6 rue Alphonse Rio · 56100 Lorient · FRANCE
+ 33 297 83 11 69 · info@ccr-s.eu
www.ccr-s.eu

Avis 135 sur la révision de la limite légale de la teneur en plomb (pb) chez la montre radiée (*Dosinia exoleta*)

Introduction

La montre radiée (*Dosinia exoleta*) est un mollusque bivalve benthique exploité en Galice. Les collectifs qui se consacrent à l'extraction de cette ressource sont les conchyliculteurs à pied et, principalement, les mareyeurs qui pêchent à bord de leur embarcation.

Le problème majeur de cette espèce dans les eaux galiciennes est constitué par les fortes concentrations en plomb (Pb) relevées dans sa chair et qui, souvent, dépassent le seuil défini dans la législation européenne pour autoriser sa consommation (soit 1,5 mg/kg de poids frais de chair au titre du règlement (CE) n° 1881/2006 de la Commission européenne du 19 décembre 2006). Cela est dû en partie au fait que la montre radiée accumule le plomb au fil des années, c'est-à-dire à mesure que sa taille s'accroît, de telle sorte que plus elle est développée, plus la teneur en plomb est élevée. Ce rapport taille-concentration n'a pas été constaté chez les autres bivalves.

C'est en mai 2006 que de tels niveaux de plomb ont été observés pour la première fois chez la montre radiée en Galice. Depuis lors, l'extraction régulière de cette espèce n'a été autorisée qu'à partir de l'année 2015 dans certaines zones de la Ría de Arousa, exclusivement pour des bivalves dont la taille oscille entre 30 et 35 mm. En effet, les analyses ont démontré que, dans ces conditions, la concentration de plomb est inférieure aux seuils fixés par la loi.

Le fait de ne pouvoir capturer que des spécimens de cette taille (30 à 35 mm) a entraîné une diminution de 89% des captures et de 77% des revenus.

Diverses études et recherches ont montré que cette espèce possède une manière particulière d'accumuler du plomb dans son organisme et que c'est précisément cette particularité qui est responsable de l'augmentation de la concentration tout au long de sa vie, mais également du fait que la transmission du plomb aux consommateurs de la montre radiée est beaucoup plus faible que dans le cas des autres bivalves ; il serait donc possible de consommer des échantillons présentant des concentrations supérieures à 1,5 mg / kg de poids frais de sa chair sans que cela entraîne un risque pour le consommateur.

Analyse

Diverses études, tant *in vivo*, en utilisant des organismes vivants tels que la crevette commune et le rat, qu'en laboratoire (*in vitro*), en simulant la digestion chez les êtres

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humains, ont montré que le plomb chez la montre radiée était très peu biodisponible pour ses consommateurs, c'est-à-dire que, même consommé, le plomb présent dans la chair n'est transféré qu'à un très faible pourcentage, de l'ordre de 0,02% pour l'homme, et que cela est dû au fait que le plomb accumulé sous forme de granulés dans l'intestin de la montre radiée est inorganique.

En comparant la biodisponibilité relative du plomb dans la montre radiée avec celle d'autres mollusques bivalves, tels que les moules, on a pu parvenir à la conclusion que celle-ci est inférieure de 50% dans le cas de la montre radiée, c'est-à-dire que, pour transférer la même quantité de plomb, la montre radiée devrait avoir une concentration de plomb deux fois supérieure à la teneur présente chez les autres bivalves.

Si nous extrapolons ces données, et compte tenu du fait que la réglementation en vigueur fixe la teneur maximale en plomb des bivalves à 1,5 mg/kg de poids frais de chair, il serait possible, comme le démontrent les études, de consommer des spécimens de montres radiées contenant le double, c'est-à-dire 3 mg/kg de poids frais de chair, sans que cela ne représente un risque pour la santé humaine.

Proposition

Compte tenu des résultats obtenus dans les différentes études et recherches, nous demandons à l'Autorité européenne de sécurité des aliments (EFSA) une révision de la limite légale actuelle de la teneur en plomb fixée pour les mollusques bivalves (soit 1,5 mg/kg de poids frais de chair au titre du règlement de la CE 1881/2006), et d'introduire une exception pour la *Dosinia exoleta* en fixant pour cette espèce une quantité maximale de plomb de 3 mg/kg de poids frais de chair.

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Lead accumulation in extracellular granules detected in the kidney of the bivalve *Dosinia exoleta*

Susana DARRIBA[1,a] and Paula SÁNCHEZ-MARÍN[2,3,b]

[1] Technological Institute for the Monitoring of the Marine Environment of Galicia (INTECMAR), Consellería do Medio Rural e do Mar., Vilagarcía de Arousa, Galicia, Spain
[2] Laboratorio de Ecoloxía Mariña (LEM), Universidade de Vigo, Vigo, Galicia, Spain
[3] Institut national de la recherche scientifique, Centre Eau, Terre et Environnement, Québec, Canada

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Abstract – Populations of the marine molluscan bivalve *Dosinia exoleta* in Galicia (northwest Spain) present lead (Pb) concentrations above the limit for human consumption. Accordingly, its collection for human consumption was forbidden since 2008. The high bioaccumulation of Pb in this species is surprising given that Pb concentrations are not very high in its environment and that other bivalve infaunal species inhabiting the same areas do not show such high Pb contents. This study reports the discovery and description of extracellular granules present in the kidney tubule lumina of this species. Large granules (20–200 μm) mainly composed of calcium phosphate represent between 50% and 75% of the dry weight of the kidneys. Metal analysis revealed that from 78 to 98% of the Pb body burden was present in the kidney, and from 87% to 92% of this Pb within the kidney was contained in metal rich granules. Most of the zinc in these bivalves was also found to be associated with these kidney granules, while other metals, such as copper and cadmium, were associated with other kidney fractions. This study confirms that the high Pb concentrations observed in *D. exoleta*, and the relationship of Pb concentration with individual size, are due to the inclusion of Pb in kidney granules that accumulate in the kidney lumen over the course of the bivalve's life.

Keywords: Metal bioaccumulation / Lead uptake / Metal rich granules / Clam / Veneridae / Bivalvia / Atlantic Ocean

1 Introduction

The bivalve mollusc *Dosinia exoleta* (Linnaeus 1758) was once very important as a commercial species harvested in the Galician Rías (NW Spain). Until 2005, its production was over one thousand tonnes per year (Anonymous 2012), but in 2006 the exploitation of this marine resource was partially interrupted due to the high Pb concentrations detected in its flesh, which were higher than the 1.5 μg g^{-1} wet weight (ww)- limit established by the European Commission for human consumption (Anonymous 2006). Then, in 2008, the collection of this species for human consumption was prohibited in Galicia.

Studies of metal concentrations in other bivalve molluscs from the Galician Rías (Besada et al. 2002; Beiras et al. 2003c; Besada and González-Quijano 2003; Saavedra et al. 2004; Blanco et al. 2008; Besada et al. 2011) showed lower levels of Pb concentrations than those detected in *D. exoleta* (Sánchez-Marín and Beiras 2008). Galician Rías have only a low level of pollution, mainly restricted to localised areas (Beiras et al. 2003a; Beiras et al. 2003b; Prego and Cobelo-García 2003). The high Pb accumulation by large individuals of *D. exoleta* from the Ría de Arousa was unusual given the low level of Pb pollution in this Ría and this was interpreted as a particularity of this species (Sánchez-Marín and Beiras 2008).

Metal accumulation by bivalves and other biota is influenced by several biological and environmental factors (size, age, season, reproductive stage, etc.). Sanchez-Marín and Beiras (2008) found a positive relationship between size and metal accumulation in *D. exoleta*. It was observed that larger individuals (>40 mm) could contain much higher concentrations of Pb than smaller ones. This pattern is often observed in bivalves that accumulate metals in metal concretions, where the metal is accumulated as a storage compartment throughout the animals' lives, so that larger –and older- animals usually contain more granules (Brown 1982; Wallace et al. 2003). The ability of bivalve molluscs to accumulate metals, such as lead, copper, cadmium and zinc is well known, but the mechanisms of detoxification are not well understood (Marigómez et al. 2002; Wang and Rainbow 2008). Aquatic invertebrates sharing the same habitat may have very different metal concentrations, depending on their uptake and elimination kinetics, assimilation efficiencies and detoxification strategies

[a] Corresponding author: sdarriba@intecmar.org
[b] Corresponding author: paula.sanchez-marin@ete.inrs.ca

(Luoma and Rainbow 2008; Wang and Rainbow 2008). A very common means of detoxification in bivalves is the inclusion of metals in insoluble granules or deposits (Mason and Jenkins 1995; Marigómez et al. 2002), which may or may not be excreted. Three types of metal-rich granules are frequently observed within bivalve tissues: copper-, calcium- and iron-containing granules (Brown 1982). Although all three may be present in bivalve tissues, calcium-containing granules have received the most attention, with descriptions in several tissues, especially in kidney (Sullivan et al. 1988a). The excretory system of bivalves includes the pericardial gland and kidney (nephridium) which is composed of tubular structures (renal tubules) that collect fluids from the pericardial gland and are connected to the excretory pore to excrete the urine.

A histological study performed in 2008 (Darriba et al. 2009) revealed the presence of extracellular granules in the lumen of the renal tubules of all *D. exoleta* individuals examined from the Galician Rías. The present study characterizes and examines the morphological aspects of granular concretions in the kidney tubule lumina of *D. exoleta* by microscopic techniques, and their relation with the high levels of lead accumulation in this species.

2 Material and methods

2.1 Samples

Dosinia exoleta individuals of uniform size (between 40 and 45 mm) were collected from the Galiñeiro natural subtidal bed in the Ría de Arousa (Galicia, NW Spain) in two sampling campaigns in March 2009 and September 2010. Samples were transported to the laboratories in isothermal freezers.

2.2 Histological and scanning electron microscope analysis

In March 2009, the kidneys of ten adults were dissected, taking one piece for histological analysis and another for scanning electron microscopy (SEM) analysis and energy-dispersive X-ray spectroscopy (EDS) from each.

For histological analysis, the kidney samples were fixed in Davidson's solution (Shaw and Battle 1957) and embedded in paraffin. Paraffin blocks were cut into 5-μm sections with a microtome. Tissue sections were deparaffinized, stained with Harris' hematoxylin and eosin and examined by light microscopy.

For SEM analysis, small pieces of tissue were fixed with 10% formalin stabilized with methanol and washed in 0.1 M cacodylate buffer with 8% sucrose, added 4 hours later. Kidney pieces were cut with a blade and shaken on a Petri dish, using dissecting forceps to help to free a high number of kidney granules. Two washes of 1 hour with Mili-Q water were then required to eliminate the cacodylate buffer. Granules were collected using a Pasteur pipette, put on stubs with carbon disc adhesive and dried at 30 °C until total water evaporation. Finally, the granules were carbon-coated in a Sputter Coater EMITECH K550X by carbon evaporation.

Morphological examination and EDS, for identification of elemental composition, were realized at 20 kV in a Philips XL 30 scanning electron microscope fitted with an EDAX DX4 energy dispersed system. Kidneys embedded in paraffin were also sectioned to obtain a cross section of the granules in the tubule lumina, which was also examined under SEM and analysed by EDS.

2.3 Metal analysis and subcellular fractionation

Ten individuals from those collected in March 2009 were dissected to separate the kidney from rest of body and kept frozen in polypropylene vials until digestion and analysis. Metal concentration in the kidneys and the rest of the tissues were measured separately for each individual.

The organisms collected in the second sampling campaign in September 2010 were used for subcellular fractionation of the kidneys. Freshly dissected kidneys from 30 individuals were put into six pre-weighed 10 ml polypropylene vials (five kidneys per vial) and homogenized in 2:1 volume of ultrapure water using a homogenizer (Ultra-Turrax). Homogenized tissue was fractionated using a method modified from Wallace et al. (2003). Briefly, the tissues were centrifuged (15 min; 1400 g), the supernatants (cytosol and organelles; S1 fraction) were collected in 10 ml pre-weighed polypropylene vials and the pellets were heated for 2 min at 100 °C after addition of 1 ml of ultrapure water. Then, 1 ml of NaOH 1 M was added to the pellets and they were heated for 1 h at 60 °C. After a second centrifugation (10 min; 4600 g) the supernatants (cellular debris; S2) were added to the S1 fraction, and the pellets (metal rich granules, MRG) were washed 3 times with 1 ml NaOH 1 M and a last time with ultrapure water. The washing solutions (supernatants) were added to the S1+S2 fractions so that two cellular fractions were finally obtained: one containing the granules and the other containing all the rest. The vials were dried to constant weight at 70 °C. Separate kidneys from 25 individuals were individually weighed to establish the relationship between wet and dry weight (dw); the resulting dw: ww ratio for the kidneys was 0.32 ± 0.08.

Blank vials were included with the samples to evaluate possible metal contamination during the fractionation procedure.

To assure that the washing of the granules was complete, the quantity of organic carbon remaining in the MRG fraction was measured in a preliminary fractionation test using the wet oxidation method (Walkley 1947) and was found to be lower than 8%.

Metal (Cu, Pb, Zn and Cd) contents of the kidneys, the rest of the clam tissues, the granules and the rest of the kidney tissue were analysed by ICP-MS (X Series, Thermo Elemental, Cheshire, UK) after digestion with HNO_3 and H_2O_2 following a microwave assisted procedure described elsewhere (Sánchez-Marín and Beiras 2008). Procedure blanks and certified reference material ERM-CE278 (mussel tissue) were included in the sample treatment and analysis. The percentage of recovery of Cu, Pb, Zn and Cd was between 95 and 105% in all cases.

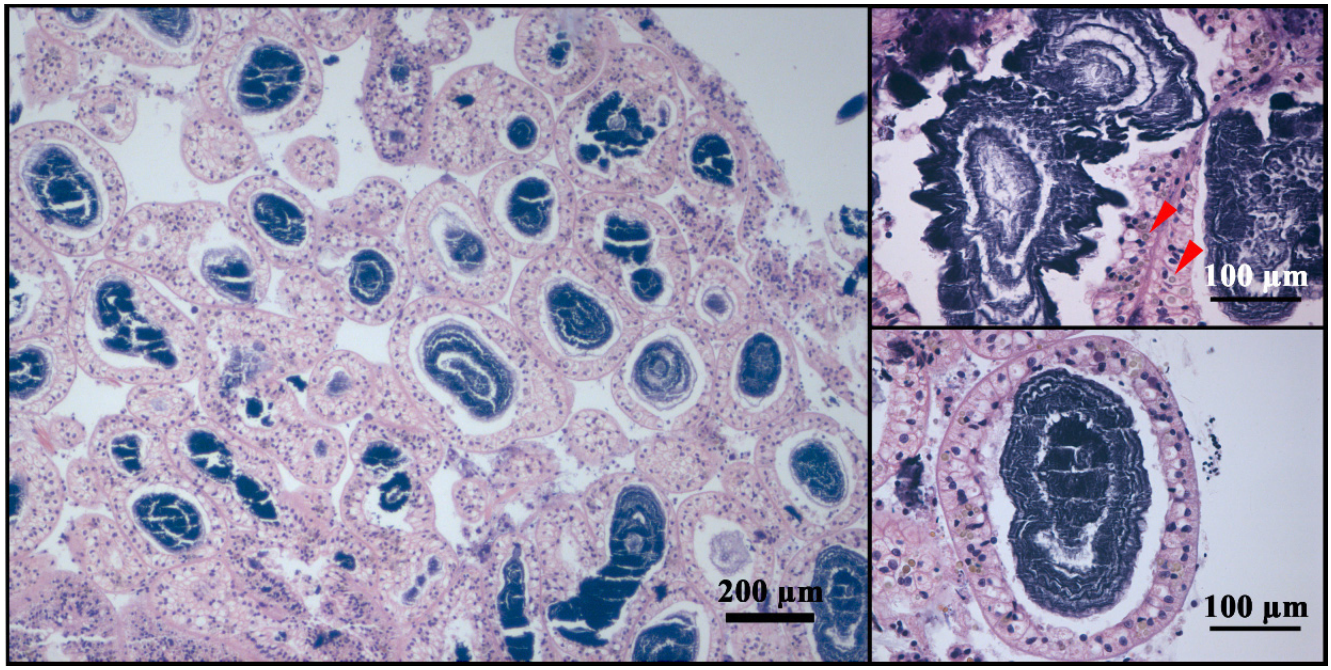

Fig. 1. Kidney lumen of *Dosinia exoleta* stained with Harris' hematoxylin and eosin observed by light microscope. Large extracellular granules (20–200 μm) are stained in blue, and small intracellular refringent granules in epithelial cells are indicated by red arrows.

3 Results

3.1 Histological and scanning electron microscope analysis

Dosinia exoleta has a large kidney in comparison with other bivalve species, such as mussels or other clams. Wet weight (ww) of dissected kidneys varied between 0.1 and 0.4 g and represented between 1.5% and 5% of the total wet weight of the soft tissues.

Light microscopy showed that kidney tubule lumina were filled with large basophilic extracellular granules of variable sizes (20–200 μm in the large axis) surrounded by epithelial cells. These granules have an irregular shape and are organized in concentric layers around a central core. Most epithelial cells contained small intracellular refringent granules of round shape and brown colour (Fig. 1).

Under the scanning electron microscope, extracellular granules showed variable size and an extremely irregular shape with a dimpled surface (Fig. 2).

X-ray microanalysis revealed that the granules were mainly composed of Ca, P and O, with a significant proportion of Mg, and a lower concentration of Cl, Na and Mn. The granules are, therefore, mainly formed by $Ca_3(PO_4)_2$ deposits. Other elements, such as Si, Zn and Fe were present in concentrations close to limit of detection of EDS, and were only detected in some of the scans performed, one of which is shown in Figure 3.

3.2 Metal distribution in the organism

The ten individuals analysed showed a high variation in their Pb content. Pb concentrations in the whole organism varied from 2.0 to 37.0 μg g^{-1} dry weight (dw). Zn concentrations varied by up to an order of magnitude of difference, ranging from 118 to 1151 μg Zn g^{-1} dw. The concentrations of Cu and Cd were more homogeneous, presenting mean concentrations of 4.7 ± 1.6 μg Cu g^{-1} dw and 0.6 ± 0.15 μg Cd g^{-1} dw.

Although the kidney was the only organ studied separately, the concentration of the four studied metals was higher there than in the rest of tissues (Table 1). However, this increased tendency for metals to be in the kidney was much more marked for Pb and Zn than for the other two metals. Of the total Pb present in the soft tissues, from 78 to 98% was found in the kidney. For Zn this amount varied between 70% and 97%, while the amount of Cd and Cu in this organ represented less than 74% and 46% of total body burden, respectively.

3.3 Metal distribution in the kidney

The MRG fraction represented between 50% and 75% of the dry weight of the kidneys, showing both the high quantity of granules and their relative importance in kidney composition, as can be seen in Figure 1.

The limit of detection of EDS (between 1 and 10 mg g^{-1}) was not enough to detect the Pb concentrations in the granules. Pb concentration in the MRG fraction measured by ICP-MS was 143 ± 15 μg g^{-1} dw (Table 2). Pb and Zn concentrations were much higher in the MRG fraction than in the rest of the kidney tissues, while the opposite distribution was observed for Cu and Cd.

From the total metal contents within the kidney, the majority of Pb (from 87% to 92%) and Zn (from 85% to 91%) were in the MRG fraction. In contrast, Cu and Cd were preferentially associated with the organic fractions of the kidney.

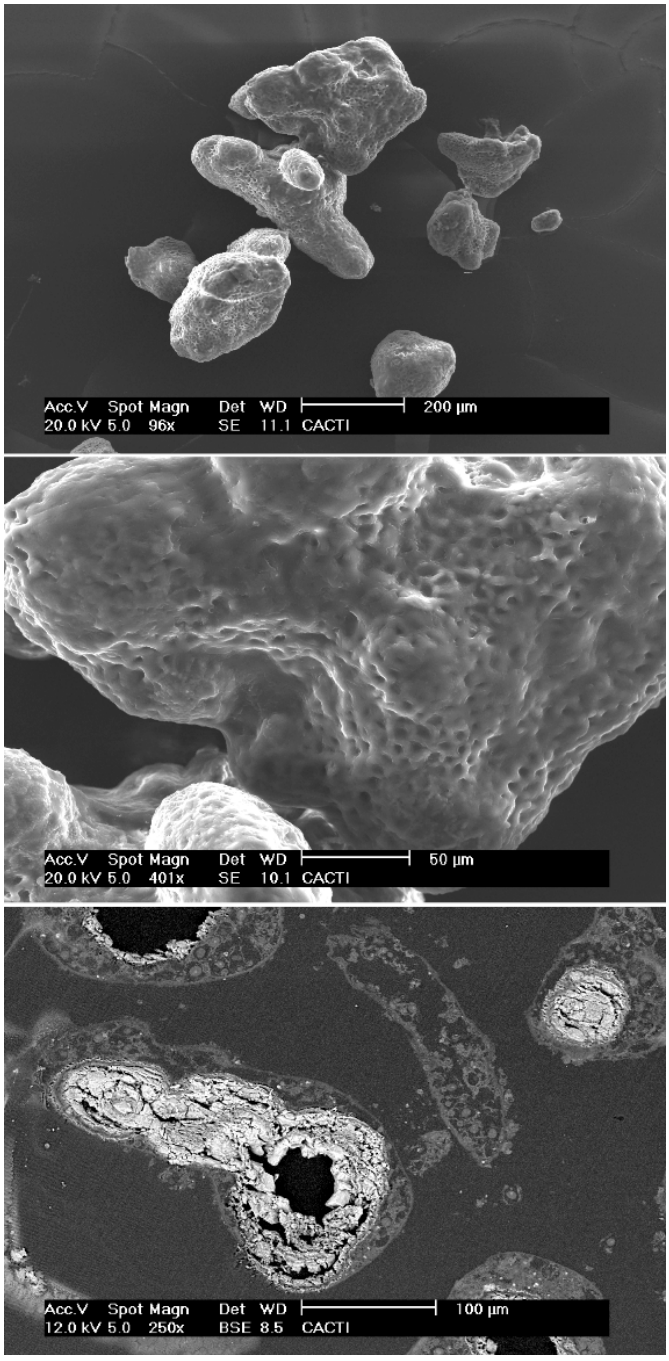

Fig. 2. Scanning electron microscopy images of isolated extracellular granules and a cross section of granules in the kidney lumen.

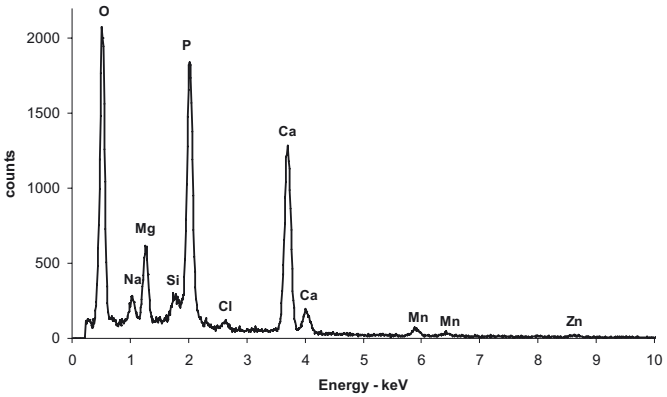

Fig. 3. Energy dispersive X-ray microanalysis of a cross section of a kidney granule showing its main elemental composition.

Table 1. Metal concentrations (μg g^{-1} dw) in whole soft tissues of *Dosinia exoleta* and distribution between the kidney and the rest of the tissues (n = 10 individuals).

		min	max	median
Pb	Whole soft tissues	2.0	36.9	5.0
	Kidney[a]	59.5	437.0	99.1
		(78%)	(98%)	(92%)
	Rest of tissues	0.1	1.0	0.5
Cu	Whole soft tissues	2.4	7.4	4.7
	Kidney[a]	11.0	76.1	35.0
		(23%)	(46%)	(35%)
	Rest of tissues	1.9	5.3	3.3
Cd	Whole soft tissues	0.3	0.8	0.6
	Kidney[a]	3.3	14.9	8.0
		(52%)	(74%)	(62%)
	Rest of tissues	0.1	0.4	0.2
Zn	Whole soft tissues	118	1151	430
	Kidney [a]	2761	14061	8384
		(70%)	(97%)	(89%)
	Rest of tissues	19.2	58.5	44.3

[a]The percentage of metal in the kidney was calculated based on the total metal content in the organism's soft tissues.

Table 2. Metal concentrations (μg g^{-1} dw) in the subcellular fractions of the kidney metal rich granules (MRG) and the rest of the kidney[a].

	Pb	Cu	Cd	Zn
MRG	143 ± 15	29 ± 13	2.4 ± 0.4	7689 ± 1683
Rest of kidney	13 ± 6	117 ± 16	16 ± 4	915 ± 324

[a] Values expressed as the arithmetic mean ± standard deviation obtained from six composite samples of five kidneys each.

The fraction of these metals that was present in the granules varied from 2% to 25% for Cu and from 9% to 12% for Cd. Mean distribution of four metals within the kidney is represented in Figure 4.

4 Discussion

By combining histological and microscope information with metal analysis, it was demonstrated that the reason for the very high Pb contents reached in large individuals of *D. exoleta* was the accumulation of Pb in inorganic concretions in the kidney. Zn was also found to be strongly associated with the kidney, forming part of the large extracellular calcium phosphate granules present in this organ. For this reason, Pb and Zn concentrations were very variable (Table 1), given that the number of granules present in the kidney were also

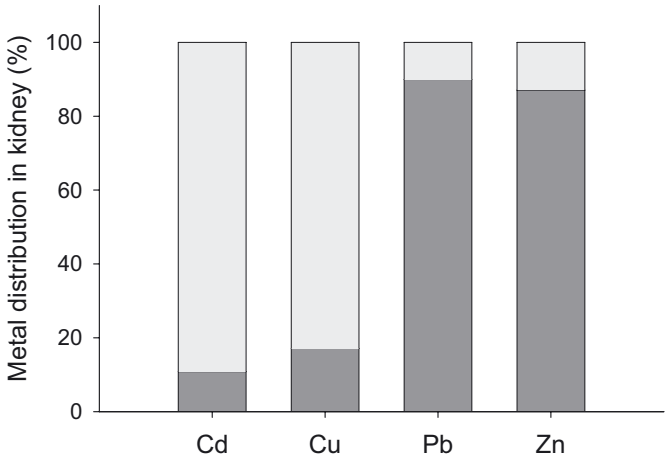

Fig. 4. Mean metal distribution in the kidney of *Dosinia exoleta* in the MRG (metal rich granules) fraction (dark grey) and the rest of the kidney (light grey). The majority (>80%) of Pb and Zn were found in the MRG fraction, while only <20% of Cu and Cd were found in this fraction.

very variable among individuals, as a great heterogeneity in the density of granules occupying the kidney lumen was observed in the several cross sections examined. In contrast, Cd and Cu were associated with other tissue fractions, and their concentrations presented less inter-individual variation.

The granules described here correspond to type B calcium-containing granules, according to the classification in Brown (1982). These granules are described as containing low purity calcium, i.e., together with magnesium, manganese, phosphorus and perhaps other metals. This type of granules, as opposed to the type A calcium-containing granules −believed to have mainly a calcium storage function−, have more dynamic functions such as excretion, storage and calcium mobilization, and/or detoxification. In a different classification made by Hopkin (1989), these same low purity calcium granules are named "Type A granules" and are known to be able to accumulate potentially toxic class A and borderline metals such as Mn, Zn and Pb, while class B metals such as Cd, Cu and Hg have not been detected in them. This agrees with our finding that Pb and Zn are preferentially found in the granules, while Cu and Cd are not, combined with the presence of significant Mn concentrations in the granules, which are high enough to be detected with EDS (Fig. 3). However, Hopkin (1989) describes type A granules as being intracellular, while the granules described here are extracellular. This is probably the result of intracellular granules being excreted into the kidney lumen, where aggregation of smaller granules or layering of intraluminal material in concentric rings would lead to the formation of extracellular kidney granules, as described in Marigómez et al. (2002). It is likely that bivalve kidney granule production and subsequent increase in size is a continuous process of lysosomal maturation, residual body release and extracellular accumulation (Sullivan et al. 1988a).

The presence of larger granules in some species is believed to reflect a longer residence in the kidney lumen and concomitant acquisition of materials, although it is not known why granules are retained and continue to grow in the kidney in some species, while in others they are quickly excreted (Sullivan et al. 1988a; Marigómez et al. 2002). Given the large size reached by the granules found in *D. exoleta*, and the relationship of Pb concentration with animal size (Sánchez-Marín and Beiras 2008), it is probable that this species retains all the granules it produces or that their excretion is very limited. Gold et al. (1982) found that the largest specimens of *M. mercenaria* had significantly higher amounts of kidney concretions than either intermediate or small clams, again suggesting a relationship between age and accumulation of granules. Also, the size of the kidney seems to play a role in granule production: bivalve species showing "kidney gigantism" have been shown to produce very large quantities of extracellular metal-sequestering granules compared with species that have normal kidneys (Reid and Brand 1989).

Large, extracellular kidney granules, similar to the ones described here, have been observed in several bivalves including *Pinna nobilis* (Ghiretti et al. 1972), *Macrocallista nimbosa* (Tiffany et al. 1980), *Donax trunculus* (Mauri and Orlando 1982), *Cyclosunetta menstrualis* (Ishii et al. 1986), *Mercenaria mercenaria* (Sullivan et al. 1988a) and *Donacilla cornea* (Regoli et al. 1992). In most cases, these granules were shown to contain high Mn concentrations, although some other trace metals were also detected, such as Fe, Zn and Pb. Sullivan et al. (1988a) reported Pb concentrations in kidney granules from *M. mercenaria* at around 150−200 μg g^{-1} dw, similar to those observed in *D. exoleta* (146 ± 16 μg g^{-1} dw). Subcellular distribution in the kidney also showed that these metals were mainly associated with concretions, while Cu and Cd were associated with the cytosolic fraction in *M. mercenaria* (Sullivan et al. 1988b).

It is not clear if the formation of renal concretions in bivalves has a detoxification role or another biological function, although it has been proposed that the excretion of concretions to the kidney lumen in some molluscs is a mechanism that contributes to the elimination of certain metals (Mason and Jenkins 1995; Marigómez et al. 2002). Mauri and Orlando (1982) observed that *D. trunculus* presented fewer renal concretions in relatively unpolluted waters than in highly polluted waters and that the formation of renal concretions in naturally concretion-free clams could be induced by exposing them to high Mn concentrations in the surrounding water. However, renal concretions containing heavy metals are also found in bivalve populations in apparently unpolluted waters. This suggests that factors other than high metal concentrations may induce their formation. According to Doyle et al. (1978), renal concretions appear to be a normal formation of the excretory process of some molluscs under reproductive, environmental, or pollutant-induced stress. This type of renal concretions might have evolved as a system involved in calcium homeostasis, to eliminate excess ionic calcium from the cells (Simkiss 1977), and the inclusion of other metals in their composition might be a secondary function (Mason and Jenkins 1995). Interestingly, induction of renal concretions was observed after exposure of *D. cornea* to sublethal concentrations of Cu and Cd (Regoli et al. 1992), but Cu and Cd were not detected in the concretions. According to these authors, the presence of these metals may have interfered with Ca metabolism (disrupting the

plasma membrane Ca-extruding systems) and the formation of renal concretions was activated to eliminate calcium excess.

In the case of *D. exoleta*, there is not enough data at present to determine wether the formation of kidney granules is induced by external factors, such as metal pollution, or not. The Galician Rías present a low to moderate degree of pollution, restricted to localized areas (Beiras et al. 2003b; Prego and Cobelo-García 2003), and Mn levels are not particularly high, and comparable to natural reference levels in other parts of the world (Prego and Cobelo-García 2003). Granules have been observed in the kidneys of individuals from other populations in other Galician Rías (unpublished data); and high levels of Pb have been detected in all analysed populations in the Galician Rías. This information may indicate that kidney granule formation and maturation may be a general process in *D. exoleta*, and in larger – and older- individuals, resulting in high Pb body burdens. Given the large size of the kidney in this species, which would make a high granule content possible, if Pb is never excreted from the body, then even not very elevated Pb concentrations in the environment could lead to final Pb body burdens over the safety limit for human consumption.

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

Paula Sánchez-Marín[a,b,*], Rafael Durán[c], Ricardo Beiras[a]

[a] Toralla Marine Science Station (ECIMAT), University of Vigo, Illa de Toralla, E-36331, Vigo, Galicia, Spain
[b] Instituto Español de Oceanografía, IEO, Centro Oceanográfico de Vigo, Subida a Radio Faro, 50, E-36390, Vigo, Spain
[c] Department of Functional Biology and Health Sciences, Faculty of Biology, University of Vigo, Campus Lagoas Marcosende, E-36310, Vigo, Spain

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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

* Corresponding author. Instituto Español de Oceanografía, IEO, Centro Oceanográfico de Vigo, Subida a Radio Faro, 50, E-36390, Vigo, Spain
E-mail address: paula.sanchez@ieo.es (P. Sánchez-Marín).

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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).

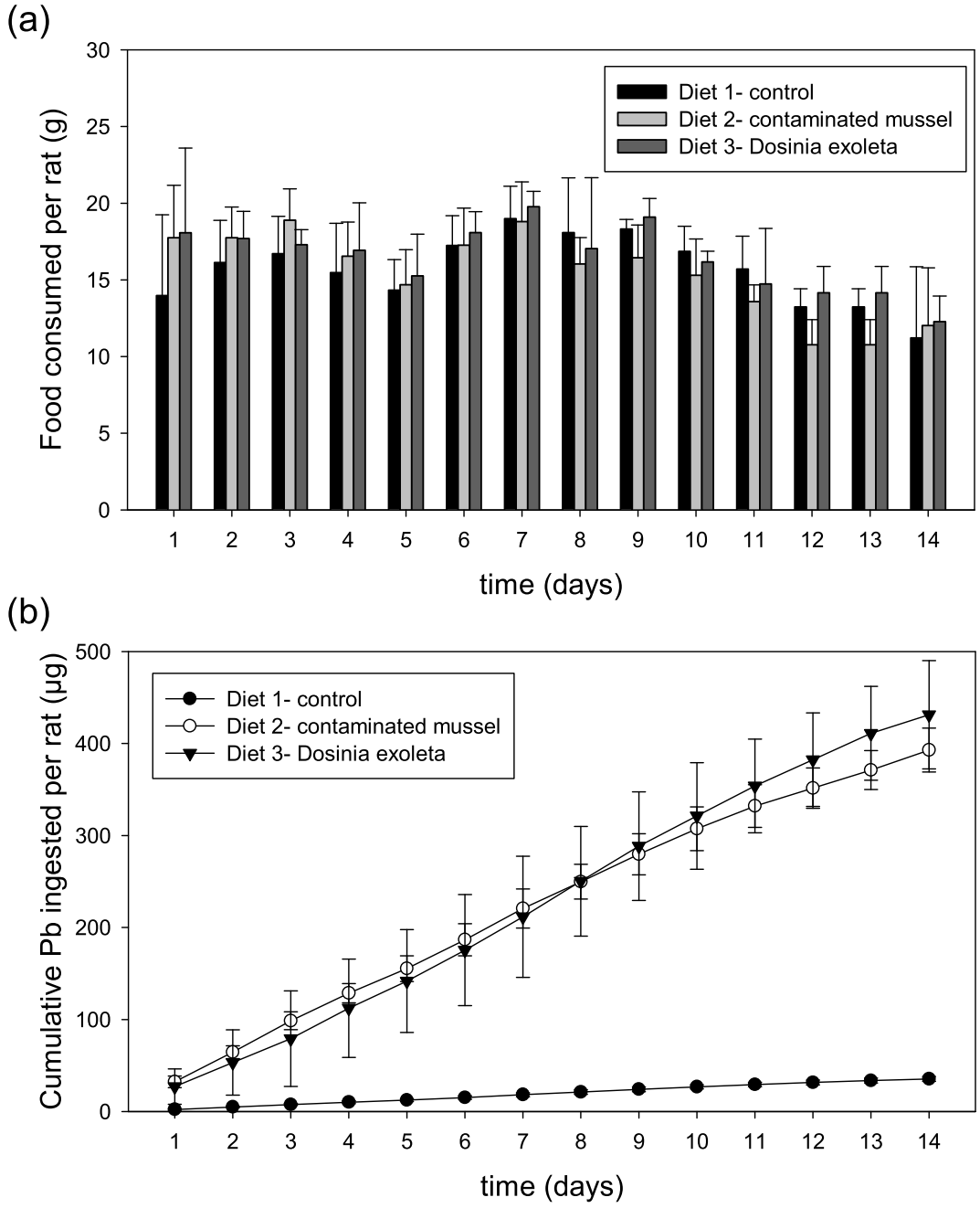

Fig. 1. Food consumed (a) and cumulative Pb ingested (b) per rat during the 14-days oral bioavailability experiment. Mean ± *SD* (*n = 4*).

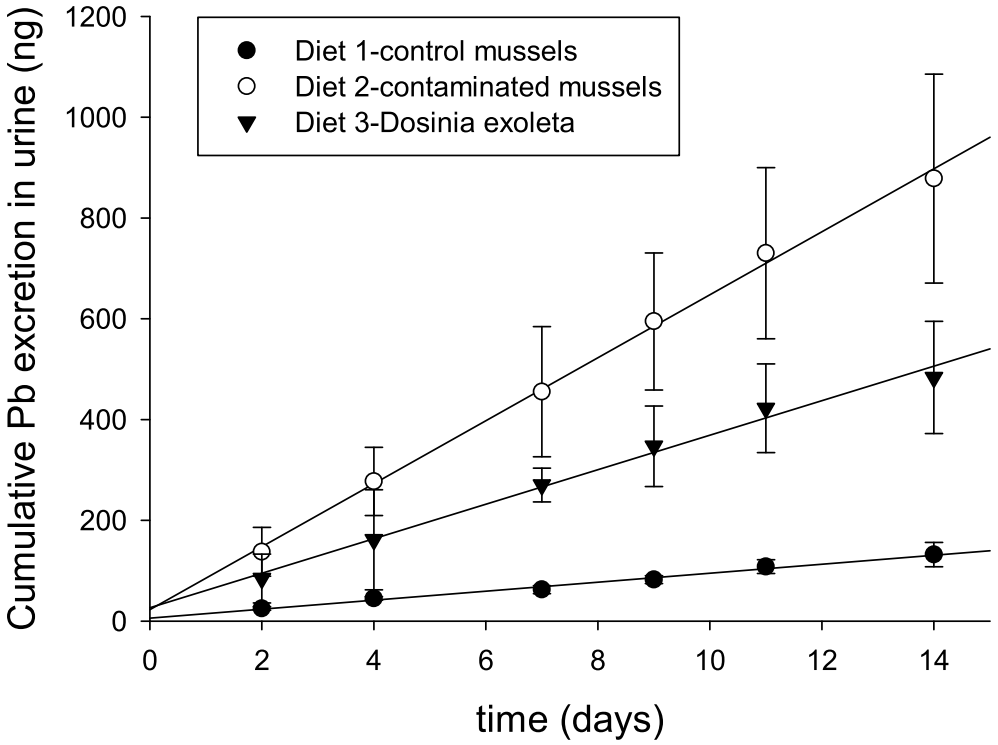

Fig. 2. Cumulative Pb excreted in urine per rat during the 14-days oral bioavailability experiment. Mean ± SD (n = 4).

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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Subcellular distribution and trophic transfer of Pb from bivalves to the common prawn *Palaemon serratus*

Paula Sánchez-Marín*, Ricardo Beiras

Department of Ecology and Animal Biology and ECIMAT, University of Vigo, 36310 Vigo, Galicia, Spain

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ABSTRACT

The edible clam *Dosinia exoleta* has been reported to accumulate high contents of lead (Pb) in soft tissues disregarding the levels of Pb in the environment. This is due to the retention of Pb in the form of metal rich granules (MRG) in their kidneys throughout the mollusc lifespan. The potential for trophic transfer of Pb in this form to predators is expected to be low, since metals in the form of MRG are generally supposed to be trophically unavailable, but this assumption is based on studies with other metals (Ag, Cd, Cu or Zn) and has not been demonstrated with Pb until now. This study was designed to test if the Pb present in *D. exoleta* in the form of MRG is available to a decapod consumer, the common prawn *Palaemon serratus,* in comparison with a mussel diet showing a different subcellular distribution of Pb. As hypothesised, despite the high Pb concentrations (15 μg g^{-1} ww) offered to the prawns as *D. exoleta* tissues, Pb was almost completely unavailable for trophic transfer, and the prawns fed with this diet during 28 days showed the same Pb accumulation as prawns fed with a control diet with a much lower Pb concentration. On the contrary, individuals fed with mussel tissues containing the same Pb concentrations as the diet based on *D. exoleta* tissues showed 10 times higher Pb bioaccumulation, corresponding to a trophic transfer factor of 1.1%. Subcellular fractionation experiments revealed that the fraction of Pb in the form of MRG was much lower for the mussel, confirming, as observed for other metals, that MRG-associated Pb is not available for trophic transfer to decapod crustaceans.

1. Introduction

Assimilation of trace metals from the diet is a significant source of metal bioaccumulation in marine organisms, including invertebrates (Luoma and Rainbow, 2008). The degree of trophic availability of metals is usually determined by the assimilation efficiency, quantified as the ratio between the amount assimilated in the gut and the amount ingested. For instance, assimilation efficiency of metals from phytoplankton or sediment particles in the digestive system of bivalves may range from 10% to 80%, and when food is significantly enriched in metal compared to water the contribution to total metal uptake by this via is very relevant (Chong and Wang, 2001; Ke and Wang, 2001).

Assimilation efficiency of metals largely depends on their chemical form in the food source, given that not all metal species are available for trophic transfer (Rainbow et al., 2011). A commonly used approach to assess how does metal speciation in the diet affect its trophic availability is the use of operationally defined separation techniques such as subcellular fractionation. These techniques separate the cell into different soluble and insoluble fractions that can subsequently be categorized to be or not within the trophically available metal (TAM) fraction (Wallace and Luoma, 2003). In the original formulation by Wallace and Luoma (2003), based on three experiments of trophic transfer of Cd from a clam and two oligochaete species to grass shrimps, three out of five operationally defined fractions, including metal associated with cytosolic proteins and metal associated to organelles, appeared to be trophically available, while other two fractions, including metal rich granules (MRG) and cell debris, appeared unavailable for trophic transfer. Additional studies have shown that the specific fractions considered as TAM may vary depending on the digestive power of the predator, the metal under study and the prey-specific particularities regarding chemical speciation of the metal in each particular fraction (reviewed by Rainbow et al., 2011).

Most studies have used decapod crustaceans as consumers and bivalves or polychaetes as preys to study the influence of the subcellular compartmentalization of metals such as Cd, Cu, Zn and Ag on their assimilation efficiency (Wallace and Lopez, 1997; Rainbow et al., 2004, 2006a; Rainbow and Smith, 2010). However, information on how subcellular compartmentalization of Pb affects its trophic transfer is lacking up to date, partly due to the lack of a suitable radio-labelled form to work with.

* Corresponding author.
E-mail address: paulasanchez@uvigo.es (P. Sánchez-Marín).

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Due to the risk posed by some metals such as Pb, Cd and Hg to human health their levels in foodstuff are regulated in the legislation, such as the European Directive EC 1881/2006 (Anonymous, 2006) that imposes for Pb a maximum of 1.5 mg Kg^{-1} of wet weight in bivalve molluscs. The edible clam *Dosinia exoleta* shows high Pb concentrations that may exceed this limit, especially in large > 40 mm individuals (Sánchez-Marín and Beiras, 2008). This occurred in populations inhabiting relatively clean areas, and the reason seems to be a distinctive feature of this species that accumulates Pb in extracellular granules in their kidneys throughout their live span (Darriba and Sánchez-Marín, 2013). The proportion of Pb present in MRG in this species was shown to range from 68% to 90% (Darriba and Sánchez-Marín, 2013), and therefore it represents a suitable organism to study the potential trophic transfer of Pb from this fraction to consumers.

The present study aims at testing the hypothesis that Pb present in the form of MRG in *Dosinia exoleta* is not available for trophic transfer to a marine consumer, the common prawn *Palaemon serratus*, in comparison to Pb present in other bivalve species (the mussel *Mytilus galloprovincialis*) showing a different subcellular distribution of Pb.

2. Materials and methods

2.1. Diet preparation

Mussels (*Mytilus galloprovincialis*), between 40 and 50 mm long were obtained from a mussel raft in the Ría de Arousa (Galicia, NW Iberian Peninsula). They were maintained in laboratory during 4 weeks in flow-through natural seawater and fed twice a week with phytoplankton. Around 100 individuals were dissected and the soft tissues were drained on a filter paper and kept frozen. This uncontaminated mussels were subsequently used as diet 1 (control diet). Another 100 individuals were exposed to 100 μg L^{-1} Pb during 48 h, as described in Sánchez-Marín et al. (2011). After the exposure period, the mussels were introduced in clean seawater during 10 min, then they were dissected and the soft tissues were again introduced in clean seawater during another 10 min to desorb weakly adsorbed Pb. After that, the soft tissues were drained on a filter paper and kept frozen. This Pb-enriched mussels were used as diet 2 in the trophic transfer experiments. This diet was prepared from laboratory exposed mussels due to the high levels of Pb desired, i.e. similar to the ones achieved in diet 3 (see below), which are not found in wild mussels.

Specimens of *Dosinia exoleta* were collected from a shellfish extraction area in the Ría de Arousa (42°31'00"N 8°51'44"W). Individuals between 40 and 50 mm long were chosen and dissected to separate the kidney from the rest of the soft tissues, that were kept frozen separately to be used for the preparation of diet 3, that contained a 1:2 wet weight proportion of kidney: rest of tissues.

Frozen tissues of mussels, clams and clam kidneys were cut with a scalpel in pieces of 3–5 mm^3, suitable for manipulation/ingestion by the prawns. The fragments were then mixed to obtain a homogeneous composite for each diet. In the case of diet 3, the kidneys and the rest of the tissue fragments were kept separately and mixed at the time of feeding only, to assure that the 1:2 ratio kidney: rest was accurately maintained.

In addition, around 100 individuals of *M. galloprovincialis* were sampled in a polluted site (Vigo harbour, 42°13'36"N 8°44'40"W) and left during 24 h in clean water to let the mussels empty the digestive system. These mussels were not used as diet in the trophic transfer experiment, but were included in the Pb subcellular distribution analyses.

2.2. Trophic transfer experiments

Living specimens of the common prawn *Palaemon serratus* were obtained from local fishermen and maintained in the laboratory during 4 days without being fed, in flow-through natural seawater at 18 °C. Forty-eight individuals of 50–60 mm length were divided in three experimental groups of 16 individuals each and introduced individually in 1 L beakers. The prawns were maintained in a continuous flow of 0.8 ± 0.2 L min^{-1} of 10 μm filtered seawater at 18 °C. The beakers were covered with a nylon mesh of 80 μm pore diameter to avoid losses of food and prevent the prawns from escaping. Prawns were fed on the different diets during 28 days. Each prawn was fed with 100 mg wet weight (ww) per day dosed twice a week, at 300 and 400 mg per dose. Normally, the prawns consumed all the food offered within less than 1 h. After the 28 days experimental period the prawns were let two more days in the experimental system without being fed, to let them empty the digestive system, although the last dose of food (400 mg) was administered 6 days previous to sampling. Several prawns moulted during the experiment, and in some cases they consumed their moult. Otherwise moults were removed from the beaker before the next feeding event.

At the end of the experiment the individuals were dissected, the digestive gland was separated from the rest of the tissues, and both samples were introduced in pre-weighed polypropylene vials for subsequent metal analysis. Digestive glands and rest of tissues of 16 individuals were also sampled at the beginning of the experiment for this purpose.

At each feeding event, two samples of 300 mg of each one of the diets were introduced in pre-weighted polypropylene vials and kept frozen for metal analysis, so that 16 samples of 300 mg were analysed for each diet.

A preliminary test was done to check whether the diet offered to the prawns was effectively ingested. Even though visual observations denoted that the animals consumed the food (it could be observed inside the stomach) we noticed that some pieces of *D. exoleta* kidney partially broke up in the water and some particles were lost. To quantify this partial loss, five individuals were fed with 300 mg of diet 3 in individual beakers and then sampled within less than 15 min after food ingestion, so that the food was still in the digestive system. The same was done with diet 2 for comparison. The prawns were introduced in polypropylene vials and frozen for subsequent metal analysis.

2.3. Subcellular distribution analysis

Subcellular fractionation of diets was performed following the methodology described in Wallace et al. (2003) and Rosabal et al. (2014). A diagram of the subcellular fractionation procedure is included in Fig. 1.

Two grams of wet weight of each one of the diets were homogenized in triplicate with 8 mL of cold 20 mM TRIS buffer (pH 7.6) using Potter-Elvehjem glass homogenizers. Homogenized tissues were transferred to 12-mL polypropylene vials and 500 μL of homogenate were taken for Pb analysis. The rest was centrifuged at 1450 *g* for 15 min, in a refrigerated centrifuge at 4 °C. The obtained pellet (P1) was digested with 8 mL of 0.5 M NaOH as described in Rosabal et al. (2014), and

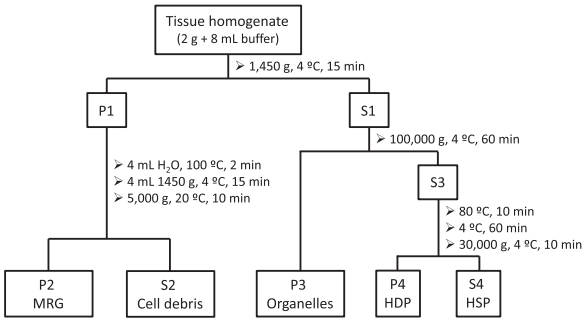

Fig. 1. Diagram showing the operational steps for the subcellular fractionation procedure used to separate the bivalve tissues into five operationally defined fractions. MRG = Metal Rich Granules; HDP = Heat-Denatured Proteins; HSP = Heat-Stable proteins.

then centrifuged at 5000 *g* (10 min; 20 °C) to obtain the P2, corresponding to the metal rich granules (MRG), and S2 (cell debris) fractions. The first supernatant (S1) containing the cytosol was transferred to ultracentrifuge polycarbonate tubes and centrifuged at 100,000 *g* during 60 min at 4 °C, to separate the organelles (P3) from the cytosol (S3). The supernatant (S3) was heated at 80 °C for 10 min and then centrifuged after cooling for 1 h in ice (30,000g; 10 min; 4 °C) to separate the heat-denatured proteins -HDP- (P4) from the heat-stable proteins -HSP- (S4). Three blanks including TRIS buffer but without tissue were included within the samples and treated in the same way as the tissue samples so that three blank vials were obtained per fraction.

2.4. Analysis of Pb in tissues and cell fractions

All tissues, cell fractions and blank samples were dried in an oven at 70 °C until constant weight and digested with nitric acid (for trace analysis, Scharlau) and hydrogen peroxide (traceselect, Fluka) following a microwave assisted procedure (Sánchez-Marín and Beiras, 2008). Reference material ERM-CE278k (mussel tissue) was included in triplicate vials. After appropriate dilution with ultrapure water, Pb was 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 94%.

2.5. Analysis of Pb in exposure water

An additional test was done to check if Pb could desorb from the diets and be available for bioaccumulation in the dissolved form. Three hundred milligrams of diets 1, 2, or 3 were introduced in 1 L of filtered seawater and stirred during 3 min. This time period was higher than the mean renewal time of water in the exposure beakers (1.45 min). After this time, agitation was stopped so that the pieces of diet were let to settle, and 50 mL of seawater were taken for analysis of dissolved Pb. The samples were acidified to pH 2 with HNO_3 (for trace analysis, Scharlau) and Pb concentration was measured by anodic stripping voltammetry using a standard additions procedure in a 797 VA Computrace (Metrohm), with a hanging mercury drop as working electrode, and a Ag/AgCl and Pt-rod as reference and auxiliary electrodes respectively.

All labware used in the experiments, sampling and analysis was previously soaked in 5% HNO_3 for 24 h followed by abundant rinsing with ultrapure water.

3. Results and discussion

3.1. Pb concentration and subcellular distribution of Pb in diets and wild mussels

Table 1 shows Pb concentrations in the three diets offered to the prawns. Control diet (diet 1) had a low Pb concentration (0.16 μg g^{-1} ww) while diets 2 and 3, comprising Pb-enriched mussel tissue and *D. exoleta* tissues with a 1:2 kidney: rest proportion respectively, had very similar Pb concentrations, of 17 and 15 μg g^{-1} ww.

The wild mussels collected in Vigo harbour, included for comparison with mussels exposed to Pb in laboratory, presented a Pb concentration of 2.8 μg g^{-1} dw (0.57 μg g^{-1} ww).

Subcellular distribution analysis was done to check for differences among the compartmentalization of Pb in the clam and mussel tissues.

Table 1
Pb concentration in the diets offered to the prawns. Mean ± SD (n).

Diet	Pb (μg g^{-1} ww)	Pb (μg g^{-1} dw)
Diet 1 – Control mussel	0.16 ± 0.08 (12)	0.69 ± 0.35 (12)
Diet 2 – Pb-enriched mussel	17 ± 7 (12)	75 ± 27 (12)
Diet 3 – D. exoleta (33% kidney)	15 ± 2 (15)	44 ± 6 (12)

The subcellular distribution of Pb in diets 2 (mussels contaminated with Pb in the laboratory) and 3 (*D. exoleta* clams) and in wild mussels from Vigo harbour is presented in Fig. 2. The sum of the Pb content of the five subcellular fractions ranged from 94–142% of the total Pb content estimated from the analysis of the homogenate. The percentage of Pb in each fraction was expressed in relation to the total Pb calculated as the sum of the five fractions.

In diet 3 (*D. exoleta*), 90% of Pb was in the form of MRG, the rest being located in cell debris (9%) and organelles (1%). In the case of diet 2 (Pb exposed mussels), the amount of Pb in the form of MRG was only 25% of the total. The majority of Pb (58%) was in the cell debris fraction, while 11% was in organelles, and the remaining 6% was associated with proteins in the cytosol (2.6% as HSP and 3.3% as HDP).

Despite the differences in total Pb concentrations achieved in the soft tissues (2.8 vs 75 μg g^{-1} dw), the subcellular distribution of Pb in wild mussels from a polluted area was remarkably similar to that of mussels original from a clean area and exposed to waterborne Pb in the laboratory during 48 h. In both cases most of the Pb was in the cell debris fraction (60%), 25% was in the form of MRG, 9% was in organelles and the remaining 6% associated to proteins in the cytosol. One remarkable difference between Pb distribution in mussels from a polluted area and mussels contaminated in the laboratory was the proportion of Pb bound to HSP, that was up to 4.5% of the total Pb, and represented a 75% of cytosolic Pb in the mussels from Vigo harbour. In laboratory exposed mussels, only 44% of cytosolic Pb was bound to HSP. This may be due to the acclimation of wild mussels to an environment with high concentrations of metals, resulting in the activation of detoxification mechanisms, such as metalothioneins, (Mourgaud et al., 2002) which are extracted in the HSP fraction (Wallace et al., 2003). It could be that wild mussels from a polluted area presented more metalothioneins than mussels exposed in the laboratory, due a longer acclimation time, or that the higher Pb concentration achieved in laboratory-exposed mussels overwhelmed the capacity of metalothioneins to sequester Pb, and a higher proportion was "spilled" onto the HDP fraction.

3.2. Trophic transfer experiments

3.2.1. Control of dissolved Pb concentrations in the exposure water

The running seawater used for the experiments, as well as the water that was in contact with diet 1 and 3 showed a Pb concentration under the limit of detection of the method (< 0.05 μg L^{-1}). Water in contact with diet 2 for 3 min presented a measurable Pb concentration (1.3 μg L^{-1}), showing that some desorption of Pb from the mussel tissue fragments occurred. However, it is highly improbable that the main source of Pb accumulation in shrimps fed with contaminated mussels may be dissolved Pb uptake. Firstly, the animals were exposed in an open circuit, with a water renewal time of 1.5 min, and secondly, food was dosed only twice per week, and its consumption was usually very fast (< 1 h). Therefore, even though some desorption of Pb from the mussel tissues could occur, this is not expected to have a relevant effect on dissolved Pb concentrations in the exposure water.

In a similar experiment by Boisson et al. (2003), where shrimps were fed on ^{210}Pb-labelled mussels, several control shrimps were kept in the same aquaria separated by a net that did not allow the transfer of food, in order to test for possible contamination of seawater with ^{210}Pb. They did not detect any significant contamination of control shrimps from seawater and their food washing protocol was not more strict than ours.

3.2.2. Pb transfer to consumers

Pb concentrations in the prawn tissues (digestive gland and rest of tissues) are presented in Table 2. One individual corresponding to diet 1 treatment died during the experiment. Otherwise, the number of replicates in the analytical results was occasionally lower than 16 due

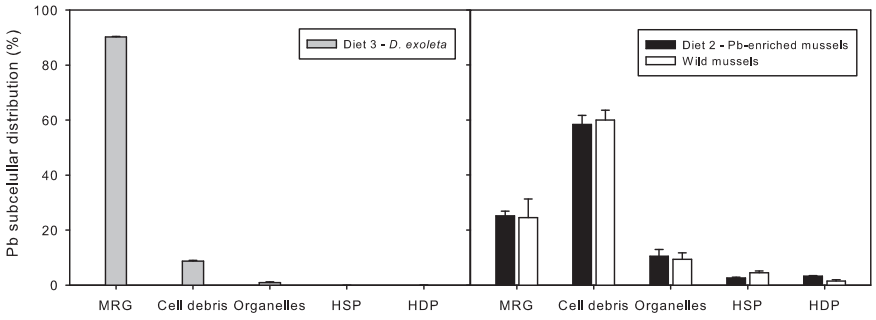

Fig. 2. Pb subcellular distribution in diet 3 (*Dosinia exoleta* tissues with a 1:2 kidney:rest proportion), diet 2 (*Mytilus galloprovincialis* exposed to Pb in laboratory) and wild mussels (*M. galloprovincialis* from a polluted area). Pb concentrations in whole tissues were 15, 17 and 0.6 μg g^{-1} ww for diet 3, diet 2, and wild mussels respectively. Mean ± SD (n = 3) is represented. MRG = Metal Rich Granules; HSP = Heat stable proteins; HDP = Heat-denatured proteins.

Table 2
Pb concentration in tissues of *Palaemon serratus* prawns from the trophic transfer experiment. Mean ± SD (n) in μg g^{-1} dw.

Treatment	Digestive gland	Rest of tissues	Whole animal
Initial	0.033 ± 0.019 (16)	0.024 ± 0.008 (16)	0.024 ± 0.007 (16)
Diet 2 – diet in stomach	n.m.[a]	n.m.	6.84 ± 0.67 (4)
Diet 3 – diet in stomach	n.m.	n.m.	4.94 ± 1.67 (5)
Diet 1 – 28 days[b]	0.33 ± 0.19 (15)	0.074 ± 0.047 (13)	0.078 ± 0.049 (13)
Diet 2 – 28 days[b]	7.4 ± 4.4 (15)	0.73 ± 0.47 (16)	0.86 ± 0.55 (15)
Diet 3 – 28 days[b]	0.43 ± 0.21 (16)	0.053 ± 0.036 (16)	0.058 ± 0.036 (16)

[a] n.m.: not measured.
[b] Individuals sampled after 28 days of experiments were let to empty their digestive system before sampling.

to accidental spillage of vials during the sample digestion process. Prawns presented a very low initial Pb concentration (0.024 μg g^{-1} dw), and after being kept in laboratory for 28 days and fed with clean mussel tissues (diet 1) the Pb concentration in their tissues was tripled (0.078 μg g^{-1} dw). This could be due to the Pb content in the diet, higher than the one present in the food consumed by shrimps in the wild, and to the bioaccumulation of dissolved Pb coming from the PVC pipes of the running seawater facilities. In any case, this Pb bioaccumulation observed in control prawns was very low compared to that achieved in exposed ones (see below). Prawns fed with diet 2 (Pb-enriched mussel) showed a relevant increase in the concentration of Pb in their tissues (0.86 μg g^{-1} dw) that was 36 times higher than the initial concentration (0.024 μg g^{-1} dw). On the contrary, prawns fed with diet 3 showed a Pb concentration at the end of the experiment (0.058 μg g^{-1} dw) that was only 2.4 times higher than the initial one, and was similar to that of prawns fed with diet 1, a diet with a remarkably lower –two orders of magnitude– Pb concentration (Table 1).

To check that prawns had effectively ingested the MRG in diet 3, five individuals were sampled with the food in their stomach, and this was done with diet 2 and 3 for comparison. Prawns that had consumed diet 2 or 3 presented very high Pb concentrations, with a mean of 6.84 μg g^{-1} dw for diet 2 and 4.94 μg g^{-1} dw for diet 3 (Table 2). This represented 88–104% of the predicted Pb concentration that the animals were expected to show according to the amount of diet 2 consumed, and 59–89% of the estimated amount for diet 3. This demonstrates that the loss of Pb from diet 2 to the dissolved phase was very low (as discussed before), and that the vast majority of Pb offered to the shrimps was effectively ingested, despite minor losses from diet 3 particles in the vessel.

Regarding tissue distribution, Pb concentrations in the prawn digestive glands were between 4 and 10 times higher than in the rest of the tissues, and they followed the same patterns as the rest of the tissues concerning bioaccumulation from diet. Pb bioaccumulation from food seems to occur preferentially in this organ, at least in the short term. Wild prawns sampled at the beginning of the experiment, on the contrary, did not show higher Pb concentrations in the digestive gland than in the rest of tissues (Table 2).

The trophic transfer factor (TTF) obtained for Pb in the present study is 0.011, calculated in a dry-weight basis for shrimps fed on diet 2 (Pb-contaminated mussels) (Table 3). For shrimps fed with diet 3, the TTF goes down to 0.001, given that very little accumulation of Pb from this diet was observed. TTFs calculated for Pb in marine organisms are generally lower than 1 (Boisson et al., 2003; Rainbow et al., 2006b; Soto-Jiménez et al., 2011), similar to other metals that do not show biomagnification, such as Cu, Cd or Zn (Amiard et al., 1980; Rainbow et al., 2006b). Reported TTFs usually depend not only on the species involved but also on the metal concentrations in the prey, given that inverse trends between TTF and dietary metal are usually observed (DeForest et al., 2007), probably because the higher the concentration of metal in the prey, the higher the fraction present as insoluble detoxified forms.

Boisson and coworkers (2003) obtained TTFs of 0.04 and 0.08 for the Pb transfer to *Palaemonetes varians* from polychaetes and mussels respectively, although this transferred Pb included some digestive system contents, and decreased to 50% after 6 days of digestive system depuration. Therefore, the Pb TTFs for *P. varians* and *P. serratus* (from mussels) seem to be very similar.

Table 3
Trophic transfer factors/coefficients calculated for the trophic transfer of Pb to *Palaemon serratus* considering different diet fractions as source for trophically available Pb (Pb_{TA}).[a]

Trophic transfer coefficient	Pb assumed as trophically available	Diet 1	Diet 2	Diet 3	SS[d]
$TTF = (Pb_{prawn} - Pb_O)/Pb_{diet}$[b]	All fractions	0.078	0.011	0.0008	10,213
$TTC = (Pb_{prawn} - Pb_O)/Pb_{TA}$[c]	All fractions	0.34	0.049	0.002	21,392
	All fractions – MRG	0.446	0.066	0.024	355
	Organelles + cytosol	2.2	0.30	0.22	118
	Cytosolic Pb	5.6	0.84	3.53	43
$TTC = (Pb_{prawn} - Pb_{O+diss})/Pb_{TA}$	Cytosolic Pb	3.2	0.81	1.07	12

[a] Pb_O = 0.024 μg g^{-1} dw; Pb_{O+diss} = 0.048 μg g^{-1} dw.
[b] TTF are calculated on a dry weight basis for both Pb concentrations in the prawn and in the diet.
[c] For calculation of TTCs, Pb_{TA} was reported on a wet weight basis.
[d] SS (sum of squares) were calculated as $\Sigma(TTC_i/TTC_j - 1)^2$, with $TTC_{i,j}$ representing all three combinations of pairs of TTCs for diet 1, 2 and 3 with the highest value in the numerator.

The low TTF observed for Pb in decapods might be due to (1) the low assimilation efficiency of the metal, in part due to the chemical form of the metal in the diet, as will be discussed in the following section and/or (2) the elimination of assimilated Pb by the consumers, by either excretion of metal ions out of the body or by accumulation in the exoskeleton and moulting (Keteles and Fleeger, 2001; Bergey and Weis, 2007). In fact, 24 out of 48 prawns were observed to moult during the experiment. Moults were not sampled for analysis since in many cases they were ingested by the prawns and only spare pieces of moults were found in the beakers. It should be noted that Boisson et al. (2003) showed that the majority of the Pb accumulated from diet by the shrimp *P. varians* was found in the exoskeleton and muscle, and not in the digestive gland, contrary to our observations with *P. serratus*. However, Boisson and coworkers did not observe any moulting during the experiment, while in our case a significant number or prawns moulted, probably eliminating some Pb in this process.

3.3. Effect of Pb subcellular partitioning in prey on Pb assimilation by consumers

It is clear from the present results that although Pb trophic transfer from bivalves to prawns is generally low (< 1%), there are significant differences depending on the diet, and these differences seem to be due to the chemical form of the metal in the diet, specially to the proportion present as metal rich granules, that are assumed to be trophically unavailable.

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 (AEs) with the sum of fractions that appeared to be trophically available. They concluded that the TAM was that associated with the organelles and cytosol, while the metal in the cell debris and MRG appeared trophically unavailable.

Contrary to TTFs that usually assume that equilibrium is achieved in the predator tissues, assimilation efficiencies (AEs) should be calculated in the short term, before elimination of assimilated metal by the organism is significant enough to affect metal body burdens. Also, accurate AEs are usually obtained using radio-labelled elements, so that initial (before consumption) and final (after consumption and production of faecal material) element concentrations can be directly measured for each individual (pulse-chase feeding technique), instead of being estimated on the basis of average values. Our experimental approach does not allow the calculation of AEs, but calculation of trophic transfer coefficients (TTCs) can be similarly used to estimate which are the trophically available fractions. Assuming that Pb elimination by the shrimps is proportional to Pb internal concentrations, and given that ingestion rates were similar for all the individuals in our experiment, then Pb accumulated by the shrimps during the 28 days period will be expected to be directly proportional to AEs. Pb accumulated by the prawns at the steady state can be predicted in the basis of the following equation (Luoma and Rainbow, 2005) (note that the dilution effect caused by growth of the individuals and included in the original equation is not considered here):

$$Pb_{prawn}=Pb_0+\frac{k_u \times Pb_{diss}}{k_e}+\frac{AE \times IR \times Pb_{food}}{k_e} \quad (1)$$

Where Pb_O is the initial Pb concentration in the prawns, and the second and third terms represent Pb accumulation from water and food respectively, with k_u and k_e representing the rates of uptake from water and elimination from the organism, AE is the assimilation efficiency, IR is the ingestion rate, and Pb_{diss} and Pb_{food} are the Pb concentrations in the water and food respectively.

Eq. (1) can be simplified by substituting the coefficients accompanying Pb_{food}, $AE \times IR/k_e$, by the trophic transfer coefficient, and combining the first two terms into one single term:

$$Pb_{prawn}=Pb_{0+diss}+TTC \times Pb_{food} \quad (2)$$

TTCs calculated assuming that Pb accumulation from water is negligible (i.e. $Pb_{O+diss}=Pb_0$) are presented in Table 3, and Pb_{food} was substituted by different Pb subcellular fractions that may be considered as trophically available (Pb_{TA}), in order to see which one of the combinations gave better predictions of TTC (equal for the three diets). Sum of squares (SS) in Table 3 represent the SS of the deviations from 1 of the three ratios obtained among the calculated TTCs; a lower SS indicates a better agreement among TTCs. For diet 1, since subcellular distribution of Pb was not directly assessed, Pb fractionation was calculated assuming that the distribution was similar to that of wild mussels from the polluted site (that showed a 5 times higher Pb concentration). The apparent TTCs present significant differences among them if Pb from all fractions in the food is considered, being two orders of magnitude higher for diet 1 than for diet 3. The differences among TTCs are much smaller if Pb in the form of MRG is excluded from Pb_{TA}, and the best agreement is observed when only cytosolic Pb is considered, although apparent TTCs for diet 1 and 3 are still higher than that of diet 2. It could be that part of the Pb accumulated by the shrimps comes from the water, and therefore TTCs in diet 1 and 3 are overestimated. To account for this, a constant amount of Pb accumulated from water was included, as described in Eq. (2), and the best value for Pb_{O+diss} was calculated from the fitting giving the best agreement among the TTCs from the three diets, using least square analysis. Using a value for Pb_{O+diss} equal to 0.048 μg g^{-1} dw, model predictions of TTCs were improved when only cytosolic Pb was considered bioavailable (Table 3).

Despite the limited statistical power of this approach, this exercise was useful to see that the fractions that are most likely to contribute to the trophic transfer of Pb from bivalves to prawns are cytosolic Pb and, to a minor extent, organelles. This is in agreement with the TAM fraction proposed by Wallace and Luoma (2003) for Cd. A previous study by Wallace and Lopez (1997), where subcellular fractions were isolated and offered as food to *Palaemonetes pugio*, showed that cytosolic Cd was absorbed with an efficiency > 85%, while Cd associated to organelles showed an AE of 70%, and Cd in the cell debris fraction seemed partially available too. In fact, it might be also possible that the cell debris fraction was partially available for trophic transfer in our experiment, but the AE of this fraction might be different for each diet. Disruption of tissues and cells using mechanical methods such as Potter-Elvehjem homogenizers is not completely efficient, and the cell debris fraction may contain not only the nucleus and membranes, but also some undisrupted cells (Lavoie et al., 2009; Rosabal et al., 2014).

The undeniable evidence derived from this study is that Pb in the form of MRG present in *D. exoleta* is not available for trophic transfer to the prawn *P. serratus* (see Fig. 3). It seems that Pb in the form of MRG in *M. edulis* is not available either, although there might be differences in

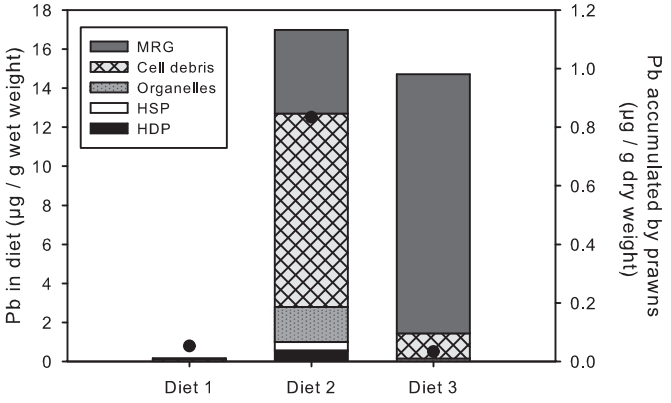

Fig. 3. Comparison of the contribution of the different subcellular fractions to Pb concentration in the diets (stacked bars) with Pb accumulated by the prawns during the 28 days experiment (black circles).

the degree of availability of the granules depending on their chemical form. *D. exoleta* presents large extracellular granules composed mainly of calcium phosphate (Darriba and Sánchez-Marín, 2013), and it is expected that Pb might be substituting Ca in the form of $Pb_3(PO_4)_2$ in these granules. In *Mytilus edulis*, small, Fe-containing, lipofuscin granules have been described in the kidney (George et al., 1982). These granules contain metals such as Cd, Pb and Zn, that seem to be bound to low molecular weight organic ligands (George, 1983). In *M. edulis* gills, Pb has been also found in extracellular carbonate deposits (Marshall and Talbot, 1979). Chemical characterization of Pb speciation in the granules would be useful to further clarify the different degrees of lability of Pb in this fraction across different prey species.

Several studies have shown different degrees of availability of MRG depending on the chemical speciation of the metals in the granules and the digestive ability of the predator. Rainbow et al. (2004) showed that Cu in the form of MRG passed through the gut of a polychaete predator undigested. Similarly, Nott and Nicolaidou showed that metal phosphate granules present in different tissues of marine invertebrate preys were found virtually intact in the faecal pellets produced by gastropods or crabs after its consumption (Nott and Nicolaidou, 1990, 1994), although some elements such as K and Mg were solubilised from the granules during the digestion process. In contrast, another type of granules composed of calcium/magnesium carbonate were completely digested by the gastropods (Nott and Nicolaidou, 1990). Also, Cheung and Wang (2005) showed that Ag, Cd and Zn associated to MRG isolated from marine invertebrates were assimilated by the neogastropod *Thais clavigera*. This was attributed to the particularly aggressive digestive ability of this species (low pH, long gut passage times), although the chemical form of the granules was unknown and it may still be possible that the most resistant metal-phosphate granules were also unavailable for the neogastropod.

3.4. Environmental relevance and implications

The present results clearly show that the Pb present in *D. exoleta* is poorly available for trophic transfer to a consumer, the prawn *P. serratus*. The present example represents an extreme case of good efficiency in Pb sequestration, that renders the high concentrations of Pb achieved in *D. exoleta* (up to 25 $\mu g\ g^{-1}$ dw; Sánchez-Marín and Beiras, 2008) almost completely unavailable for trophic transfer to consumers. Other examples such as the metal-resistant annelid populations described in the several studies by Rainbow and coworkers and Wallace and coworkers presented significant fractions of different metals in the form of MRG: 30% of Cd for the olygochaete *Limnodrilus hoffmeisteri* inhabiting Cd-polluted Foundry Cove (Wallace et al., 1998); 68% of Cu and 45% of Ag in the polychaete *Nereis diversicolor* inhabiting metal-contaminated Restronguet Creek (Rainbow et al., 2004, 2006a). However, in all these studies, the metals were also significantly present in other –more soluble– fractions, and they were therefore transferable to consumers and could represent a risk in the food chain (Rainbow et al., 2004). The extremely low transfer of Pb observed for *D. exoleta* should be seen as an exception, since Pb accumulated in other invertebrate species, such as mussels and polychaetes (Boisson et al., 2003), might represent a risk for trophic transfer that deserves further attention.

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Note

Lead concentrations and size dependence of lead accumulation in the clam *Dosinia exoleta* from shellfish extraction areas in the Galician Rías (NW Spain)

Paula Sánchez-Marín[a] and Ricardo Beiras

Laboratorio de Ecoloxía Mariña (LEM), Facultade de Ciencias do Mar, Universidade de Vigo, Campus Universitario, 36310 Vigo, Galicia, Spain

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Abstract – To protect public health, the European Commission established maximum levels of certain contaminants permitted in foodstuffs. The maximum amount of lead allowed in bivalve mollusc is 1.5 μg g^{-1} wet weight. In the Galician Rías, which are important areas of shellfish production in Spain, high levels of lead have been detected in the commercial bivalve *Dosinia exoleta* (Veneridae). Given the environmental and socio-economical problems this could represent, Pb concentration was tested in *D. exoleta* from two Rías, and the relation of lead accumulation with body size studied in detail. Implications for fisheries management are also discussed in this paper. Results showed a strong dependence of Pb accumulation on body size, with lead concentrations increasing exponentially with shell length. Larger animals presented a 5-fold increase in lead concentrations above the maximum permitted level. The size limit (length beyond which *D. exoleta* should not be extracted for commercial purposes) was initially established at 40 mm; but a more comprehensive geographical study of Pb concentrations in individuals from 35 to 40 mm long revealed that this size limit was not protective enough, and 35 mm is proposed as a safer limit.

Key words: Metal accumulation / Pollution / Size effect / Lead / Clam / *Dosinia exoleta*

1 Introduction

Lead is one of the metals most frequently enriched by man because of its use in piping, building materials, storage batteries, paints and other chemicals (Sadiq 1992). Large amounts of Pb and its compounds have been emitted into the atmosphere in past decades as a result of leaded gasoline combustion. Consequently, Pb has become ubiquitous in our environment, presenting a potential hazard for both animals and humans. One of the well known toxic effects of inorganic lead is its inhibition of hemoglobin synthesis, leading to the development of anemia. Since Pb ions have an ionic structure similar to calcium ions, they may be taken into nervous system cells and mitochondria in a similar manner to Ca (Chang and Cockerham 1994), and may hence reduce cognitive development and intellectual performance in children, and cause blood pressure and cardiovascular diseases in adults. In order to protect public health, and with the objective of further lowering the mean levels of lead in foodstuffs, the European Commission established limits on the maximum levels of Pb permitted in different food sources for humans. In the case of bivalve molluscs, the maximum established level was 1.5 μg g^{-1} wet weight (Anonymous 2002).

Shellfish production in Galicia (NW Spain) represents more than 90% of total shellfish production in Spain, in which the clam *Dosinia exoleta* represents an important fraction. In 2005, *D. exoleta* production reached 1452 tons. This is equivalent to 16% of the total extracted bivalves, which include 17 different species (Anonymous 2005). According to work by Beiras et al. (2003a,b) and Prego and Cobelo-García (2003), the Galician Rías present a low degree of pollution, mainly restricted to localised areas. However, Pb is the metal with the narrowest safety margin in the Galician Rias, and concentrations exceeding legal standards have been reported in non-commercial wild mussels from the inner parts of the Rías (Beiras et al. 2003c).

The present study was incited by the fishers' associations of the Rías of Pontevedra and Arousa, important shellfish production areas in Galicia, after local authorities found high

[a] Corresponding author: paulasanchez@uvigo.es

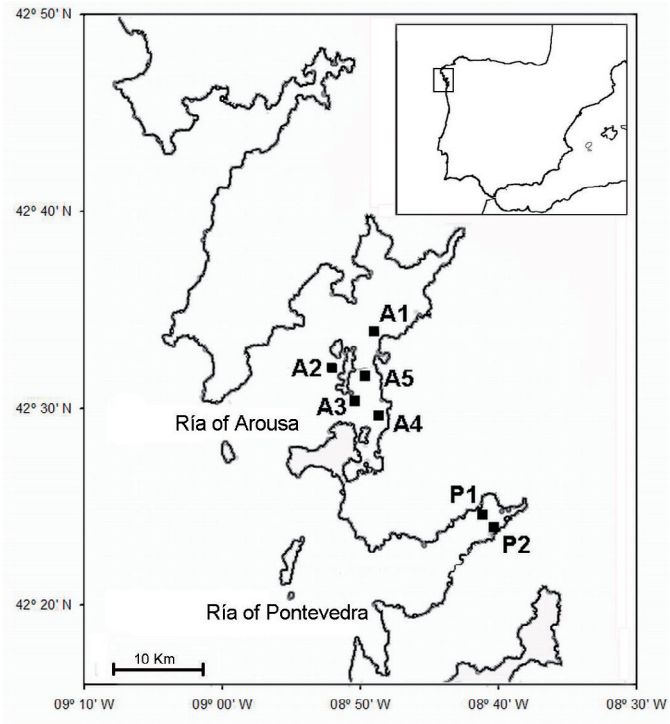

Fig. 1. Position of the Rias of Arousa and Pontevedra on the Atlantic coast of the Iberian Peninsula and localisation of sampling sites.

levels of Pb in this clam. Three different studies were conducted. The first tested whether the average Pb concentration in *D. exoleta* from representative banks in the Galician Rías conformed to European legislation. The second study investigated the quantitative relationship between body size and Pb concentration in a commercial bank, in order to identify the size class whose Pb concentration corresponded to the maximum permitted by EC standards. The third study focused on the geographical variation of the Pb concentration in that size class, in order to establish a maximum length whose Pb concentration would not exceed EC standards.

2 Material and methods

2.1 Collection and pre-treatment of the samples

Samples of *D. exoleta* were collected on three sampling dates, in May, October and November 2006 in different shellfish extraction areas of the Rías of Pontevedra and Arousa. Each sampling date corresponded to one of the three studies performed. Samples were immediately transported to the laboratory, and conserved frozen until their treatment and analysis.

The preliminary study (May 2006) covered 6 locations: A1, A2, A3 and A4 in Arousa and P1 and P2 in Pontevedra (Fig. 1). Seven individuals of a similar size (from 35 to 45 mm) were chosen from each location.

For the second study (on Pb concentration in clams of different sizes), a total of 50 individuals with the largest range of sizes possible were sampled at one location (A5, Fig. 1) in October 2006.

The clams were opened with a scalpel, and rinsed with artificial seawater (free of metals) and ultrapure water. Excess water was drained from the clams and they were allowed to air-dry for 5 min on drying paper. The tissue was separated from the valves using plastic tools, and deposited in pre-weighed polypropylene vials. Both wet and dry weight were determined, the latter after drying for 72 h in an oven at 90 °C.

In the third study (November 2006), instead of analyzing each individual separately, a homogenized pool was obtained in order to conform with the methodology described in the EC directive (Anonymous 2001). Therefore, 20 individuals between 35 and 40 mm were used for each of three homogenized pools, corresponding to locations A1, A2 and A4. Clam tissues were deposited on nylon meshes, lyophilized for 72 h and ground using a tungsten-blade grinder. Three replicates of 0.5 g were deposited in polypropylene vials for digestion and analysis.

2.2 Sample digestion and metal analysis by ICP-Mass spectrophotometry

Samples were digested with 1 ml of HNO_3 for trace analysis (Scharlau Chemie, S.A, Barcelona, Spain) and 200 μl of H_2O_2 (Tracepur, Merck, Darmstadt, Germany) per 100 mg of dried sample, using a microwave-assisted technique, following a method modified from De Wit and Blust (1998). After addition of HNO_3, samples were left to react for 48 h at room temperature. After this period, H_2O_2 was added and samples were left at room temperature for 24 h. Occasional agitation was applied using a vortex. Then samples were put in a microwave oven inside an airtight polycarbonate Bio-Safe carrier box (Nalgene, Nalge, Rochester, NY, USA). The microwave oven was operated according to the following procedure: 1 min (×2) at 90 W, 2 min (×2) at 90 W. The same was then repeated at 160 and 350 W. The final step, of 2 min at 350 W, was repeated several times until the samples were completely digested (start of ebullition, translucent colour and non-formation of foam).

After digestion, samples were diluted to a 3% acid concentration with a mixture of ultrapure water and internal standards, to obtain a final concentration of 5 μg L^{-1} of the internal standards. Pb was measured in all analyses from each sampling. In the final study, Cu, Zn and Cd were also measured. Internal standards used were: Tl (for Pb correction), Rh (for Cd) and Ge (for Cu and Zn). Samples were analyzed by inductively coupled plasma mass spectrometry using an X Series ICP-MS, (Thermo Elemental, Cheshire, UK). Procedure blanks and certified reference material ERM-CE278 (mussel tissue) were included in the sample treatment and analysis.

All the material in contact with the samples had been previously washed with 10% nitric acid and rinsed with ultrapure water.

2.3 Data analysis and statistics

Normality and homocedasticy of the data were first verified using Kolmogorov-Smirnov and Levene tests respectively. Statistical differences between lead concentrations in different

Table 1. Comparison of measured and certified values of ERM-CE278 (mussel tissue).

	Pb (μg g^{-1})	**Cu** (μg g^{-1})	**Zn** (μg g^{-1})	**Cd** (μg g^{-1})
n	11	3	3	3
Measured	1.88 ± 0.07	9.35 ± 0.09	82.73 ± 0.01	0.35 ± 0.001
Certified	2.00 ± 0.04	9.45 ± 0.13	83.1 ± 1.7	0.35 ± 0.001
%Recovery	94%	99%	100%	100%

Values expressed as arithmetic mean ± standard deviation; "*n*": number of samples measured.

locations were analysed by one-way ANOVA, applying post hoc HSD Tukey tests with the software SPSS version 15.0.1 for Windows (2006, SPSS Inc). To compare two means, or a mean to a known value, a *t*-test was used. The null hypothesis was accepted at a level of significance of 0.05. Curve fitting was done by least squares regression analysis using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA).

3 Results

Measured values of Pb, Cu, Zn and Cd in the ERM-CE278 reference (mussel tissue) are shown in Table 1. Recovery was 94% for Pb, 99% for Cu and 100% for Zn and Cd. Metal concentration in the blanks was below 1% of metal concentration in the samples. Certified reference material was measured at the beginning and at the end of each run to detect the effectiveness of internal standards for drift correction. The correction was satisfactory in all cases.

3.1 Preliminary study

Mean Pb concentrations in *D. exoleta* from the six sampled locations are shown in Table 2. Pb concentrations in clams showed no significant differences between sampling points within the same Ría. However, clams from the Ría of Pontevedra showed on average a higher ($p < 0.001$, *t*-test) Pb concentration (1.29 ± 0.63 μg g^{-1} ww, $n = 14$) than clams from Arousa (0.63 ± 0.39 μg g^{-1} ww, $n = 28$). Even though the mean concentration did not exceed the maximum permitted level in any of the studied Rías, results showed high inter-individual variability. In the Pontevedra Ría, 3 of the 14 clams analyzed presented Pb concentrations higher than 1.5 μg g^{-1} ww; and in the Arousa Ría, 2 of the 28 analyzed individuals presented concentrations above 1.5 μg g^{-1} ww.

3.2 Study of Pb concentration in clams of different size

The exponential increase in Pb concentration with size in *D. exoleta* individuals from a commercial bank in the Ría of Arousa is shown in Figure 2. For small individuals, from 20 to 40 mm long, Pb concentration increased slowly with length: the mean Pb concentration in small (<40 mm) clams was 0.7 ± 0.9 μg g^{-1} ww ($n = 24$). For individuals larger than 40 mm though, Pb concentration increased very sharply with size. Values in large clams reached 5 μg g^{-1} ww, more than five times higher than younger clams living in the same site.

The fitted curve followed the equation: $[Pb] = a \exp(b \cdot D)$; where D is shell length. Adjusted parameters were: $a = 0.015 \pm 0.010$ μg g^{-1} ww and $b = 0.116 \pm 0.010$ ($R^2 = 0.67$). The size that would have a Pb concentration of 1.5 μg g^{-1} ww was calculated to be 39.9 mm, thus agreeing with the initial observation of 40 mm as the limit size. The mean Pb concentration in individuals just below this limit (35–40 mm) was 1.1 ± 0.3 ($n = 10$), which is significantly lower

Table 2. Lead concentrations in *Dosinia exoleta* in different locations in the Rías of Arousa (A) and Pontevedra (P).

Location	**Pb** (μg g^{-1} **ww**)
A1	0.82 ± 0.15
A2	0.30 ± 0.07
A3	0.60 ± 0.45
A4	0.80 ± 0.51
P1	1.33 ± 0.87
P2	1.24 ± 0.33

Values given as mean ± sd of 7 individuals between 35 and 45 mm length.

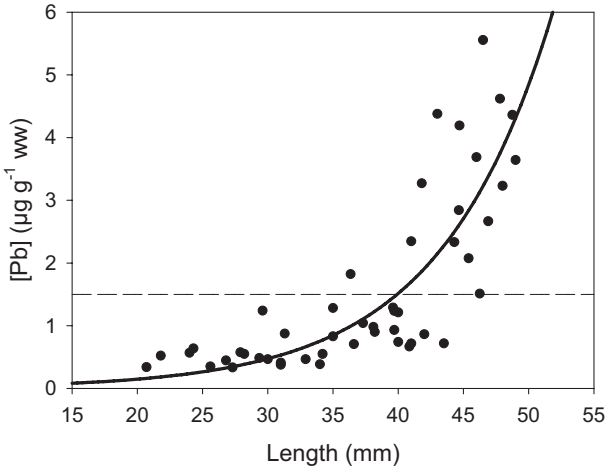

Fig. 2. Pb concentrations in *Dosinia exoleta* (μg g^{-1} ww) in relation to individual length (mm). Each point represents a single individual. The solid line represents the fitted curve following the equation: [Pb] = 0.015·exp(0.116·D), D: shell length, $R^2 = 0.67$. The horizontal dashed line marks the 1.5 μg g^{-1} ww limit for Pb in shellfish for human consumption, established by the European Commission.

Table 3. Metal concentrations (μg g^{-1} dw) in *Dosinia exoleta* (35–40 mm long) from different locations in the Ría de Arousa.

Location	Cu	Zn	Cd	Pb	
				dw	*ww*
A1	8.67 ± 0.54	203 ± 14	0.29 ± 0.03	4.65 ± 0.34	0.93 ± 0.07
A2	9.13 ± 0.72	176 ± 14	0.25 ± 0.01	2.45 ± 0.21	0.49 ± 0.04
A4	16.10 ± 1.58	301 ± 9	0.75 ± 0.06	7.89 ± 0.28	1.58 ± 0.06

Values given as mean ± sd (n = 3) of a homogenized pool of 20 *D. exoleta* individuals. *Pb* values are expressed in μg g^{-1}dw (dry weight) and also in μg g^{-1} ww (wet weight) to allow comparison with the EC norms.

than the 1.5 limit (p = 0.01, t–test). However, the security margin is very narrow, and variations in the Pb pollution level between different sites could influence clam flesh Pb concentration. For this reason, a spatial study of clams between 35 and 40 mm long was performed.

3.3 Geographical verification of Pb concentration within the selected size class

Results of the analysis of Pb, Cu, Zn and Cd in homogenized pools of *D. exoleta* from 35 to 40 mm long in three commercial banks of the Arousa Ría (A1, A2 and A4) are shown in Table 3. Results are expressed on the basis of dry weight (*dw*) since the digestion procedure is optimal using dried material. In the case of Pb results were transformed to μg g^{-1} wet weight (*ww*), to allow comparison with the EC standards, by multiplying by a factor of 0.19, (dw = 0.19 ww, R^2 = 0.98; n = 50).

In clams from the A1 and A2 locations, Pb concentrations were below the EC standard. However, in location A4, mean Pb concentration in individuals between 35 and 40 mm long was above the critical threshold, though this was not significant (t-test; p = 0.05). These results show that Pb concentration is dependent not only on size but also on location (t-test; $p < 0.001$). The location presenting the lowest Pb concentration, A2, is the most external of the ría,; while A4, the most polluted site, is closer to the river Umia. This river is known to carry a charge of urban and industrial effluents, in which local Pb inputs have been detected (Prego and Cobelo-García, 2003). The other metals measured showed the same pattern, with maximum levels in A4, and minimum in A2 (Table 3).

4 Discussion

Lead concentrations in *D. exoleta* from the two studied Galician Rías ranged between 1.5 to 8.5 μg g^{-1} dw for individuals under 40 mm in length, but reached concentrations up to 20 μg g^{-1} dw in individuals around 45 mm. Pb concentrations in the Ría of Pontevedra were significantly higher than those in the Ría of Arousa, which is consistent with previous findings (Besada et al. 2002; Beiras et al. 2003a, 2003b; Prego and Cobelo-García 2003).

Pb concentrations reported in different species of infaunal bivalve (*Macoma balthica*, *Macoma nasuta*, *Chione subrugosa, Cerastoderma edule*) around the world are normally between 1 and 4 μg g^{-1} *dw*, reaching values of 8 μg g^{-1} in some moderately polluted sites (Szefer et al. 1998; Sokolowski et al. 2002, 2007; Lu et al. 2005; Jung et al. 2006). Very high Pb concentrations, up to values of few hundreds of μg g^{-1} *dw*, have been reported for clams inhabiting extremely polluted estuaries (Bryan et al. 1985; Southgate et al. 1983). In the Galician Rías, Pb concentrations in *Venerupis pullastra* gills ranged from 0.2 to 1.9 μg g^{-1} dw (Sánchez-Marín et al., unpublished data) similar to those found for small *D. exoleta* in the present study. Similar concentrations were also reported by Saavedra et al. (2004) in *Venerupis pullastra* and *Cerastoderma edule*. The high Pb accumulation by large individuals of *D. exoleta* observed in our present study is unusual given the low degree of Pb pollution in the Ría of Arousa, and suggests that this may be a specific feature of this particular species.

Metal accumulation by bivalves and other biota is influenced by several biological and environmental factors, such as season, metal compartmentalization in the sediment, size or age of the individual and reproductive status. Several studies have related size with metal accumulation in bivalves, and three different patterns (negative relationship, positive relationship and lack of relationship) have been found depending on the species and the metal considered (reviewed by Wallace et al. 2003). The positive relationship, as found in the present work, has often been observed in bivalves inhabiting contaminated sites, and the storage of metals in metal concretions has been proposed as a possible mechanism to explain these observations (Johnels et al. 1967; Strong and Luoma 1981). The retention of metal in granular deposits has been observed to result in extremely high metal body burdens (Brown 1982). Given the long life span of *D. exoleta*, it is possible that this detoxification mechanism could be responsible for the high Pb concentration found in larger (older) clams.

From the present study, it could be inferred that clams less than 40 mm long in the Ría of Arousa are suitable for human consumption with regards to Pb levels, given that none of the studied locations presented Pb concentrations significantly higher than 1.5 μg g^{-1} ww in clams from 35 to 40 mm long. However, this limit cannot be considered as safe enough because Pb concentration in one location was just above the limit (though not significantly), and other factors (such as seasonal variations) could increase Pb concentrations in any of the studied areas. Furthermore, extension of the study to more contaminated Rías (e.g. Ría of Pontevedra) would most probably result in an increase in Pb values for the same size class. For these reasons, we propose 35 mm as a safer limit until more detailed studies can be made.

5 Conclusion

Pb accumulation in *D. exoleta* has been shown to be highly size dependant, with larger individuals reaching Pb concentrations of 25 μg g^{-1} *dw* (5 μg g^{-1}*ww*). This high Pb concentration threatens the commercialization of this species for human consumption, and makes it essential to fix a maximum size that can be extracted and commercialized without health risks for consumers.

This size limit was observed to be 40 mm for one studied location. However, when the study was extended to other more polluted sites, it was seen that the 40 mm limit would not offer sufficient protection: 35 mm is therefore advised as a safer limit.

This work highlights the necessity for detailed study on Pb concentrations in *D. exoleta* in relation with size for all areas where this species is extracted, in order to establish a specific size limit for each area or to define a safe limit size for all areas based on a significant amount of data. Other factors that can influence metal accumulation, such as the effect of season or physiological status, should also be studied.

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