio male:female = 1.6:1) were recorded. All specimens exceeded 6 cm in TL and all of them were, therefore, aged as adults and considered to be sexually mature (Nagy et al., 2015). The exoskeleton was recorded as fully formed and hardened in all the specimens, which indicates that they were in an intermolt phase of the molting process (McClain and Romaire, 2007).

The crayfish were dissected in two parts according to the different potential risk that they may represent for food safety. One part was the abdominal muscle (the white muscle in the tail), which is consumed by humans. Official food safety regulations setting Maximum Residue Levels (MRLs) of Pb or Hg in crustacean products and health risk assessments derived from crayfish consumption therefore refer to the contents in this tissue. The other part was the remainder of the crayfish, the carcass, which included the exoskeleton tissue (carapace, tergum, mouthparts, chelipeds, antennae, thoracic and abdominal appendages, etc.), the internal tissues and organs in the cephalothoracic cavity (hepatopancreas, gills, digestive tract, glands, etc.) and the hindgut removed from the upper part of the abdomen. The carcass is not directly consumed as such, but is sold by the crayfish food industry as a condiment for flavouring for its use in fish and seafood dishes, including stews, pasta and rice, or it is simply present during the cooking of the whole crayfish. Part of the metals contained in this body part of crayfish may, therefore, be released during cooking and subsequently consumed.

Once dissected, the abdominal muscle was weighed (mean ± SD = 2.07 ± 0.75 g; which represents ≈ 12 % of the BW) and minced, and all the tissues and organs constituting the carcass were fully crushed and homogenized in a mortar. The abdominal muscles and carcasses of 45 specimens (15 from each river course) were separately and individually transferred into Petri dishes and freeze-dried (Christ Alpha 1–2, Braun Biotech) to a constant weight. The muscles and carcasses of the remaining four individuals from each study site were processed as described above, and then pooled to obtain a single muscle and a single carcass sample per site in order to obtain sufficiently large sample amounts with which to conduct the *in vitro* GI bioaccessibility and cooking assays. These pooled samples were homogenized and two aliquots per sample were prepared: one aliquot was kept fresh and stored at −80 °C for the subsequent GI simulation and cooking treatment, while the other was freeze-dried to a constant weight. The freeze-dried samples of the individual and pooled abdominal muscle and carcass samples (0.2–0.4 g) were acid-digested in Pyrex tubes using a heating

block (Micro, for 40 tubes, Selecta) with an electronic controller (RAT-2, Selecta), following the methodology described by Rodríguez-Estival et al. (2011). Digest solutions were diluted to 25 ml with Milli-Q grade water and stored at 4 °C until elemental analysis.

2.3. In vitro gastrointestinal (GI) simulation

The oral bioaccessibility of Hg and Pb in the abdominal muscle and carcass was investigated through the use of a GI simulation following the standardised static *in vitro* method for GI extraction described by Minekus et al. (2014), although slight adjustments were made in accordance with our previous methods (Martinez-Haro et al., 2009; Mateo et al., 2011). Briefly, 1 g of fresh abdominal muscle or carcass was mixed with 1 ml of simulated salivary fluid (15 mM KCl, 14 mM $NaHCO_3$; pH = 7) for 5 min at 37 °C, after which 2 ml of simulated gastric fluid (47 mM NaCl, 25 mM $NaHCO_3$, 7 mM KCl, 4000 U/ml of porcine pepsine, pH = 2) were added, the pH was adjusted to 3, and the tubes were placed on an orbital shaker (Excella E24) for 2 h at 37 °C. Next, 4 ml of simulated small intestine fluid (85 mM $NaHCO_3$, 38 mM NaCl, 7 mM KCl, pH = 7) containing bile extract and porcine pancreatine were added to effective concentrations in the final digestion mixture of 0.35 % and 0.035 %, respectively. The pH was then adjusted to 7, and the tubes were again placed on an orbital shaker for 2 h at 37 °C. The final homogenates (n = 9) were centrifuged at 3350 *g* for 10 min. The supernatant liquids, whose metal content represent the absolute *in vitro* bioaccessible fraction at intestinal level with respect to the total amount ingested, were separated from the solid pellets and 1 ml was acid-digested as described above. The digest solutions were diluted to 25 ml with Milli-Q grade water and stored at 4 °C until elemental analysis. GI digestion blanks (n = 3) were obtained by adding the digestive fluids to 1 ml of Milli-Q water and were processed as the samples.

The assay was conducted in triplicate for each study site and crayfish body part using aliquots of the pooled samples. The assay was performed in 50 ml polypropylene centrifuge tubes. Simulated digestive fluids used in the oral, gastric and small intestinal phases were pre-warmed to 37 °C before use. The pH adjustments were made independently for each replicate with saturated solutions of HCl and NaOH. The total volume added to each replicate for pH adjustments (100–300 μl) was taken into account when calculating the final metal concentration in the supernatants. In each of the different digestion phases, the tube headspace was purged under a stream of N_2 in order to minimise the amount of oxygen present.

Known concentrations of Hg chloride ($HgCl_2$; [Hg] = 0.84 g/L) and Pb acetate ($Pb(CH_3CO_2)_2 \cdot 3H_2O$; [Pb] = 1.17 g/L) were also processed as described above for the subsequent calculation of *in vitro* metal bioaccessibility in relative terms (see Subsection 2.6 for further details). All reagents were of an analytical grade, and were purchased from Sigma-Aldrich, Panreac, and Merck.

2.4. Culinary treatment

The cooking process of whole crayfish was simulated under laboratory conditions using fresh aliquots of the pooled abdominal muscle and carcass. Both parts were mixed up and homogenized, taking into account that the abdominal muscle represents about 12 % of the BW, in order to simulate the whole body of crayfish at the time of cooking. About 5 g of fresh crayfish homogenate (≈ 4.4 g of carcass + ≈ 0.6 g of abdominal muscle) from each study site were heated (boiled) in Pyrex tubes using a heating block for 40 min at 100–105 °C with 2 ml of crushed tomatoes, 4 ml of white wine, and 2 ml of water. These cooking conditions, ingredients and their proportions mimic the key steps in one of the most typical Spanish recipes for crayfish, which results in a dipping tomato sauce that is consumed along with the cooked abdominal muscle. Variations to this recipe may include other steps (such as frying the crayfish in olive oil before boiling and the addition of certain spices and vegetables), but we focused on the boiling process with acidic ingredients such as tomatoes and wine because this is expected to be the part of the cooking process that most contributes to metal extractability (Mateo et al., 2011). Cooking was carried out in triplicate for each study site. The Pyrex tubes were sealed to prevent water loss during the process. The pH was measured in the cooking solutions at the beginning (3.66–3.70) and at the end (3.79–3.80) of the treatment. Once finished, the cooking homogenates (n = 9) were transferred to 50 ml polypropylene centrifuge tubes and centrifuged at 3350 *g* for 10 min. The supernatant liquids, whose metal content represents the extractable and potentially bioavailable fraction that is released from the crayfish body into the sauce during cooking, were separated from the solid pellets, and 1 ml was acid-digested as described previously. The digest solutions were diluted to 25 ml with Milli-Q grade water and stored at 4 °C until elemental analysis. Cooking blanks (n = 2) were obtained by adding crushed tomatoes, white wine and water to 5 ml of Milli-Q grade water and were processed as the samples.

2.5. Elemental analysis and quality control

The total Hg in digest solutions was analyzed using a cold vapour technique using a mercury/hydride generation system (MHS-15, PerkinElmer) coupled to an atomic absorption spectroscopy system (AAS; AAnalyst 800, PerkinElmer), whereas the Pb was analyzed using a graphite furnace coupled to the AAS system. Procedural blanks (n = 9) and certified reference material (lobster hepatopancreas, TORT-3, National Research Council, Canada; n = 8) were also processed and analyzed for quality control. The calibration standards were prepared by means of the dilution of Hg and Pb certified commercial stock solutions (Panreac). Limits of detection (LOD, μg/g d.w.; back-calculated to in tissue concentrations for each batch of analysis using the blank data) were 0.062 and 0.034 (individual abdominal muscle and carcass) and 0.029 and 0.041 (pooled samples of abdominal muscle and carcass, and supernatants from the GI simulation and the cooking treatment) for Hg and Pb, respectively. Recoveries (%, mean ± SD) of the certified reference material ranged between 89.4 ± 6.8 and 112.8 ± 9.8 for Hg, and 87.0 ± 6.1 and 115.6 ± 10.5 for Pb. Duplicate analyses were conducted in three samples randomly selected in each batch of analysis in order to measure repeatability, which was in all cases within ranges of the relative standard deviation lower than ± 7.0 % for both metals.

Food safety regulations establish the MRLs of chemical contaminants in foodstuffs on a wet weight (w.w.) basis. The GI simulations and the cooking treatment were, therefore, carried out using fresh samples as a proxy to more realistic conditions. Total metal concentrations in the abdominal muscle and carcass of crayfish (which were analyzed using freeze-dried samples) were reported in μg/g w.w. by applying tissue-specific dry residue values (mean ± SD: 17.4 ± 1.8 % for abdominal muscle and 35.1 ± 3.3 % for carcass). These dry residue values were also applied in order to derive a wet to dry weight (d.w.) conversion factor of 5.75 (w.w. × 5.75 = d.w.) for abdominal muscle and of 2.85 (w.w. × 2.85 = d.w.) for carcass, in order to compare our results in w.w. with published data expressed on a d.w. basis. The total body burden of metals was calculated by means of the sum of the metal content in the abdominal muscle and carcass, considering that they represent 12 % and 88 % of the BW, respectively.

2.6. Calculations of in vitro metal bioaccessibility and cooking extractability, and estimation of in vivo metal bioavailability

The absolute *in vitro* bioaccessibility (IVBAC) of Pb and Hg (%) at the intestinal level was defined as 100 × (amount of metal in supernatant after GI simulation) / (total amount of metal in the abdominal muscle or carcass before GI simulation). Cooking extractability (%) was defined as 100 × (amount of metal in supernatant after cooking) / (total amount of metal in the crayfish homogenate before cooking). The

results from these calculations were obtained using the Hg data from the Hg-AREA and the Pb data from the Pb-AREA, since the Hg and Pb levels were, in all other cases, too low (very low or below the respective LOD for each element) to allow accurate estimations.

Several *in vitro-in vivo* validation studies have shown that the extent of Pb solubilization in a simulated GI environment is predictive of the oral bioavailability of this metal in animal models (e.g., Juhasz et al., 2009, and references therein). In these cases, *in vitro* Pb bioaccessibility is commonly expressed in relative terms, i.e., with respect to values obtained for highly soluble pure compounds such as Pb acetate. As this reference compound has been experimentally used *in vivo* to calculate its oral bioavailability in mammals, the bioavailability of another Pb source can be calculated from its relative bioaccessibility to Pb acetate. In this study we can, therefore, estimate a relative *in vitro* bioaccessibility (Rel-IVBAC) for Pb from crayfish tissues by dividing our observed IVBAC values for this metal in crayfish abdominal muscle or carcass by the IVBAC value obtained for Pb acetate. Age is a well-established determinant of Pb absorption, and it is considered that adults normally absorb up to 15 %, whereas children absorb approximately 50 % (Ruby et al., 1999). It is consequently possible to estimate the absolute *in vivo* bioavailability (ABA) of Pb in crayfish tissues by multiplying the Rel-IVBAC values by a correction factor obtained from *in vivo* bioavailability of 0.15 in adults or 0.5 in children (Mateo et al., 2011).

Although estimations of *in vivo* bioavailability of Hg from *in vitro* simulations have not yet been accurately validated (Bradley et al., 2017), estimates of relative Hg bioaccessibility from *in vitro* extraction/dissolution tests are still considered to be an adequate surrogate for relative Hg bioavailability (Paustenbach et al., 1997). It is thus possible to tentatively follow the same rationale as that employed for Pb to estimate ABA values for Hg in crayfish abdominal muscle and carcass. For this metal, the Rel-IVBAC values were calculated by using the IVBAC obtained for Hg chloride, which is considered a highly soluble reference mercuric compound in toxicological studies. The Rel-IVBAC values were then multiplied by a factor of 0.8 to obtain the ABA for Hg in a worst-case scenario, since the oral absorption rates (*in vivo* bioavailability) of methylmercury (MeHg) are around 80 % (Berntssen et al., 2004;EFSA , 2012). MeHg is the form of Hg that is most efficiently accumulated in aquatic crustaceans, including *P. clarkii*, and ranges between 70 % and 100 % (Hothem et al., 2007; Kouba et al., 2010; Carrasco et al., 2011; Peng et al., 2017), and the total Hg content that we have determined in crayfish tissues can, therefore, be considered as a proxy for this chemical form of Hg (Johnson et al., 2014). Finally, variations in MeHg absorption kinetics have also been related to age in animal studies, but a further gap exists as regards clearly defined differences between adults and children (Mergler et al., 2007). We, therefore, conservatively considered that Hg absorption at intestinal level is equally efficient in both age groups.

2.7. Risk calculations

The potential risks to human health as a result of exposure to Hg and Pb through the consumption of crayfish abdominal muscle were evaluated by determining the exposure levels above which these metals are likely to be associated with adverse, non-carcinogenic health effects, as recommended by the U. S. Environmental Protection Agency (USEPA. 2001). We calculated target hazard quotients (THQ) as the ratio between the estimated daily intakes (EDI) as a consequence of crayfish consumption and the toxicity reference values (TRV). Values of exposure higher than the reference values (i.e. THQ > 1) indicate that exposures are likely to cause chronic systemic effects. Health risks were evaluated for two subpopulation groups, children and adults, in 5 different scenarios of crayfish consumption (Scenarios 1 to 5: 2, 4, 17, 35, and 70 days/year). The THQ (unitless) for a specific metal *i* (Hg or Pb) was, therefore, quantified according to Equation (1):

$$THQ_i = EDI_i / TRV_i \quad (1)$$

where EDI is expressed in μg/kg-day, the TRV value for Hg is 0.1 μg/kg-day, and the TRV value for Pb is (0.02 μg/kg-day). The criteria employed to select these TRV values, which allow a conservative risk assessment, are detailed in Table S1 of the Supplementary material. With regard to the different exposure scenarios, we took into account that recreational crayfish fishing and consumption are strongly seasonal, with the peak season in Spain generally running from June through September (122 days/year). The frequency of crayfish consumption can, therefore, be expressed by considering both the whole year (days/year) and the four months of the peak season (days/week or days/month) (see Table S1 of the Supplementary material for further details). The cumulative hazard quotient, which is calculated using the sum of the THQ values of the individual metals, was not estimated here owing to the very low levels of Hg and Pb in crayfish outside the Hg- and Pb-AREA, respectively.

The EDI values were obtained by multiplying the averaged daily consumption rates over a specified duration of exposure by the corresponding metal concentrations in the edible part of the crayfish (the abdominal muscle), expressed as a function of body weight, according to Equation (2):

$$EDI_i = (C_i \times IR \times ED \times EF)/(BW \times AT) \quad (2)$$

where: C_i is the concentration (μg/g, w.w.) of metal *i* in the abdominal muscle of the crayfish; IR is the ingestion rate (g of abdominal muscle/day, assuming a daily meal size of 72 g for children and 168 g for adults); ED is the exposure duration (years, considering a lifetime of 6 years for children and 78 years for adults); EF is the exposure frequency (days/year, assessing 2, 4, 17, 35, and 70 days/year as different scenarios of exposure); BW is the body weight (kg, assuming 20 and 70 kg for children and adults, respectively), and AT is the averaging time (days) (days, ED × 365 days/year). The criteria employed to select these values are detailed in Table S1 of the Supplementary material.

Furthermore, we estimated maximum safe monthly consumption rates (CR_{mm}; USEPA, 2000), i.e., the maximum allowable number of crayfish meals that could be consumed per month over a year that would not be expected to cause any chronic effect owing to exposure to a specific metal *i* (Hg or Pb), according to Equation (3):

$$CR_{mmi} = (TRV_i \times BW \times T_{ap})/(C_i \times MS) \quad (3)$$

where: the TRV values for the metal *i* (Hg or Pb, expressed in mg/kg-day) and BW (kg) were as detailed in Table S1 of the Supplementary material; T_{ap} (expressed in days/month) is the average time period in a month (4.3 weeks/month × 7 days/week); C_i is the concentration (expressed in mg/kg, w.w.) of metal *i* in the abdominal muscle of the crayfish, and MS is the meal size (as detailed in Table S1, but expressed in kg). Number of meals < 0.5/month indicates that consumption is not recommended since there is an obvious risk of exposure that might have negative effects on health, whereas number of meals > 16/month signifies that consumption can be considered safe and unrestricted (USEPA, 2000).

The toxicity reference values are based on critical toxicity studies in which reference compounds (Hg chloride and Pb acetate) are used. The THQ and CR_{mm} values calculated as detailed above, therefore, implicitly assume that the ingested dose of metals through crayfish consumption is equal to the absorbed dose, providing a conservative estimate of the health risk. However, Hg and Pb in crayfish tissues may not solubilize in GI fluids as highly soluble reference compounds under *in vivo* conditions. A number of factors (e.g., matrix properties, the chemical form of the metal, age, nutritional status) determine that only a fraction of ingested metals is solubilized in GI fluids (*in vivo* bioaccessible fraction, which represents a combined contribution of human and bacterial digestion to physiological solubilization), and that only a fraction of the latter is truly absorbed at a gastrointestinal level (Ruby et al., 1999). We, therefore, also calculated these risk indices by adjusting dietary exposure in Equations (2) and (3) with the values of absolute *in vivo* bioavailability (ABA) for Hg and Pb in the crayfish

abdominal muscle, estimated as detailed in Subsection 2.6, in order to provide a more realistic risk assessment.

2.8. Statistical analysis

For statistical purposes, Hg and Pb concentrations < LOD were assigned a value of half of the respective LOD for each element. Variables that did not have a normal distribution after testing for normality (Shapiro-Wilk normality test) and homogeneity of variance (Levene test) were log transformed (natural logarithm). Differences among the three study areas as regards metal bioaccumulation in crayfish and the risk that crayfish consumption represents for humans were studied through the use of General Linear Models (GLMs), including the area (REF-AREA vs. Hg-AREA vs. Pb-AREA) as a predictor term and Pb or Hg concentrations in abdominal muscle, carcass or total body burden as dependent variables. When a significant site-related difference was detected, specific differences between areas were studied by means of post-hoc Tukey tests. Gender (males vs. females) was also tested in the models in order to consider potential biases. A backward stepwise procedure, consisting of eliminating those terms that did not produce significant effects by themselves or when interacting with others, was used to discover the best-fit model. Relationships among body measurements and metal bioaccumulation were explored by including TL, CTL and BW separately as covariates in the models. Significant relationships were then studied for each area separately by calculating Pearson linear correlation coefficients. Tests were performed using IBM SPSS Statistics 23.0 software. Significance was set at the 0.05 level.

3. Results

Crayfish from the Hg-AREA had about 37-fold and 15-fold higher Hg levels in the abdominal muscle and carcass, respectively, than those from the REF-AREA and the Pb-AREA (all $F_{2,42} \geq 135.22$, $p \leq 0.001$; Table 1). However, crayfish from the Pb-AREA had about 175-fold and 2000-fold higher Pb levels in abdominal muscle and carcass, respectively, than those from the REF-AREA and the Hg-AREA (all $F_{2,42} \geq 148.87$, $p \leq 0.001$; Table 1). Similar differences among the study areas were also found when the total body burden of metals was considered (all $F_{2,42} \geq 171.85$, $p \leq 0.001$; Table 1). In the pooled samples, Hg and Pb levels in abdominal muscle were within the ranges observed in the individual samples (Table 1).

The interaction "Study area × Gender" had a significant effect in the model, explaining site-related differences for Hg levels in carcass ($F_{2,39} = 6.01$, $p = 0.005$), showing that Hg accumulation in the carcass of crayfish from the Hg-AREA was higher in females (0.20 ± 0.06 μg/g w.w.) than in males (0.13 ± 0.04 μg/g w.w.). The accumulation of Hg in abdominal muscle and its interaction with the study area also had a significant effect when included in this model (both $F_{2,36} \geq 4.18$, $p \leq 0.048$), showing a positive relationship between Hg levels in carcass and abdominal muscle in crayfish from the Hg-AREA (Fig. 2a). Finally, the BW, abdominal muscle weight, CTL and TL also made a significant contribution to the site-related differences for Hg levels in carcass through an interacting effect with the study area (all $F_{2,39} \geq 3.82$, $p \leq 0.031$), revealing negative correlations between Hg accumulation in this body part and these morphometric parameters in crayfish from the Hg-AREA (Fig. 2b).

The observed IVBAC values of Pb and Hg in the abdominal muscle and carcass of *P. clarkii*, the calculated values of Rel-IVBAC and the estimated ABA for both metals in both crayfish body parts are provided in Table 2. The IVBAC of Hg chloride and Pb acetate were 58.33 ± 0.96 and 25.04 ± 0.67 %, respectively. The cooking procedure extracted 8.92 ± 2.13 and 1.68 ± 0.29 % (means ± SD) of the total Hg and Pb burden, respectively, in the homogenates of pooled abdominal muscle and carcass.

Hg and Pb in the abdominal muscle of crayfish from outside the Hg- and Pb-AREA showed that, in all cases, the THQ values were well below 1, and that the CR_{mm} values were well above 16 meals/month for both adults and children. When considering the total Hg levels in the abdominal muscle of crayfish from the Hg-AREA, the THQ values exceeded the critical threshold of 1 in Scenarios 4 and 5 for both adults and children (Fig. 3). When they were adjusted by the ABA of Hg in this body part, the THQ values were still above 1 in Scenario 5 for children (Fig. 3). Notwithstanding, the THQ for Hg for adults in Scenario 5 was very close to 1 (mean ± SD: 0.87 ± 0.20). When considering the total Pb levels in the abdominal muscle of crayfish from the Pb-AREA, the THQ values exceeded the critical threshold of 1 from Scenario 2 onwards for adults and in all exposure scenarios for children (Fig. 4). Notwithstanding, the THQ for Pb for adults in Scenario 1 was very close to 1 (mean ± SD: 0.93 ± 0.57). When the THQ values were adjusted by the ABA of Pb in abdominal muscle, they were still above 1 from Scenarios 3 and 2 onwards for adults and children, respectively (Fig. 4).

The CR_{mm} values for Hg for crayfish from the Hg-AREA were above the critical threshold of 0.5 meals/month when both the total and

Table 1
Concentrations of Hg and Pb in abdominal muscle, carcass and whole body of red swamp crayfish (*Procambarus clarkii*) captured at the Bullaque (REF-AREA), Valdeazogues (Hg-AREA) and Montoro (Pb-AREA) rivers.

Metal	Tissues		Study area *		
			REF-AREA	Hg-AREA	Pb-AREA
		N individual/N pool	15/4	15/4	15/4
Hg (μg/g w.w.)	Abdominal muscle	Geometric mean (95% CI)	< LOD [b]	0.436 (0.388–0.490) [a]	0.014 (< LOD - 0.023) [b]
		Range	< LOD - 0.044	0.298–0.625	< LOD - 0.071
		Pool	0.024	0.301	0.021
	Carcass	Geometric mean (95% CI)	0.012 (0.010–0.014) [b]	0.167 (0.140–0.199) [a]	0.010 (0.009–0.012) [b]
		Range	0.009–0.030	0.091–0.310	0.009–0.021
		Pool	0.017	0.288	0.020
	Whole body	Geometric mean (95% CI)	0.012 (0.010–0.014) [b]	0.204 (0.174–0.239) [a]	0.012 (0.010–0.013) [b]
		Range	0.009–0.028	0.122–0.368	0.009–0.021
Pb (μg/g w.w.)	Abdominal muscle	Geometric mean (95% CI)	0.006 (< LOD - 0.010) [b]	< LOD [b]	1.051 (0.753–1.467) [a]
		Range	< LOD - 0.023	< LOD - 0.017	0.287–2.801
		Pool	0.018	0.018	0.871
	Carcass	Geometric mean (95% CI)	0.032 (0.013–0.079) [b]	0.009 (< LOD - 0.019) [b]	28.22 (25.09–31.74) [a]
		Range	< LOD - 0.176	< LOD - 0.065	18.73–38.86
		Pool	0.093	0.053	42.01
	Whole body	Geometric mean (95% CI)	0.031 (0.013–0.071) [b]	0.008 (< LOD - 0.017) [b]	24.29 (21.59–27.32) [a]
		Range	< LOD - 0.160	< LOD - 0.053	15.64–34.47

* Superscript letters indicate significant differences (Tukey tests: p ≤ 0.05) between study areas for each parameter.

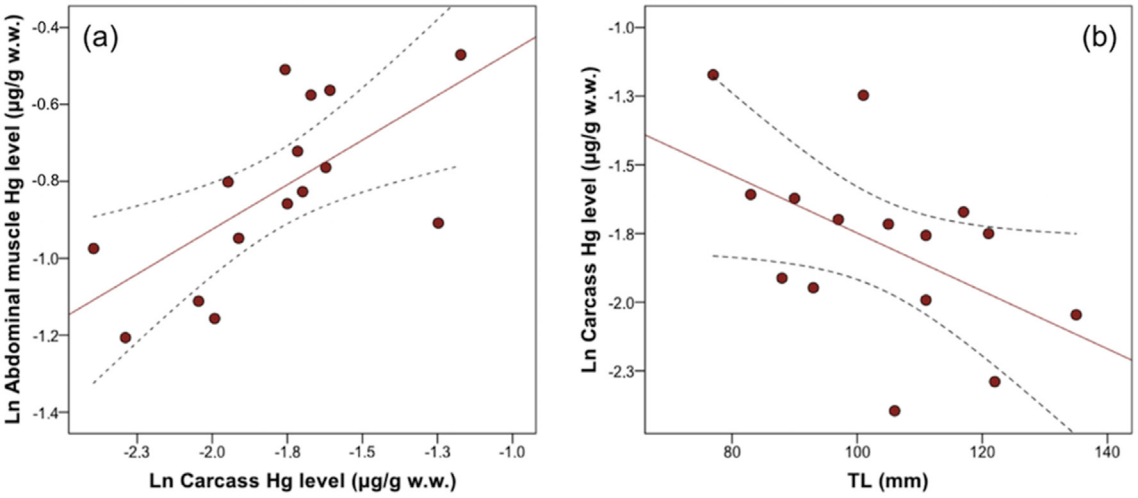

Fig. 2. Relationship between Hg levels in abdominal muscle and carcass (a) ($y = 0.46\ x + 0.004$; $r = 0.661$, $p = 0.007$) and Hg levels in carcass and TL (b) ($y = -0.01\ x - 0.69$; $r = -0.551$, $p = 0.049$) in crayfish from the Hg-AREA.

Table 2
Absolute *in vitro* bioaccessibility (IVBAC, %; mean ± SD), relative *in vitro* bioaccessibility (Rel-IVBAC, %), and absolute *in vivo* bioavailability (ABA, %) of Hg and Pb in abdominal muscle and carcass of red swamp crayfish (*Procambarus clarkii*).

			Crayfish body part	
Metal	Parameter		Abdominal muscle	Carcass
Hg	IVBAC		27.86 ± 4.05	52.14 ± 10.44
	Rel-IVBAC		47.76	89.39
	ABA		38.21	71.51
Pb	IVBAC		33.73 ± 5.91	1.02 ± 0.30
	Rel-IVBAC		134.70	4.07
	ABA	Adults	20.21	0.61
		Children	67.35	2.04

bioavailable levels of Hg in abdominal muscle were considered for both adults and children (i.e., values < 0.5 indicate that consumption is not recommended), although they were well below the safe threshold level of 16 meals/month (i.e., values > 16 indicate that consumption may be unrestricted) (Fig. 5). When the total Pb levels in crayfish abdominal muscle were considered, the CR_{mm} values for crayfish from the Pb-AREA were below 0.5 meals/month for both adults and children (i.e., consumption is not recommended). When calculations were adjusted by the ABA of Pb in abdominal muscle, the CR_{mm} for adults reached a value of 1.48 ± 1.08 (mean ± SD), whereas it was still below the critical threshold of 0.5 for children (Fig. 5).

4. Discussion

4.1. Interpreting metal residues in crayfish

P. clarkii is considered a good bioindicator for metal pollution, as the accumulation of metals in its tissues is dose- and time-dependent, thus reflecting the levels of metals in the surrounding freshwater environment (Kouba et al., 2010). Our results indicated that populations of crayfish in the Valdeazogues (Hg-AREA) and Montoro (Pb-AREA) rivers respectively take in and assimilate the Hg and Pb pollution that still persists in these two historical mining districts. The levels of Hg and Pb in the abdominal muscle of crayfish from the Hg- and Pb-AREA, respectively, were well above those commonly found in the muscle tissue of freshwater crustaceans from reference (unpolluted) aquatic environments worldwide (Hg: 0.27–0.88 µg/g d.w. ≈ 0.05–0.15 µg/g w.w.; Pb: 0.06–2.25 µg/g d.w. ≈ 0.01–0.39 µg/g w.w.; Kouba et al., 2010), and are in line with those found in other research into the bioaccumulation of metals in crayfish from metal-polluted environments. For example, in previous research carried out in the Valdeazogues River (Hg-AREA), Higueras et al. (2006) found Hg levels in abdominal muscle ranging from 2.38 to 9.06 µg/g d.w. (≈ 0.41–1.58 µg/g w.w.) in crayfish near an important open pit located about 9 km upstream of our sampling site. Mercury levels in the abdominal muscle of crayfish from the Hg-AREA were also very similar to those found in the *P. clarkii* population inhabiting the Flix reservoir in the Ebro River basin (NE Spain), which is considered to be highly impacted by industrial Hg pollution, where the total Hg concentration in abdominal muscle was 0.59 ± 0.28 µg/g w.w. (mean ± SD), ranging from 0.39 to 0.79 µg/g w.w. (Carrasco et al., 2011). Lead levels in the abdominal muscle of crayfish from the Pb-AREA were in the range of those found by Stanek et al. (2017) in a Polish lake strongly affected by industrial pollution (mean ± SD: 14.94 ± 3.36 µg/g d.w.; ≈ 2.60 ± 0.58 µg/g w.w.). The carcasses of *P. clarkii* from the Hg-AREA and the Pb-AREA also reflected the absorption of high levels of environmental pollution. For example, the total Hg levels found in the carcass of crayfish from the Hg-AREA in the present study were, on average, about 4-fold higher than those found by Hothem et al. (2007) in the carcass of crayfish from several relatively clean aquatic ecosystems (mean = 0.04 µg/g w.w.).

In crustacean species, a number of factors including gender and body size may have an influence on metal bioaccumulation (Mancinelli et al., 2018). In the present study, Hg accumulation in the carcasses of female crayfish from the Hg-AREA was higher than in males, and the bioaccumulation of Hg in carcass correlated positively with Hg in abdominal muscle and negatively with several morphometric parameters. These results could be associated with male and females' differential abilities to withstand exposure to Hg or with increasing body size in larger mature individuals. Tunca et al. (2013) suggested that high metal accumulation in crayfish may be linked to growth retardation, that specimens with high metal accumulation may perish before attaining large sizes, or that small crayfish may have higher exposure rates than large individuals because of differences in feeding habits. Whatever the case may be, our observed gender- and size-related differences in Hg contents occurred in carcass, and their impact on food safety is, therefore, expected to be limited. In this regard, it is worth noting that our results showed different metal bioaccumulation patterns depending on their toxicokinetics in crayfish. The bioaccumulation of Hg in crayfish from the Hg-AREA was, on average, 2.6-fold higher in abdominal muscle than in carcass, which is consistent with the fact that muscle tissue has a relatively high protein content and that Hg has a high affinity for the sulphur-containing amino acid cysteine in proteins

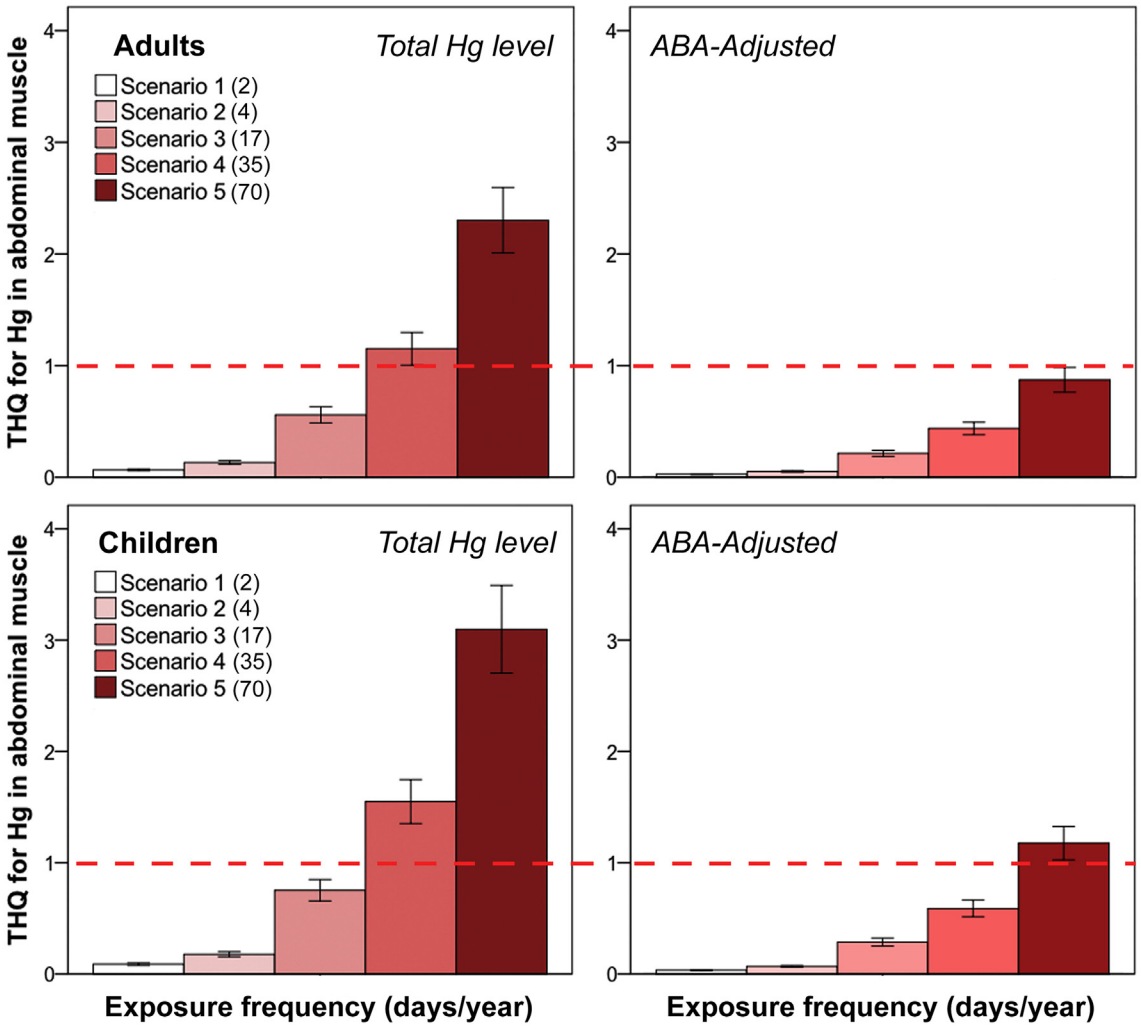

Fig. 3. Target hazard quotients (THQ) for Hg in abdominal muscle of red swamp crayfish (*Procambarus clarkii*) from the Valdeazogues River (Hg-AREA), based on total metal concentration and its estimated absolute *in vivo* bioavailability (ABA), for adults and children under different scenarios of crayfish consumption (Scenarios 1 to 5: 2, 4, 17, 35, and 70 days/year). The red dashed line shows the critical threshold value of 1 established for this risk parameter. Error bars represent 95 % confidence intervals.

(Henriques et al., 2014). This signifies that Hg tends to be largely accumulated in the muscle in comparison to other tissues (Hothem et al., 2007). In contrast, Pb in crayfish from the Pb-AREA was 27-fold higher in carcass than in abdominal muscle, which is attributable to the presence of the hepatopancreas, the gills, the exoskeleton and viscera in this body part, as these tissues accumulate the highest amounts of most metals, including Pb, in crayfish (Alcorlo et al., 2006; Kouba et al., 2010).

4.2. Total metal concentrations in food safety risk assessment

The levels of Hg and Pb in crustacean food products are strictly regulated in most countries worldwide through the establishment of official Maximum Residue Levels (MRLs). Foodstuffs not complying with the established MRLs shall not be used as food ingredients nor be placed on the market, as they could pose a health risk for consumers. In the present study, the MRLs established by the European Union legislation on food safety for total Hg and Pb in the muscle meat of crustaceans (0.5 μg/g w.w. for both metals; Commission Regulation (EC) 1881/2006, and its amendment Commission Regulation (EU) 420/2011) were exceeded in 27 % and 87 % of the crayfish abdominal muscle from the Hg-AREA and the Pb-AREA, respectively. This indicates that the consumption of crayfish from these mining districts might pose a health risk for human consumers. This is of special concern for crayfish from the Montoro River (Pb-AREA), where average Pb levels in abdominal muscle duplicated the maximum concentration allowed for human consumption.

When considering the total levels of metals in a particular food matrix in food safety risk assessments, ingested dose is a valid approach for exploratory screenings aimed at identifying potential health risks for consumers (Peng et al., 2016a; Gusso-Choueri et al., 2018). In this regard, the total Hg levels in the abdominal muscle of crayfish from the Hg-AREA make it advisable not to consume crayfish more than 35 days/year (Scenario 4, which is equivalent to 2 days/week throughout the peak season) in the case of both adults and children. According to our estimated safe consumption rates, adults and children should not eat more than 2.94 ± 0.68 and 1.96 ± 0.46 (mean ± SD) meals of crayfish from the Hg-AREA per month, respectively. The total Pb levels in the edible part of crayfish from the Pb-AREA make it advisable not to consume it more than 4 days/year (Scenario 2, which is equivalent to 1 day/month throughout the peak season) for adults, whereas children should not even reach a consumption rate of 2 days/year (Scenario 1). Our estimated safe consumption rates suggest that the consumption of the abdominal muscle of crayfish from the Pb-AREA should be completely restricted for both adults and children in order to avoid unnecessary risks of undergoing subtle adverse effects on their health.

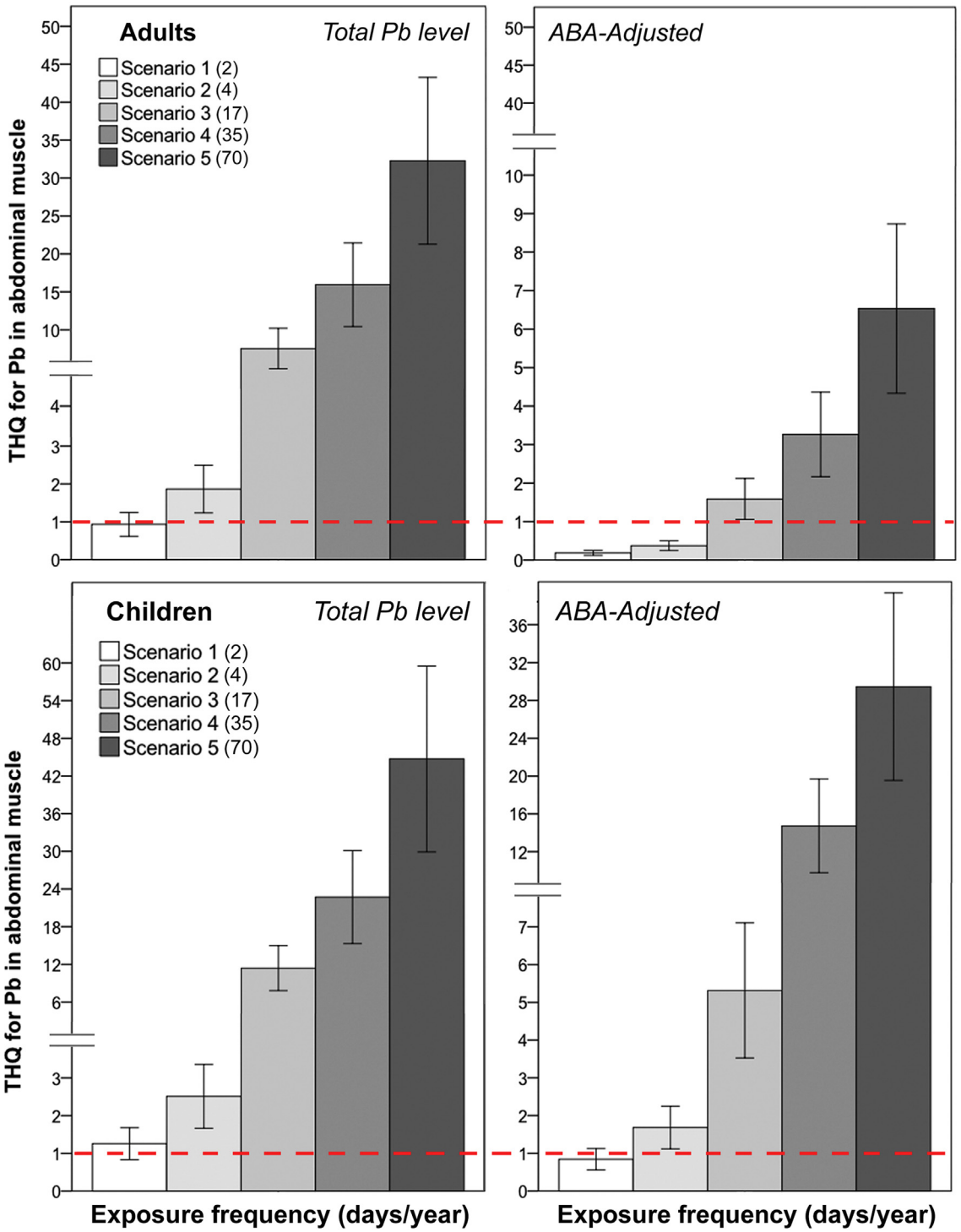

Fig. 4. Target hazard quotients (THQ) for Pb in abdominal muscle of red swamp crayfish (*Procambarus clarkii*) from the Montoro River (Pb-AREA), based on total metal concentration and its estimated absolute *in vivo* bioavailability (ABA), for adults and children under different scenarios of crayfish consumption (Scenarios 1 to 5: 2, 4, 17, 35, and 70 days/year). The red dashed line shows the critical threshold value of 1 established for this risk parameter. Error bars represent 95 % confidence intervals.

4.3. Accounting for bioavailability of metals in food safety risk assessment

Nevertheless, health risk assessments of dietary exposure to metals may be overestimated when based on total levels in foodstuffs. Metals in crayfish tissues may not solubilize completely during gastrointestinal passage, which would determine that only a fraction of the ingested dose is truly absorbed at intestinal level. The bioaccessibility or bioavailability of metals in crayfish must, therefore, be taken into account in order to provide more accurate and realistic risk assessments (Ruby et al., 1999; Bradley et al., 2017). The *in vitro* bioaccessibility of metals in foodstuffs is, on numerous occasions, used in exposure assessments as a surrogate for *in vivo* bioaccessibility or maximum *in vivo* bioavailability (Amiard et al., 2008; Laird and Chan, 2013; Peng et al., 2016b). However, this may also lead to the misdiagnosis of health risks, since *in vitro* bioaccessibility may not be precisely equal to *in vivo* bioavailability. *In vitro* results can, therefore, be used to estimate or predict the

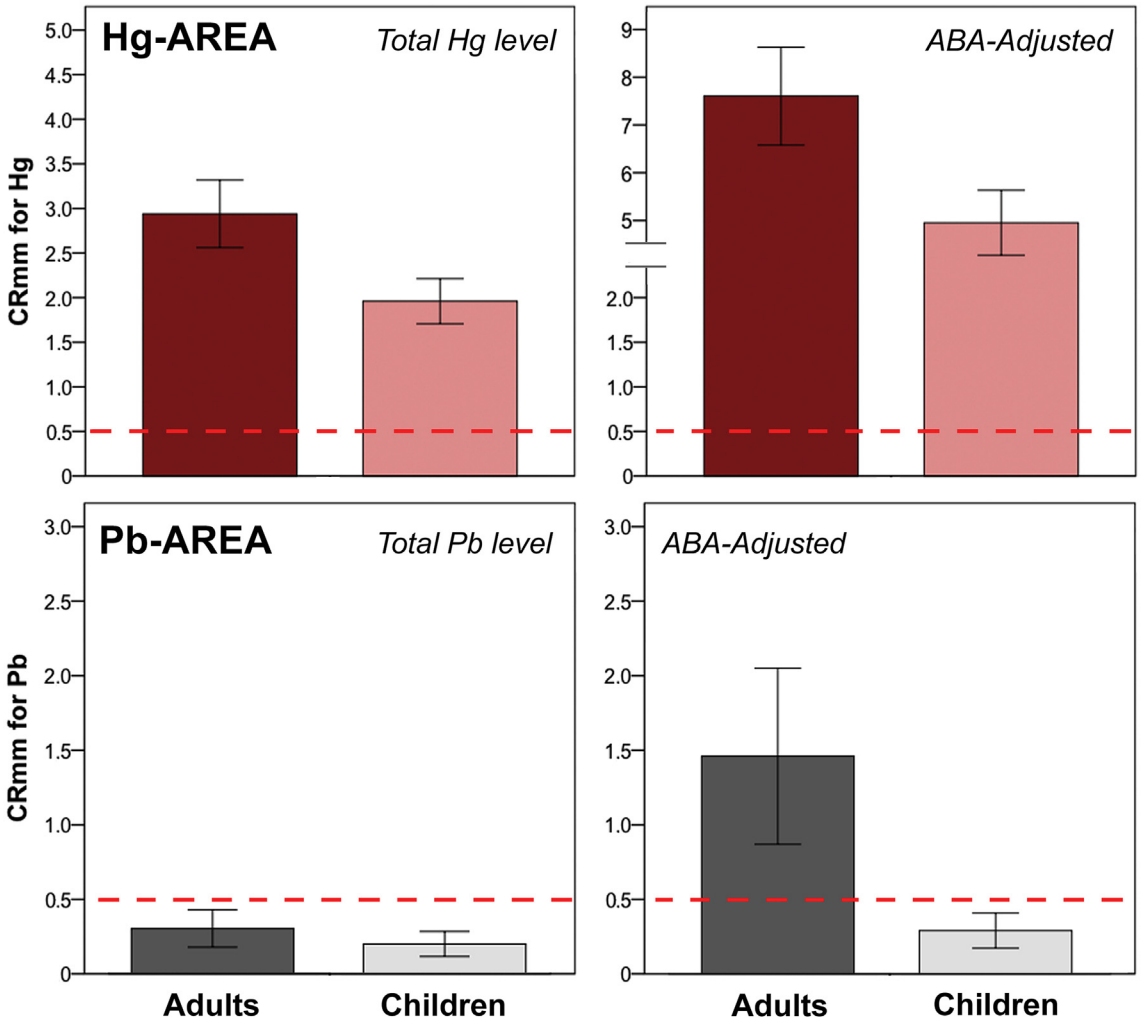

Fig. 5. Maximum allowable monthly consumption rates (CR_{mm}; meals/month) for Hg and Pb levels in abdominal muscle of red swamp crayfish (*Procambarus clarkii*) from the Valdeazogues (Hg-AREA) and Montoro (Pb-AREA) rivers, respectively, based on total metal concentration and its estimated absolute *in vivo* bioavailability (ABA), for adults and children. The red dashed line shows the critical threshold value of 0.5 established for this risk parameter. Error bars represent 95 % confidence intervals.

expected *in vivo* outcomes with good confidence when they are based on reliable *in vitro-in vivo* validations, which include the consideration of bioaccessibility and bioavailability in relative terms with respect to reference materials (Drexler and Brattin, 2007; Juhasz et al., 2009; Mateo et al., 2011).

The IVBAC values observed here for Hg and Pb in the abdominal muscle of crayfish coincided with those attained in other investigations using *in vitro* extracting methods that mimic the metal solubilization from shellfish and fish by digestive fluids. Peng et al. (2017) reported that Hg bioaccessibility (indicated by the digestive solubilization rate of MeHg) in the tail muscle of *P. clarkii* ranged from 3.7 % to 39.3 %. Amiard et al. (2008) reported values of Pb bioaccessibility in seven species of shellfish, ranging from 19 % to 52 % (median = 33%), while Intawongse et al. (2012) found that the GI bioaccessibility of Pb ranged from 28 % to 51 % in oysters from an estuary in southern Thailand affected by metal contamination. These relatively low to moderate IVBAC values may be associated with modes of metal storage in the muscle tissue of crayfish that hinder solubilization in the digestive fluids (Amiard et al., 2008; He and Wang, 2013; Peng et al., 2017; Bradley et al., 2017). Our estimated ABA values for both Hg and Pb also coincided with the ranges of bioavailability commonly observed for these metals in food under both *in vitro* and *in vivo* conditions (Berntssen et al., 2004; Ruby et al., 1999). With regard to Hg, it is worth noting that our estimated ABA value supports a body of emerging research suggesting that Hg may be less than 100% bioavailable from seafood to human consumers, ranging from 12 % to 79 % for MeHg (Bradley et al., 2017). Notwithstanding, our results for Hg must be viewed with caution, since estimations of the *in vitro-in vivo* bioavailability of this metal in the form of Hg chloride or MeHg have not yet been validated accurately. Moreover, *in vitro* simulations with MeHg may be necessary in order to discover the differences among different Hg species as regards oral bioavailability. They are, therefore, subjected to the uncertainty inherent to potential variations associated with the transport and uptake of Hg through the gastrointestinal tract. For instance, the apparent permeability in intestinal epithelium cell cultures for MeHg can be lower than for Hg (II), especially owing to the higher intracellular retention and the accumulation on the mucous layer of MeHg (Vázquez et al., 2013). Here, we have assumed 80% bioavailability of the total bioaccessible Hg in the intestine, which may be an adequate value when considering the high absolute bioavailability of this organic form (Berntssen et al., 2004; EFSA , 2012).

The bioavailability-adjusted risk indices for Hg in the abdominal muscle of crayfish suggest that adults should not consume more than 7.73 ± 1.80 (mean ± SD) meals of crayfish from the Hg-AREA per month. In the case of children, the THQ values make it advisable for them not to consume crayfish from the Hg-AREA more than 70 days/year (Scenario 5, which is equivalent to 4 days/week throughout the peak fishing season), whereas estimated safe consumption rates suggest

that children should not eat more than 5.16 ± 1.20 (mean ± SD) meals of crayfish per month. In the case of crayfish from the Pb-AREA, the bioavailability-adjusted THQ values suggest that adults and children should not consume them more than 17 days/year (Scenario 3, which is equivalent to 1 day/week throughout the peak season) and 4 days/year (Scenario 2, which is equivalent to 1 day/month throughout the peak season), respectively. The estimated safe consumption rates indicate that adults should not eat more than 1.48 ± 1.08 (mean ± SD) meals of crayfish from the Pb-AREA per month, whereas the consumption of crayfish should still be completely restricted for children. Overall, our results clearly suggest that, even after adjusting for bioavailability, it is not safe to consume crayfish from the Valdeazogues (Hg-AREA) and the Montoro (Pb-AREA) rivers because of their high levels of Hg and Pb, respectively.

4.4. Cooking extractability of metals in crayfish

The bioavailability and risk parameters reported here are based on data from raw samples of the abdominal muscle of crayfish, although this food item is commonly consumed cooked. Cooking is another factor that may contribute to defining the risk that chemical substances, including metals, represent for food safety, since the boiling, steaming, frying or grilling processes can induce some chemical and/or physical changes that may modify the metal content and/or bioaccessibility (Maulvault et al., 2012; He and Wang, 2013; Alves et al., 2018; Atia et al., 2018). However, Peng et al. (2016b) found no significant differences in the bioaccessibility of several metals (including Pb) in the tail muscle of *P. clarkii* cooked by either stewing or frying; and Peng et al. (2017) found that frying and stewing had no significant effects on the MeHg digestive solubilization rate in the abdominal muscle of *P. clarkii*, although values in cooked samples (9.8 ± 0.8 and 7.8 ± 3.9 %, respectively) tended to be lower than in raw tissues (39.3 ± 1.8 %). From these and other previous works, we can draw the conclusion that the effect of cooking on metals in food products (and their risk for consumers) are very variable and highly dependent on the metal, the species and the culinary procedures, which are in turn greatly influenced by cultural factors (Perelló et al., 2008; Mateo et al., 2011; Gao and Wang, 2014). Current limits for the presence of metals (MRLs) are consequently set for raw products, and most human health risk assessments prefer to consider trace elements in raw products. Irrespective of the need for more research to determine how to adjust food safety regulations and surveillance procedures in order to include cooked products and reduce this uncertainty and bias in risk assessments (Laird and Chan, 2013), we therefore assumed that cooking has a minor effect on the levels of metals in crayfish abdominal muscle.

Nevertheless, we evaluated the effect of cooking on the concentration of metals in the crayfish body burden in the context of the traditional Spanish cuisine for this food item. In contrast to what we were expecting owing to the significant metal burden in the carcass, the cooking procedure extracted relatively small amounts of the total Hg and Pb body burden in crayfish (means ± SD: 8.92 ± 2.13 and 1.68 ± 0.29 %, respectively), especially in the case of Pb. This may, for both metals, be associated with the high proportion of crayfish exoskeleton in the cooking mixture. Crayfish carapace is rich in amine and sulphur functional groups that provide donor ligands and chelation sites for the adsorption and retention of metals, including Pb. In this regard, crayfish carapace contains large amounts of calcium carbonate and chitin, which contribute to retaining or removing Pb ions in aqueous solutions (Zheng et al., 2010; Wang et al., 2016). This argument is supported by the very low bioavailability estimated for Pb in the carcass of crayfish (0.61–2.04%). In the case of Hg, a higher cooking extractability might be linked to the relatively high bioavailability of Hg in the carcass (71.51 %) in comparison to the muscle tissue, since cysteine (a strong MeHg-protein binding agent in crayfish muscle) is expected to be much less abundant in the exoskeleton than in muscle (Peng et al., 2017; Bradley et al., 2017).

Overall, our results suggest that cooking has a limited impact on the total Hg and Pb concentrations in crayfish, at least in the context of the traditional Spanish cuisine. However, the extractable and potentially bioavailable fractions of metals that are released into the dipping sauce (which is consumed along with the crayfish tail muscle) might be also of concern. In the present study, the concentrated dipping sauce resulting from cooking crayfish from the Hg-AREA and the Pb-AREA, respectively, would have Hg and Pb levels of 0.02 and 0.41 μg/g w.w., respectively, which are well below the maximum standard limits recommended for Hg (0.1 μg/g w.w.) and Pb (1.5 μg/g w.w.) by the Joint FAO/WHO Codex Alimentarius Commission (CODEX STAN 193–1995) for food grade products (e.g., processed tomato) and its consumption may not, therefore, be of toxicological concern. Notwithstanding, the relatively high bioavailability of Hg and the high total Pb level (despite its very low bioavailability) in the carcass of crayfish from the Hg- and Pb-AREA, respectively, signifies that it is not recommendable to use this body part of crayfish from the study areas as a condiment for flavouring.

5. Conclusions & final remarks

Our results indicate that naturalized populations of the crayfish *P. clarkii* from the Valdeazogues River in the former Almadén Mining District (Hg-AREA) and the Montoro River in the former Alcudia Valley Mining District (Pb-AREA), both in central Spain, bioaccumulate high levels of Hg and Pb pollution, respectively. Risk indices indicate that their consumption may pose a health risk for human consumers, especially in the case of crayfish from the Pb-AREA, even when the health risk assessment is adjusted by the estimated absolute bioavailability of these metals in the edible part of crayfish. Cooking had a very limited effect on the total concentration of metals in the crayfish body burden when following a traditional Spanish recipe, especially for Pb, signifying that the metal fraction released into the dipping tomato sauce resulting from the cooking process may not be of major food safety concern. Nevertheless, according to the high bioavailability of Hg and the high total Pb level in the carcass of crayfish from the Hg- and Pb-AREA, respectively, it may not be recommendable to use the carcass of crayfish from these mining districts as a condiment for flavouring.

Metal pollution in these aquatic environments does not seem to be dispersed homogeneously in either space or time (see e.g., García-Ordiales et al., 2017, and reference therein) and the consumption of crayfish from different river sections or different periods may, therefore, involve different levels of food safety risks. It is also important to note that our human health risk assessment considered only crayfish as a food item, but other fish species from these river courses, which might also be bioaccumulating high levels of metals, are also part of the diet of local communities in this rural area. Further research is, therefore, encouraged to better understand the overall potential food insecurity situation linked to mining pollution in these aquatic environments. To date, recreational fishing is an important measure for the control and eradication of *P. clarkii* in Spain and its practice should not, therefore, be forbidden. Alternatively, the implementation of priority advisory measures as regards communicating the risks of crayfish and metal consumption to the local population, and especially to the fishing sector, would be highly recommendable.

Finally, from an ecotoxicological perspective, attention should be paid to the role of *P. clarkii* as a major vector of environmental pollutants to higher trophic levels. In this regard, it is worth highlighting that *P. clarkii* has become a highly important prey for tens of mammal and bird species within the predator community in the invaded ecosystems (e.g., Tablado et al., 2010). It may, therefore, potentially increase the bioaccumulation and biomagnification of metal pollution in top predators and compromise their conservation. This highlights the need for an ecological risk assessment that would, together with the results from the present work, contribute to future restoration plans focused on improving water quality and the health of the aquatic ecosystems in

these historical mining districts.

Acknowledgements

We are grateful to Irene Lorente Espadas for her valuable help during the laboratory work. We also appreciate the assistance of Mónica Martinez-Haro and Pablo R. Camarero with field and laboratory work, respectively. J. Rodríguez-Estival was supported by a Research and Innovation Strategy for Smart Specialisation (RIS3) postdoctoral contract (2017/11747) from the Junta de Comunidades de Castilla-La Mancha. The study was financially supported by the Junta de Comunidades de Castilla-La Mancha (Project PPII-2014-028-P).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109682.

Conflicts of interest

The authors have no competing interests to declare.

Financial support

The study was financially supported by the Junta de Comunidades de Castilla-La Mancha (Project PPII-2014-028-P). The funding source had no involvement in study design, collection, analysis and interpretation of data, writing of the report, or the decision to submit this article for publication.

References

Ackefors, H., 1999. The positive effects of established crayfish. In: Gherardi, F., Holdich, D.M. (Eds.), Crayfish in Europe as Alien Species. How to Make the Best of a Bad Situation? A.A. Balkema, Rotterdam, The Netherlands, pp. 49–61.

Alcorlo, P., Otero, M., Crehuet, M., Baltanás, A., Montes, C., 2006. The use of the red swamp crayfish (*Procambarusclarkii*, Girard) as indicator of the bioavailability of heavy metals in environmental monitoring in the River Guadiamar (SW, Spain). Sci. Total Environ. 366, 380–390.

Alcorlo, P., Baltanás, A., 2013. The trophic ecology of the red swamp crayfish (*Procambarusclarkii*) in Mediterranean aquatic ecosystems: a stable isotope study. Limnética 32, 121–138.

Allert, A.L., Distefano, R.J., Fairchild, J.F., Schmitt, C.J., McKee, M.J., Girondo, J.A., Brumbaugh, W.G., May, T.W., 2013. Effects of Historical Lead-Zinc Mining on Riffle-Dwelling Benthic Fish and Crayfish in the Big River of Southeastern Missouri. Ecotoxicology 22, USA, pp. 506–521.

Alves, R.N., Maulvault, A.L., Barbosa, V.L., Fernández-Tejedor, M., Tediosi, A., Kotterman, M., van den Heuvel, F.H.M., Marques, A., 2018. Oral bioaccessibility of toxic and essential elements in raw and cooked commercial seafood species available in European markets. Food Chem. 267, 15–27.

Amiard, J.-C., Amiard-Triquet, C., Charbonnier, L., Mesnil, A., Rainbow, P.S., Wang, W.-X., 2008. Bioaccessibility of essential and non-essential metals in commercial shellfish from Western Europe and Asia. Food Chem. Toxicol. 46, 2010–2022.

Atia, A.S., Darwish, W.S., Zaki, M.S., 2018. Monitoring of heavy metal residues, metal-metal interactions and the effect of cooking on the metal load in shellfish. J. Anim. Plant Sci. 28, 732–743.

Bradley, M.A., Barst, B.D., Basu, N., 2017. A review of mercury bioavailability in humans and fish. Int. J. Env. Res.Pub. Health 14, 169.

Berntssen, M.H.G., Hylland, K., Lundebye, A.-K., Julshamn, K., 2004. Higher faecal excretion and lower tissue accumulation of mercury in Wistar rats from contaminated fish than from methylmercury chloride added to fish. Food Chem. Toxicol. 42, 1359–1366.

Campos, J.A., Esbrí, J.M., Madrid, M.M., Naharro, R., Peco, J., García-Noguero, E.M., Amorós, J.Á., Moreno, M.M., Higueras, P., 2018. Does mercury presence in soils promote their microbial activity? The Almadenejos case (Almadén mercury mining district, Spain). Chemosphere 201, 799–806.

Carrasco, L., Benejam, L., Benito, J., Bayona, J.M., Díez, S., 2011. Methylmercury levels and bioaccumulation in the aquatic food web of a highly mercury-contaminated reservoir. Environ. Int. 37, 1213–1218.

CODEX STAN 193-1995. General Standard for Contaminants and Toxins in Food and Feed. Adopted in 1995. Revised in 1997, 2006, 2008, 2009. Amended in 2010, 2012, 2013, 2014, 2015. Joint FAO (Food and Agriculture Organization of the United Nations)/WHO (World Health Organization) Codex Alimentarius International Food Standards. Rome, Italy. Pp. 59.

Commission Regulation (EC) 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs.

Commission Regulation (EU) 420/2011 of, 29 April 2011. Amending Regulation (EC) 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs.

Conde, A., Domínguez, J., 2015. A proposal for the feasible exploitation of the red swamp crayfish *Procambarusclarkii* in introduced regions. Conserv. Lett. 8, 440–448.

Cremades, O., Parrado, J., Alvarez-Ossorio, M.-C., Jover, M., Collantes de Teran, L., Gutierrez, J.F., Bautista, J., 2003. Isolation and characterization of carotenoproteins from crayfish (*Procambarusclarkii*). Food Chem. 82, 559–566.

Drexler, J.W., Brattin, W.J., 2007. An in vitro procedure for estimation of lead relative bioavailability: with validation. Hum.Ecol. Risk Assess. 13, 383–401.

EFSA (Panel on Contaminants in the Food Chain, CONTAM), 2012. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. EFSA J. 10, 2985.

El-Kady, A.A., Abdel-Wahhab, M.A., 2018. Occurrence of trace metals in foodstuffs and their health impact. Trends Food Sci. Technol. 75, 36–45.

El-Sherif, S.A.E.H., El-Ghafour, S.A., 2015. Nutritive value of canned River Nile Crayfish (*Procambarusclarkii*) products. Egypt. J.Aquat. Res. 41, 265–272.

FAO (Food and Agriculture Organization of the United Nations), 2018. FAO Yearbook of Fishery and Aquaculture Statistics 2016. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 104.

Fernández Díaz, C., Macho Rivero, G., Ríos Giráldez, J., 2016. Red swampcrawfish: Guadalquivir delta. Fishsource – sustainable fisheries partnership. URL. https://www.fishsource.org/fishery_page/4207, Accessed date: 25 October 2018.

Gao, S., Wang, W.-X., 2014. Oral bioaccessibility of toxic metals in contaminated oysters and relationships with metal internal sequestration. Ecotox. Environ. Saf. 110, 261–268.

García-Ordiales, E., Loredo, J., Covelli, S., Esbrí, J.M., Millán, R., Higueras, P., 2017. Trace metal pollution in freshwater sediments of the world's largest mercury mining district: sources, spatial distribution, and environmental implications. J. Soils Sediments 17, 1893–1904.

Gherardi, F., 2006. Crayfish invading Europe: the case study of *Procambarusclarkii*. Mar. Freshw. Behav. Physiol. 39, 175–191.

Gherardi, F., Panov, V., 2006. Data sheet *Procambarusclarkii*. DAISIE (delivering alien invasive species inventories for Europe). URL. http://www.europe-aliens.org/speciesFactsheet.do?speciesId=53452, Accessed date: 24 October 2018.

Gusso-Choueri, P.K., Araújo, G.S.D., Cruz, A.C.F., Stremel, T.R.D.O., Campos, S.X.D., Abessa, D.M.D.S., Oliveira Ribeiro, C.A.D., Choueri, R.B., 2018. Metals and arsenic in fish from a Ramsar site under past and present human pressures: consumption risk factors to the local population. Sci. Total Environ. 628–629, 621–630.

He, M., Wang, W.X., 2013. Bioaccessibility of 12 trace elements in marine molluscs. Food Chem. Toxicol. 55, 627–636.

Henriques, J.F., Tavares, P.C., Correia-Dos-Santos, M.M., Trancoso, M.A., Santos-Reis, M., Branquinho, C., 2014. Monitoring Hg and Cd contamination using red swamp crayfish (*Procambarusclarkii*): implications for wetland food chain contamination. Water AirSoil Poll. 225, 2210.

Hernández, A., Jébrak, M., Higueras, P., Oyarzun, R., Morata, D., Munhá, J., 1999. The Almadén mercury mining district, Spain. Miner. Depos. 34, 539–548.

Higueras, P., Oyarzun, R., Lillo, J., Sánchez-Hernández, J.C., Molina, J.A., Esbrí, J.M., Lorenzo, S., 2006. The Almadén district (Spain): anatomy of one of the world's largest Hg-contaminated sites. Sci. Total Environ. 356, 112–124.

Higueras, P., Oyarzun, R., Iraizoz, J.M., Lorenzo, S., Esbrí, J.M., Martínez-Coronado, A., 2012. Low-cost geochemical surveys for environmental studies in developing countries: testing a field portable XRF instrument under quasi-realistic conditions. J. Geochem. Explor. 113, 3–12.

Higueras, P., María Esbrí, J., Oyarzun, R., Llanos, W., Martinez-Coronado, A., Lillo, J., López-Berdonces, M.A., García-Noguero, E.M., 2013. Industrial and natural sources of gaseous elemental mercury in the Almadén district (Spain): an updated report on this issue after the ceasing of mining and metallurgical activities in 2003 and major land reclamation Works. Environ. Res. 125, 197–208.

Higueras, P.L., Amorós, J.Á., Esbrí, J.M., Pérez-de-los-Reyes, C., López-Berdonces, M.A., García-Navarro, F.J., 2016. Mercury transfer from soil to olive trees. A comparison of three different contaminated sites. Environ. Sci. Pollut. Res. 23, 6055–6061.

Hothem, R.L., Bergen, D.R., Bauer, M.L., Crayon, J.J., Meckstroth, A.M., 2007. Mercury and trace elements in crayfish from northern California. Bull. Environ. Contam. Toxicol. 79, 628–632.

Huner, J., Bonham, V., 2011. *Procambarusclarkii* (Red Swamp Crayfish). CABI (Centre for Agriculture and Biosciences International) Invasive Species Conpendium. URL. https://www.cabi.org/isc/datasheet/67878, Accessed date: 24 October 2018.

Intawongse, M., Sriraksa, S., Dean, J.R., Kongchana, P., 2012. Estimation of bioaccessibility of heavy metals in oysters using the physiologically based extraction test. Instrum. Sci. Technol. 40, 372–383.

Johnson, B.L., Willacker, J.J., Eagles-Smith, C.A., Pearl, C.A., Adams, M.J., 2014. Invasive crayfish as vectors of mercury in freshwater food webs of the Pacific Northwest. Environ. Toxicol. Chem. 33, 2639–2645.

Juhasz, A.L., Weber, J., Smith, E., Naidu, R., Marschner, B., Rees, M., Rofe, A., Kuchel, T., Sansom, L., 2009. Evaluation of SBRC-gastric and SBRC-intestinal methods for the prediction of in vivo relative lead bioavailability in contaminated soils. Environ. Sci. Technol. 43, 4503–4509.

Kouba, A., Buřič, M., Kozák, P., 2010. Bioaccumulation and effects of heavy metals in crayfish: a review. Water Air Soil Poll. 211, 5–16.

Lacal, M., Lominchar, M.A., García, E., Sierra, M.J., Rodríguez-Alonso, J., Giaggio, R., Díez, S., Loredo, J., Millán, R., 2013. Bioacumulación de mercurio en organismos acuáticos de Almadén (Ciudad Real, España). 9° Congreso Ibérico y 6° Iberoamericano de Contaminación y Toxicología Ambiental. (Valencia, España).

Laird, B.D., Chan, H.M., 2013. Bioaccessibility of metals in fish, shellfish, wild game, and seaweed harvested in British Columbia, Canada. Food Chem. Toxicol. 58, 381–387.

Loureiro, T.G., Anastácio, P.M., Araujo, P.B., Souty-Grosset, C., Almerão, M.P., 2015. Red

swamp crayfish: biology, ecology and invasion – an overview. Nauplius 23, 1–19.
Mancinelli, G., Papadia, P., Ludovisi, A., Migoni, D., Bardelli, R., Fanizzi, F.P., Vizzini, S., 2018. Beyond the mean: a comparison of trace- and macroelement correlation profiles of two lacustrine populations of the crayfish *Procambarusclarkii*. Sci. Total Environ. 624, 1455–1466.
Martinez-Haro, M., Taggart, M.A., Green, A.J., Mateo, R., 2009. Avian digestive tract simulation to study the effect of grit geochemistry and food on Pb shot bioaccessibility. Environ. Sci. Technol. 43, 9480–9486.
Mateo, R., Baos, A.R., Vidal, D., Camarero, P.R., Martinez-Haro, M., Taggart, M.A., 2011. Bioaccessibility of Pb from ammunition in game meat is affected by cooking treatment. PLoS One 6, e15892.
Maulvault, A.L., Anacleto, P., Loureno, H.M., Carvalho, M.L., Nunes, M.L., Marques, A., 2012. Nutritional quality and safety of cooked edible crab (*Cancer pagurus*). Food Chem. 133, 277–283.
McClain, W.R., Romaire, R.P., 2007. Cultured aquatic species information program – *Procambarusclarkii*. Rome, Italy. In: FAO Fisheries and Aquaculture Department, URL. http://www.fao.org/fishery/culturedspecies/Procambarus_clarkii/en#tcNA00EA, Accessed date: 23 October 2018.
Mergler, D., Anderson, H.A., Chan, L.H.M., Mahaffey, K.R., Murray, M., Sakamoto, M., Stern, A.H., 2007. Methylmercury exposure and health effects in humans: a worldwide concern. Ambio 36, 3–11.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food-an international consensus. Food Funct. 5, 1113–1124.
Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romaris-Hortas, V., Moscoso-Perez, C., Lopez-Mahia, P., Muniategui-Lorenzo, S., Bermejo-Barrera, P., Prada-Rodriguez, D., 2011. In-vivo and in-vitro testing to assess the bioaccessibility and the bioavailability of arsenic, selenium and mercury species in food samples. Trac. Trends Anal. Chem. 30, 324–345.
Nagy, R., Fusaro, A., Conard, W., 2015. *Procambarusclarkii* (Girard, 1852): U. S. Geological Survey, Nonindigenous Aquatic Species Database. Gainesville, Florida. URL. http://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=217, Accessed date: 24 October 2018 Revision date: 04/04/2018.
Ng, J.C., Juhasz, A., Smith, E., Naidu, R., 2015. Assessing the bioavailability and bioaccessibility of metals and metalloids. Environ.Sci.Pollut. R. 22, 8802–8825.
Ortiz-Santaliestra, M.E., Rodríguez, A., Pareja-Carrera, J., Mateo, R., Martinez-Haro, M., 2019. Tools for non-invasive sampling of metal accumulation and its effects in Mediterranean pond turtle populations inhabiting mining areas. Chemosphere (in press).
Palero-Fernández, F.J., Martín-Izard, A., 2005. Trace element contents in galena and sphalerite from ore deposits of the Alcudia Valley mineral field (Eastern Sierra Morena, Spain). J. Geochem. Explor. 86, 1–25.
Pareja-Carrera, J., Mateo, R., Rodríguez-Estival, J., 2014. Lead (Pb) in sheep exposed to mining pollution: implications for animal and human health. Ecotoxicol. Environ. Saf. 108, 210–216.
Paustenbach, D.J., Bruce, G.M., Chrostowski, P., 1997. Current views on the oral bioavailability of inorganic mercury in soil: implications for health risk assessments. Risk Anal. 17, 533–544.
Peng, Q., Greenfield, B.K., Dang, F., Zhong, H., 2016a. Human exposure to methylmercury from crayfish (*Procambarusclarkii*) in China. Environ. Geochem. Health 38, 169–181.
Peng, Q., Nunes, L.M., Greenfield, B.K., Dang, F., Zhong, H., 2016b. Are Chinese consumers at risk due to exposure to metals in crayfish? A bioaccessibility-adjusted probabilistic risk assessment. Environ. Int. 88, 261–268.
Peng, Q., Greenfield, B.K., Dang, F., Gong, Y., Bu, W., Zhong, H., 2017. Mechanistic understanding of low methylmercury bioaccessibility from crayfish (*Procambarusclarkii*) muscle tissue. Sci. Total Environ. 603–604, 453–460.
Perelló, G., Martí-Cid, R., Llobet, J.M., Domingo, J.L., 2008. Effects of various cooking processes on the concentrations of arsenic, cadmium, mercury, and lead in foods. J. Agric. Food Chem. 56, 11262–11269.
Reglero, M.M., Monsalve-González, L., Taggart, M.A., Mateo, R., 2008. Transfer of metals to plants and red deer in an old lead mining area in Spain. Sci. Total Environ. 406, 287–297.
Rodríguez-Estival, J., Martinez-Haro, M., Monsalve-González, L., Mateo, R., 2011. Interactions between endogenous and dietary antioxidants against Pb-induced oxidative stress in wild ungulates from a Pb polluted mining area. Sci. Total Environ. 409, 2725–2733.
Ropero, M.J.P., Rodríguez Fariñas, N., Mateo, R., Berzas Nevado, J.J., Rodríguez Martín-Doimeadios, R.C., 2016. Mercury species accumulation and trophic transfer in biological systems using the Almadén mining district (Ciudad Real, Spain) as a case of study. Environ. Sci. Pollut. Res. 23, 6074–6081.
Ruby, M.V., Schoof, R., Brattin, W., Goldade, M., Post, G., Harnois, M., Mosby, D.E., Casteel, S.W., Berti, W., Carpenter, M., Edwards, D., Cragin, D., Chappell, W., 1999. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. Environ. Sci. Technol. 33, 3697–3705.
Safruk, A.M., Berger, R.G., Jackson, B.J., Pinset, C., Hair, A.T., Sigal, E.A., 2015. The bioaccessibility of soil-based mercury as determined by physiological based extraction tests and human biomonitoring in children. Sci. Total Environ. 518–519, 545–553.
Schmitt, C.J., Stricker, C.A., Brumbaugh, W.G., 2011. Mercury bioaccumulation and biomagnification in Ozark stream ecosystems. Ecotoxicol. Environ. Saf. 74, 2215–2224.
Stanek, M., Dąbrowski, J., Różański, S., Janicki, B., Długosz, J., 2017. Heavy metals bioaccumulation in tissues of spiny-cheek crayfish (*Orconectes limosus*) from lake GopŁo: effect of age and sex. Bull. Environ. Contam. Toxicol. 98, 740–746.
Strzebońska, M., Jarosz-Krzemińska, E., Adamiec, E., 2017. Assessing historical mining and smelting effects on heavy metal pollution of river systems over span of two decades. Water Air Soil Pollut. 228, 141.
Tablado, Z., Tella, J.L., Sánchez-Zapata, J.A., Hiraldo, F., 2010. The paradox of the long-term positive effects of a North American crayfish on a European community of predators. Conserv. Biol. 24, 1230–1238.
Taggart, M.A., Reglero, M.M., Camarero, P.R., Mateo, R., 2011. Should legislation regarding maximum Pb and Cd levels in human food also cover large game meat? Environ. Int. 37, 18–25.
Tunca, E., Ucuncu, E., Ozkan, A.D., Ulger, Z.E., Tekinay, T., 2013. Tissue distribution and correlation profiles of heavy-metal accumulation in the freshwater crayfish *Astacusleptodactylus*. Arch. Environ. Contam. Toxicol. 64, 676–691.
USEPA (U. S. Environmental Protection Agency), 2000. Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories. Vol. 2 – Risk Assessment and Fish Consumption Limits, third ed. Office of Science and Technology, United States Environmental Protection Agency, Washington DC EPA 823-B-00-008.
USEPA (U. S. Environmental Protection Agency), 2001. Risk Assessment Guidance for Superfund: Vol. III – Part A, Process for Conducting Probabilistic Risk assessment.Office of Emergency and Remedial Response. United States Environmental Protection Agency, Washington DC EPA 540-R-02-002.
Vázquez, M., Calatayud, M., Vélez, D., Devesa, V., 2013. Intestinal transport of methylmercury and inorganic mercury in various models of Caco-2 and HT29-MTX cells. Toxicology 147–153.
Vedia, I., Miranda, R., 2013. Review of the state of knowledge of crayfish species in the Iberian Peninsula. Limmetica 32, 269–286.
Wang, Y., Wang, S., Sun, H., Lei, J., Ye, T., Lin, L., Wang, L., 2016. Crayfish carapace powder adsorbing heavy metal ions from aqueous solution: capacity, characterization, mechanism. J. Aquat. Food Prod. Technol. 25, 122–130.
Zheng, X., Li, B., Zhu, B., Kuang, R., Xu, B., Ma, M., 2010. Crayfish carapace micropowder (CCM): a novel and efficient adsorbent for heavy metal ion removal from wastewater. Water 2, 257–272.