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In vivo oral bioavailability of Pb sequestered in metal rich granules in bivalves

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ABSTRACT

The present study was designed to evaluate *in vivo* the oral bioavailability of lead (Pb) present in the marine bivalve *Dosinia exoleta*. This infaunal clam, despite inhabiting in clean areas, presents Pb concentrations that are over the 1.5 mg kg^{-1} wet weight limit for human consumption set by the European Commission. However, Pb is accumulated in this clam in the form of metal rich granules, and it has been shown to be unavailable for trophic transfer to a marine decapod, so it was hypothesised that it might be unavailable for human consumers as well. Twelve Sprague Dawley rats were fed during 14 days with a diet including control mussels *(Mytilus galloprovincialis)*, *D. exoleta*, or mussels enriched in Pb to the same levels as those found in *D. exoleta*. Pb accumulation in different rat tissues (blood, bone, kidneys and liver) was analysed. It was observed that Pb assimilation from *D. exoleta* was about half of Pb assimilation from *M. galloprovincialis*, and absolute bioavailabilities were around 2% for *M. galloprovincialis* and 1% for *D. exoleta*. These results suggest that it might be possible to increase the limit for human consumption for this bivalve to 3 mg kg^{-1} wet weight without representing an increase in the risk for consumers.

1. Introduction

The presence of lead (Pb) in foodstuff is a matter of concern due to the well known neurotoxic effects of this metal (Chang, 1996), its nephrotoxicity and cardiovascular effects (EFSA, 2010). European legislation has established maximum levels of this metal in foodstuff, including bivalve mollusks (EC, 2006).

The edible bivalve *Dosinia exoleta* was extracted for commercial purposes in Galicia (NW Spain) until the discovery of high Pb concentrations in their tissues, that occasionally exceed the 1.5 mg kg^{-1} wet weight (ww) limit for human consumption established by the European legislation (Sánchez-Marín and Beiras, 2008). Previous studies have shown that from 68 to 90% of the Pb present in *D. exoleta* is accumulated in the form of metal-rich granules in the kidney (Darriba and Sánchez-Marín, 2013), and it is not available for trophic transfer to invertebrates such as the prawn *Palaemon serratus* (Sánchez-Marín and Beiras, 2017). On this basis, it is hypothesised that Pb in the form of metal rich granules in *D. exoleta* may be also poorly bioavailable to human consumers. On the basis of oral Pb bioavailability studies oriented to assess the risk associated with soil ingestion by children, it is currently accepted that oral Pb bioavailability depends on its chemical form and solubility (Hettiarachchi and Pierzynski, 2004; Ng et al., 2015).

The present study was designed with the aim of evaluating the relative bioavailability of Pb from *D. exoleta in vivo*, using the rat as a model organism. Bioavailability of Pb from *D. exoleta* was compared with that from other bivalve (the mussel *Mytilus galloprovincialis*) showing a different subcellular partitioning of Pb in its tissues.

2. Materials and methods

2.1. Rat diet preparation

Rat diet consisted of 80% commercial maintenance pellets (A04; SAFE) that were finely ground and mixed with 20% bivalve tissue powder prepared as follows. For diet 1 (control diet), the bivalve tissue powder consisted of 100% uncontaminated mussels (*M. galloprovincialis*) obtained from aquaculture, that were lyophilised and ground to a fine powder. The bivalve tissue for diet 2 was prepared with a mixture of uncontaminated mussels with Pb-enriched mussels, obtained after 72-h exposure to 100 µg L^{-1} of dissolved lead, as described in Sánchez-Marín et al. (2011). After exposure, mussels were placed in

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clean running seawater during 30 min, opened and rinsed with filtered seawater to desorb weakly adsorbed Pb, lyophilised and ground. The resulting Pb-contaminated powder, that was enriched to 78 μg g^{-1} dry weight (dw) of Pb, was mixed with uncontaminated mussel powder in a 1:7 ratio in order to obtain a homogeneous mixture with a Pb concentration equal to that present in *D. exoleta* used for diet 3. The bivalve tissue for diet 3 was prepared with lyophilised and ground tissue of *D. exoleta* from 38 to 47 mm length, collected from the field by local fishermen.

Around 400 g of the three bivalve tissue powders were prepared and stored in polypropylene containers. At the beginning of the trophic transfer experiment and every time that new diets were needed, 40 g of each one of these bivalve powders were mixed with 160 g of the ground maintenance pellets in polypropylene flasks for preparation of the final rat diets. Six times throughout the experiments, 2 g of each diet were sampled and introduced in 50 mL polypropylene vials for Pb analysis.

2.2. Trophic transfer of Pb

Twelve female Sprague Dawley rats, between 200 and 250 g weight, obtained from Janvier Labs, were introduced in metabolic cages (dimensions 35.5 × 27.9 × 48.3 cm) after a training period of one week designed to minimize stress due to isolation. Four individuals were assigned to each treatment (diet 1, 2 or 3), and rats were fed *ad libitum* with its corresponding diet during 14 days. Water was also provided *ad libitum*. The quantity of food ingested was recorded daily, at the same hour, and all faeces and urine produced were collected thrice per week in 50 mL polypropylene vials. The rest of food left uneaten by rats was also sampled on day 2 for Pb analysis in order to check if rats could be selective on the particles ingested (avoiding mineral particles, for instance).

Animals were housed at 21 °C, 41–55% humidity and air renovation of 15–20 h^{-1}, with artificial light (50 lux) at a photoperiod of 12:12 h. Animal health was monitored daily and body weight was monitored weekly.

After the 14 days experimental period, rats were anaesthetised by intraperitoneal injection of ketamine (75 mg kg^{-1}) and medetomidine (0.5 mg kg^{-1}) and sacrificed by exsanguination. Blood, liver, kidneys and bone (right femur) were dissected and introduced in 50 mL preweighed polypropylene vials for Pb analysis.

The experiments were performed according to Directive 2010/63/EU on the protection of animals used for scientific purposes, and the project was authorized by Consellería de Medio Rural (Xunta de Galicia, Spain) with reference ES360570215601/16/TOX/CON.AMB./08/RB/01.

2.3. Pb analysis

Samples of food, rat tissues and faeces were dried at 70 °C until constant weight (checking was done every 24 h). Faeces were weighed, ground to a powder and homogenized, and 0.5 g of each homogenized sample was transferred to a new vial for Pb analysis. Urine samples were homogenized and 5 mL of each sample were transferred to a new vial and dried at 70 °C until constant weight. Liver samples were ground and bone samples were crushed in a porcelain mortar, previous to digestion. Samples were digested with 7 mL of HNO_3 (69%, trace metal grade, Scharlau) and 1.4 mL of H_2O_2 (30%, for trace analysis, Sigma-Aldrich) per gram of dry sample, using a microwave-assisted procedure (Sánchez-Marín and Beiras, 2008). Blank vials and reference material (ERM-CE278k; mussel tissue) were included in the digestion process. Samples were diluted with ultrapure water and analysed by inductively coupled plasma mass spectrometry (ICP-MS) using a X Series, Thermo Elemental ICP-MS (Cheshire, UK). The percentage of recovery for Pb was 97%. The limit of detection was calculated as 3 × SD from the analysis of 10 blank samples, and corresponded to 0.003 mg kg^{-1} dw for rat tissues and 0.58 μg L^{-1} for blood samples.

2.4. Data treatment and statistics

Diet 1 was used as control, and therefore Pb concentrations in rats fed with diet 1 were used as background for calculations of relative and absolute bioavailabilities. The rationale for this (instead of sacrificing rats at the beginning of the experiment) was to account for changes in Pb concentration due to other sources (drinking water, dust), and for changes in the quantity of food and water consumed by rats (as well as on the weight gains or losses during the experiment) caused by the presence of a 20% of bivalve in the diet.

The relative bioavailability (RBA) of Pb from *D. exoleta* compared to *M. galloprovincialis* was calculated for each rat tissue as:

$$RBA_{bivalve} = ([Pb]_{diet3} - [Pb]_{diet\ 1})/([Pb]_{diet2} - [Pb]_{diet1}) \quad (1)$$

with [Pb] being the concentration of Pb in the corresponding tissue.

Note that relative bioavailability is denoted as $RBA_{bivalve}$ to avoid confusion with usually reported RBAs in the literature, based on the use of Pb-acetate in drinking water as reference dose.

Absolute bioavailability (ABA) of Pb was calculated as the sum of the amount of Pb accumulated in bone, kidney, liver and blood and the accumulated excretion in urine and bile divided by the total amount of Pb ingested during the 14 days time period, using the following equation:

$$ABA = \frac{\sum_{i=1}^{6} (Pb\ content_{diet\ 2\ or\ 3} - Pb\ content_{diet\ 1})}{Pb\ ingested} \quad (2)$$

Where i = 1–6 are the different tissues or biological fluids analysed (or estimated in the case of bile). For calculation of Pb contents, the weight of the skeleton was assumed to be a 3.5% in dry weight of the live weight of the rat (Spichtin, 1970) and total blood volume was calculated according to Lee and Blaufox (1985). Cumulative Pb eliminated through bile in faeces was assumed to be 9 times the cumulative Pb excreted in urine (Klaassen and Shoeman, 1974).

Significant differences among means were tested by standard *t*-test at a $p < 0.05$ level of significance.

3. Results

3.1. Rats health monitoring

All individuals were healthy during the study, showing only mild to moderate signs of stress due to isolation (hair alterations and chromodacryorrhea). Body weight at the end of the experiment varied between 97% and 109% of initial weight. There were not differences in weight or health status depending on the treatment.

3.2. Pb ingested by rats

Pb concentration in *D. exoleta* was 10.5 mg kg^{-1} dw, equivalent to 2.1 mg kg^{-1} ww (assuming 80% humidity), exceeding the legal limit for human consumption (1.5 mg kg^{-1} ww). Pb concentration in maintenance diet was 0.066 ± 0.005 (n = 3) mg kg^{-1}. Pb concentration in experimental rat diets was 0.16 mg kg^{-1} for diet 1, 1.81 mg kg^{-1} for diet 2 and 1.97 mg kg^{-1} for diet 3 (Table 1). These concentrations are referred to the diet as offered (7% of humidity). Rest of uneaten food presented the same Pb concentration as offered food (*t*-test, $p < 0.001$), showing that rats were not able of selecting the particles ingested from any of the offered diets.

Rats ingested in average 16 g of food per day, and no differences were observed depending on the treatment. A slight decrease in the quantity of food ingested per day was observed from 10th day of isolation on, reaching 12 g in the last day of the experiment. This decrease occurred in all treatments (Fig. 1a). On the basis of the concentration of Pb in the food and the quantity of food consumed per day, the quantity of Pb consumed per rat during the 14 days of exposure was 393 μg and

Table 1
Pb concentration in diets and in rat tissues after 14 days of feeding on experimental diets. Mean ± SD (n = 6 for diets and n = 4 for rats).

	Diet 1 Control *Mytilus galloprovincialis*	Diet 2 Contaminated *Mytilus galloprovincialis*	Diet 3 *Dosinia exoleta*	$RBA_{bivalve}$ (Dosinia/Mytilus)
Pb in diet ***(mg kg^{-1})*** [a]	0.161 ± 0.014	1.814 ± 0.255	1.970 ± 0.188	–
Pb in kidney ***(mg kg^{-1} dw)***	0.02 ± 0.01	0.27 ± 0.02	0.13 ± 0.01	**0.45**
Pb in liver ***(mg kg^{-1} dw)***	0.004 ± 0.006	0.019 ± 0.005	0.013 ± 0.004	0.61
Pb in bone ***(mg kg^{-1} dw)***	0.040 ± 0.003	0.117 ± 0.010	0.093 ± 0.024	0.68
Pb in blood ***(μg L^{-1})***	0.66 ± 0.13	4.23 ± 0.19	2.70 ± 0.26	0.57

[a] Concentration in the diet as offered (7% of humidity).

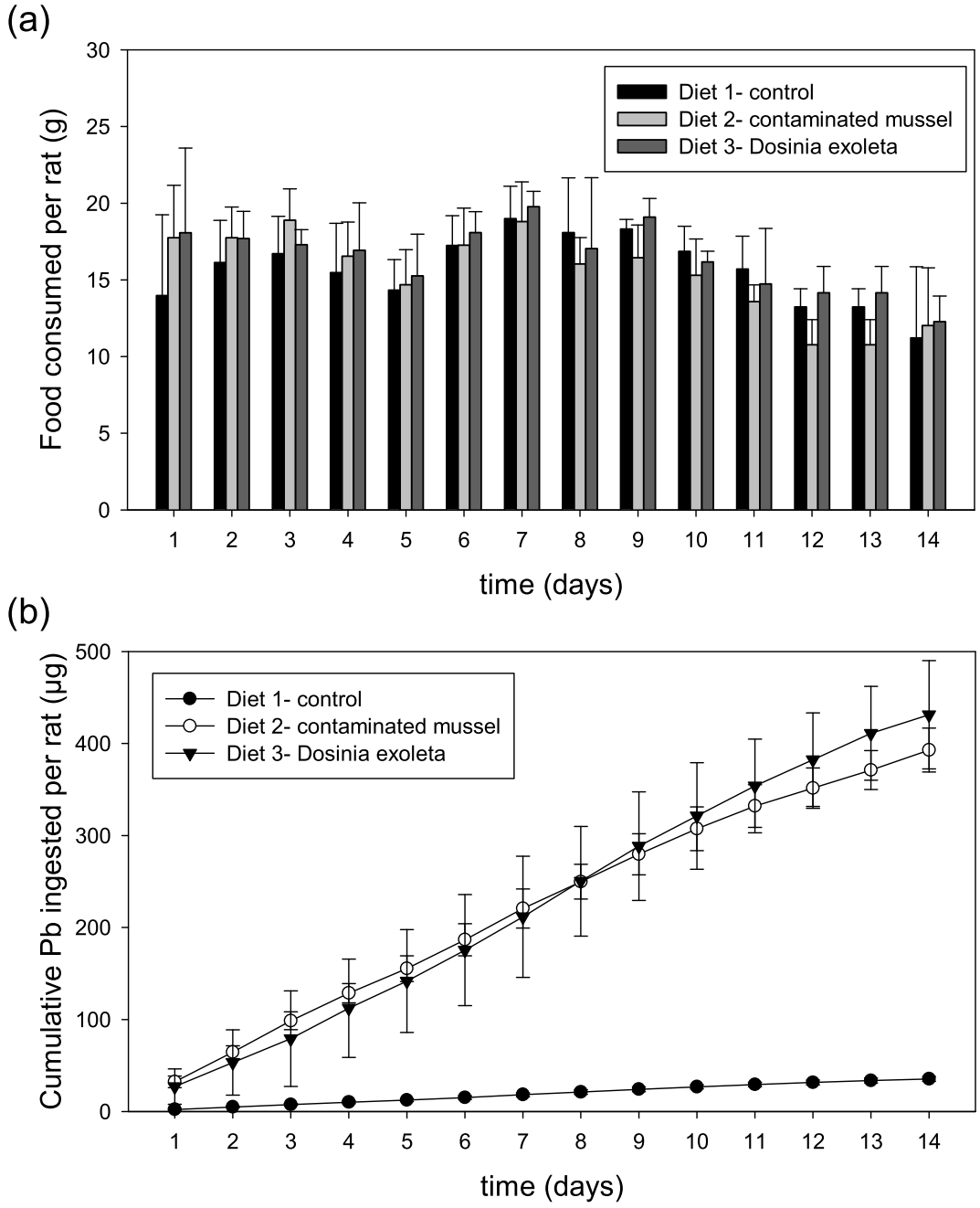

Fig. 1. Food consumed (a) and cumulative Pb ingested (b) per rat during the 14-days oral bioavailability experiment. Mean ± *SD* (*n = 4*).

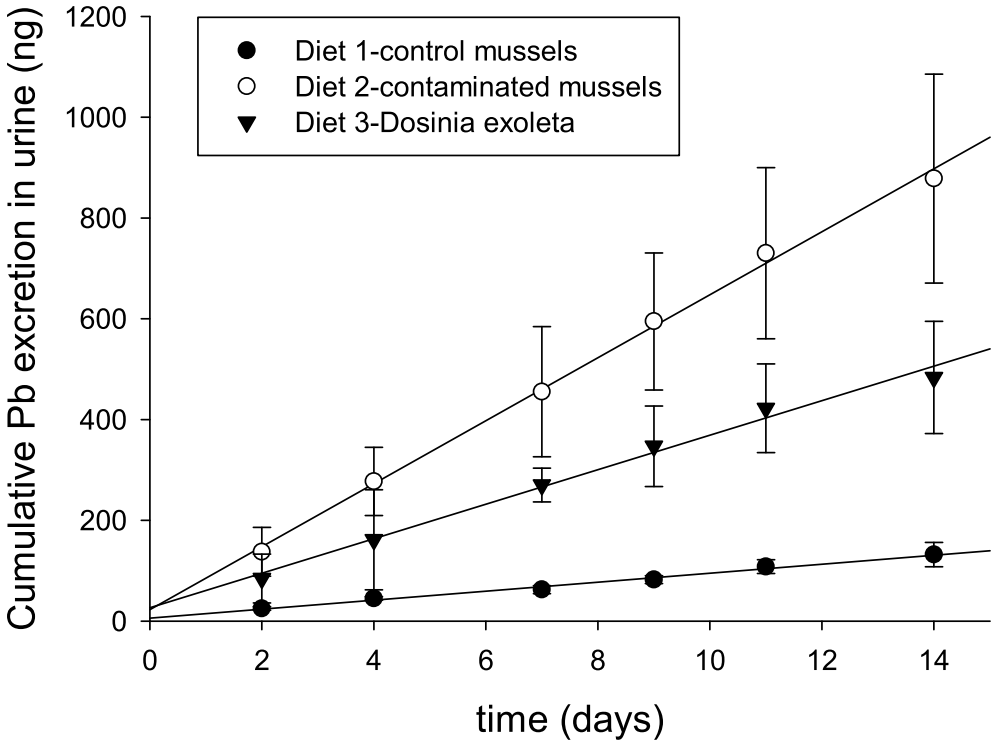

Fig. 2. Cumulative Pb excreted in urine per rat during the 14-days oral bioavailability experiment. Mean ± SD (n = 4).

431 μg for diets 2 and 3 respectively, and only 35 μg for diet 1 (Fig. 1b).

3.3. Relative Pb bioavailability

Rats fed with diets 2 and 3 accumulated Pb in all analysed tissues in comparison with rats fed with control diet (Table 1). The tissue showing the highest Pb concentration was the kidney, followed by the bone. Kidney was also the tissue showing the highest Pb enrichment when comparing Pb-enriched *vs* control fed rats, followed by blood. Despite the quantity of Pb ingested by rats was similar for diets 2 and 3, Pb bioacummulation differed, varying for diet 3 between 50% and 79% of that from rats fed with diet 2, depending on the tissue considered. The concentration of Pb in kidney and blood was significantly lower in rats fed with diet 3 compared to diet 2 (*t*-test, $p < 0.05$). The kidney was chosen as the most appropriate tissue for estimation of the relative Pb bioavailability in *D. exoleta* because of the higher concentrations of Pb observed in control rats (well over the analytical LOD), and the lower inter-individual variability in Pb concentrations, leading to more precise data. Therefore, considering Pb bioaccumulation in kidney, the relative bioavailability of Pb in *D. exoleta* compared to *M. galloprovincialis* was 0.45. For comparison, the relative bioavailability obtained with the other tissues was also calculated, and it ranged from 0.57 to 0.68 (Table 1), being the average of all tissues 0.58.

Pb was eliminated in urine at a rate of 9, 63 and 34 ng day^{-1} for diets 1, 2 and 3 respectively (Fig. 2), and the total amount of Pb eliminated in urine during the 14 days experimental period summed up 132 (± 24) ng for rats fed diet 1, and 878 (± 208) and 483 (± 111) ng for rats fed diets 2 and 3 respectively. These results are in agreement with those of Pb accumulation in tissues, showing that Pb elimination –proportional to Pb assimilation– was also around half for rats fed diet 3 (*D. exoleta*) in comparison with rats fed diet 2 (contaminated mussels).

3.4. Absolute Pb bioavailability

Food consumed during the last 24 h before sacrifice is expected to be mainly in the rat digestive system, according to mean transit times of food (ca. 15 h) (Varga, 1976) and the nocturnal behaviour of rats (sacrificed in the morning). Therefore, Pb faecal excretion was compared with Pb ingestion during the first 13 days of feeding, and it represented 95 (± 6) % of ingested Pb (Table 2). Given the ca. 10% error in estimation of Pb concentrations in the diets, and a similar 10% batch to batch variability in Pb concentration in faeces, it was not possible to determine Pb ABA using mass balance calculations. For this to be possible, Pb ABA would have to be higher than 20%.

ABA calculated according to eq. (2) was on average 2.1% for mussels and 0.9% for *D. exoleta* (Table 2). Despite Pb accumulated in other tissues different from the ones analysed was not considered for calculations of assimilated Pb, this is not expected to result in a significant underestimation of assimilated Pb. Freeman et al. (1996) assumed that Pb accumulated in other rat tissues represented 4% of Pb accumulated in blood, bone, liver, and kidney. Ellickson et al. (2001), in a study evaluating Pb bioavailability from soil, included the analysis of Pb contents in rat muscle, spleen, heart, and lung, in addition to blood, bone, liver and kidney, and showed that > 99% of Pb body content was in bone, blood and kidney, with less than 0.6% accumulated in muscle, and non significant values reported for the other tissues. Furthermore, Winiarska-Mieczan and Kwiecień (2016) reported that 99% of the Pb accumulated in soft tissues were in liver and kidney, and less that 1% in other organs (brain, spleen, lungs, heart).

Also, despite the use of *M. galloprovincialis* from a clean area in the control diet, Pb concentration in diet 1 was higher than Pb concentration in maintenance diet (0.16 *vs* 0.07 mg kg^{-1}). Therefore, it is possible that ABA might be slightly underestimated by using these organisms to calculate the background Pb in tissues. If background Pb was assumed to be 0 in eq. (2) –what would lead to an overestimation of ABA values–, then Pb ABA would be 2.6% for diet 2 and 1.3% for diet 3.

4. Discussion

The present data show that Pb assimilation from *D. exoleta* is approximately half of that from *M. galloprovincialis*, as observed in Pb

Table 2
Pb ingested, excreted and accumulated in different rats tissues (ng) during the 14-days experimental period and calculation of absolute bioavailability (ABA).

Treatment group	Rat nº.	14-days ingested Pb	13-days ingested Pb	Faeces-Pb	Urine-Pb	Liver-Pb	Kidneys-Pb	Bone-Pb	Blood-Pb	Bile-Pb[a]	Total assimilated Pb	Back. corr. total assim. Pb [b]	**ABA%**
Diet 1	1	38,133	37,072	37,538	108	24	10	364	9	968	1482	–	–
	2	32,049	30,559	30,004	122	1	10	291	9	1099	1532	–	–
	3	34,906	33,038	33,009	134	< LOD	13	382	12	1206	1746	–	–
	4	36,586	33,769	34,609	164	< LOD	11	414	14	1479	2082	–	–
Diet 2	5	416,774	393,919	345,605	1012	62	129	1206	71	9109	11,588	9878	2.4%
	6	373,186	361,196	310,553	586	35	100	909	65	5278	6974	5263	1.4%
	7	371,390	346,704	340,376	1040	62	126	965	63	9361	11,617	9906	2.7%
	8	410,117	382,437	346,958	874	48	104	1119	73	7869	10,087	8376	2.0%
Diet 3	9	476,386	449,531	430,340	599	53	58	838	45	5394	6988	5278	1.1%
	10	483,124	457,944	420,734	535	44	60	1226	49	4818	6731	5021	1.0%
	11	404,373	383,883	363,316	459	32	57	697	37	4128	5410	3699	0.9%
	12	360,910	352,871	308,333	340	24	66	640	46	3062	4177	2466	0.7%

LOD = Limit of detection.
[a] Estimated as 9 × Urine-Pb.
[b] Background corrected total assimilated Pb.

accumulation in all analysed tissues and in Pb excretion in urine, which were in all cases approximately half for rats fed with a similar dose of Pb from *D. exoleta* in comparison with Pb-contaminated mussels.

Subcellular distribution of Pb in similar samples was done in a previous study (Sánchez-Marín and Beiras, 2017), where it was shown that 90% of Pb in *D. exoleta* is in the form of metal rich granules (MRG), while this fraction only accounts for 25% of Pb content in *M. galloprovincialis*. It was also shown that wild mussels collected from a harbour location and subjected to *in situ* long-term Pb exposure presented the same subcellular distribution as laboratory exposed mussels (Sánchez-Marín and Beiras, 2017). Therefore, the differences in subcellular distribution and assimilation by rats cannot be attributed to the exposure type (long term field exposure vs short term laboratory exposure) but to the interspecific differences in Pb compartmentalization. Wallace and Luoma (2003) investigated how the subcellular fractionation of Cd accumulated in the tissues of bivalves and oligochaetes affected its trophic transfer to the decapod crustacean *Palaemon macrodactylus* by comparing assimilation efficiencies with the sum of fractions that appeared to be trophically available. They concluded that the available metal fraction was that associated with the organelles and cytosol, while the metal in the cell debris and MRG appeared trophically unavailable. MRG in *D. exoleta* were described in Darriba and Sánchez-Marín (2013). Energy dispersive X-ray spectroscopy showed that they are mainly composed of Ca, P and O (Darriba and Sánchez-Marín, 2013), so they have been postulated as being calcium phosphate granules, where Pb might be substituting Ca in the form of $Pb_3(PO_4)_2$. Pb in this form appeared to be completely unavailable for trophic transfer to a marine invertebrate (Sánchez-Marín and Beiras, 2017), but the present study shows that it is partially assimilated in a vertebrate digestive system. According to the bioavailability of Pb from soils with different composition, it was proposed that Pb-phosphate compounds have medium bioavailability, in a mineral ranking from lower to higher bioavailability: anglesite, galena, Pb(M) oxide < Pb-phosphate, Pb-oxide < cerrusite (USEPA, 2017), which is in agreement with the partial bioavailability observed for Pb-phosphate containing granules. The main chemical form of Pb in MRG from *M. galloprovincialis* is not known, although Pb has been found in lipofuscin granules and extracellular carbonate deposits in *Mytilus edulis* (Marshall and Talbot, 1979; George, 1983). It may be possible that Pb in MRG from *M. galloprovincialis* is in a more bioavailable form than in *D. exoleta* ones, although the lower fraction of Pb in MRG in the mussel would also explain the higher bioavailability of Pb in this species.

No other studies have been found evaluating the bioavailability of Pb from bivalves *in vivo*, except one study where it was shown that Pb from polluted mussels was accumulated in mice, but the degree of bioavailability was not quantified (Regoli and Orlando, 1994). *In vitro* studies have shown that differences in bioaccessibility (i.e. the fraction of metal that is released from the food matrix to the digestive tracts) among bivalves depend on the subcellular distribution of the metal (He and Wang, 2013; Gao and Wang, 2014). He and Wang (2013) showed a negative correlation between Pb bioaccessibility and the fraction of Pb present as MRG in 11 marine mollusc species, which would be confirmed by the present *in vivo* data.

ABA of Pb from bivalves was roughly estimated to be around 2% of dose for *M. galloprovincialis* and 1% for *D. exoleta*. This estimation was done summing up Pb contents in all analysed tissues and cumulative Pb excreted in urine and bile (this last was estimated) as a function of dose. Using a similar approach (sum of accumulated Pb in target organs as a fraction of dose), Pb ABA to rats was previously reported to be around 15% from Pb-acetate incorporated in the diet and from 0.8 to 9% from soils (Freeman et al., 1996). Ellickson et al. (2001) reported an ABA of 0.7% from a soil source, using also rats. Pb ABA to minipigs was reported to be 3% from Pb-acetate and from 0.5 to 1.9% from soils (Marschner et al., 2006). Therefore, the ABAs reported for soils in similar studies are in the same order of magnitude of those estimated here for bivalve molluscs.

Another approach for estimation of ABA is to compare Pb accumulation in a target organ (normally blood Pb levels) in animals that have been fed Pb-containing food with those subjected to intravenous Pb administration (assumed to be 100% bioavailable). Comparing with intravenous administration of Pb, ABA of Pb-acetate to rats varied depending on the organ considered between 7 and 15% and that of mine waste soil was 0.4–2.7% (Freeman et al., 1994).

Therefore, despite differences in experimental procedures, such as duration of the experiment, doses administered, etc., our data seems to be in agreement with previous data of Pb bioavailability to animal models, and the bioavailability of Pb from bivalves is in the same range as that reported earlier for soils. Whether such a low Pb bioavailability is general for all food sources or is specific to bivalve tissues cannot be elucidated given the almost complete lack of *in vivo* studies on Pb bioavailability for other food sources not being soils. Only one study was found evaluating ABA of Pb in milk to suckling rats, that varied from 36% to 45% depending on milk source, and it was 50% from Pb administered in water (Hallén and Oskarsson, 1995).

In addition to chemical speciation of Pb in food, other factors have great influence in the bioavailability of Pb, namely the concentration of competitors of Pb uptake and the presence of chelators such as phytic acid (Mushak, 1991; Schroder et al., 2004). Pb absorption has been shown to vary from 1 to 20% depending on the different levels of Ca and Fe in the accompanying food (Kostial and Kello, 1979). Since both

Ca and Fe are expected to be at high levels in marine bivalve tissues as compared with other food sources, this factor might also contribute to the low bioavailability of Pb in bivalves.

The European Food and Safety Administration (EFSA) has not established a maximum safe Pb intake, and it has revoked the provisional tolerable weekly intake (PTWI) of 25 μg kg^{-1} body weight proposed by the WHO in 1986 (EFSA, 2010). On this basis, the recommendation is to consume the least Pb as possible, although it should be noted that cereals, vegetables and tap water are the most important contributors to Pb exposure in the European population (EFSA, 2010). Given that the RBA of Pb in *D. exoleta* is around 50% compared to a common food such as *M. galloprovincialis* mussels, and the low frequency of consumption of this type of food, an increase in the maximum permitted level of Pb in this bivalve to the double of the present limit (from 1.5 to 3 μg g^{-1} ww) would not increase risk for consumers.

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