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

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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

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(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.

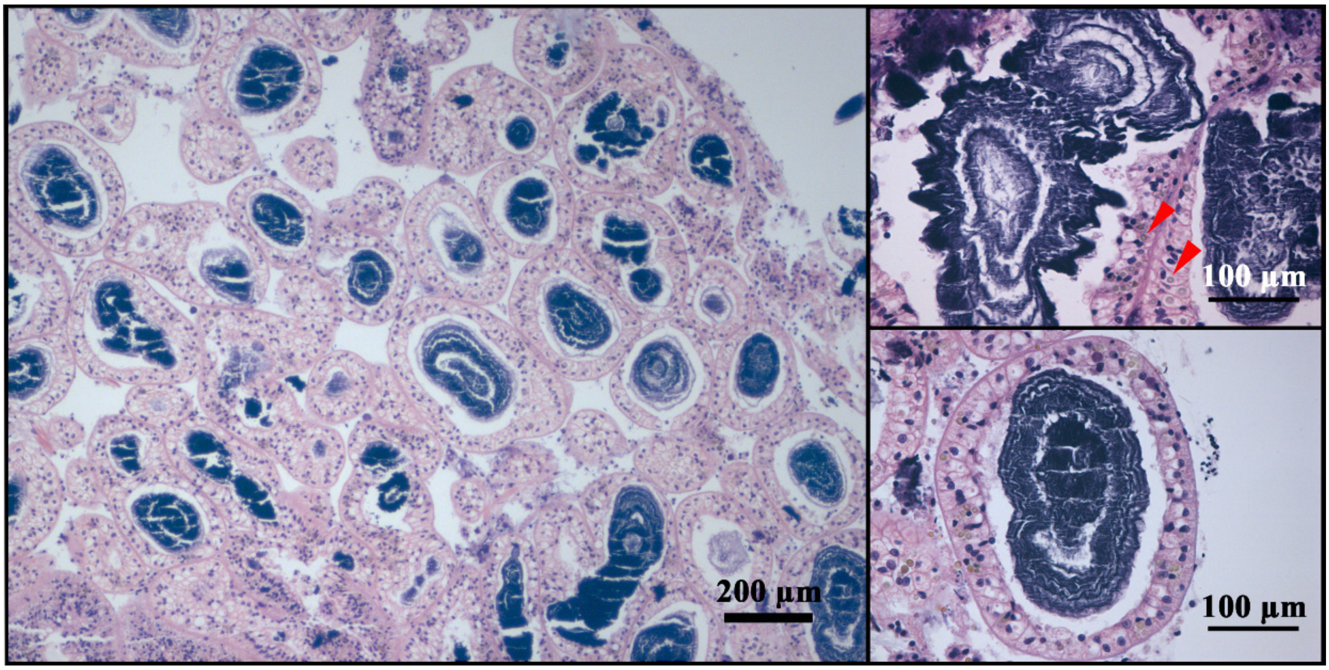

Fig. 1. Kidney lumen of *Dosinia exoleta* stained with Harris' hematoxylin and eosin observed by light microscope. Large extracellular granules (20−200 µm) are stained in blue, and small intracellular refringent granules in epithelial cells are indicated by red arrows.

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 $\mu g\ g^{-1}$ 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 $4.7 \pm 1.6\ \mu g$ Cu g^{-1} dw and $0.6 \pm 0.15\ \mu g$ 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 $\mu g\ g^{-1}$ 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.

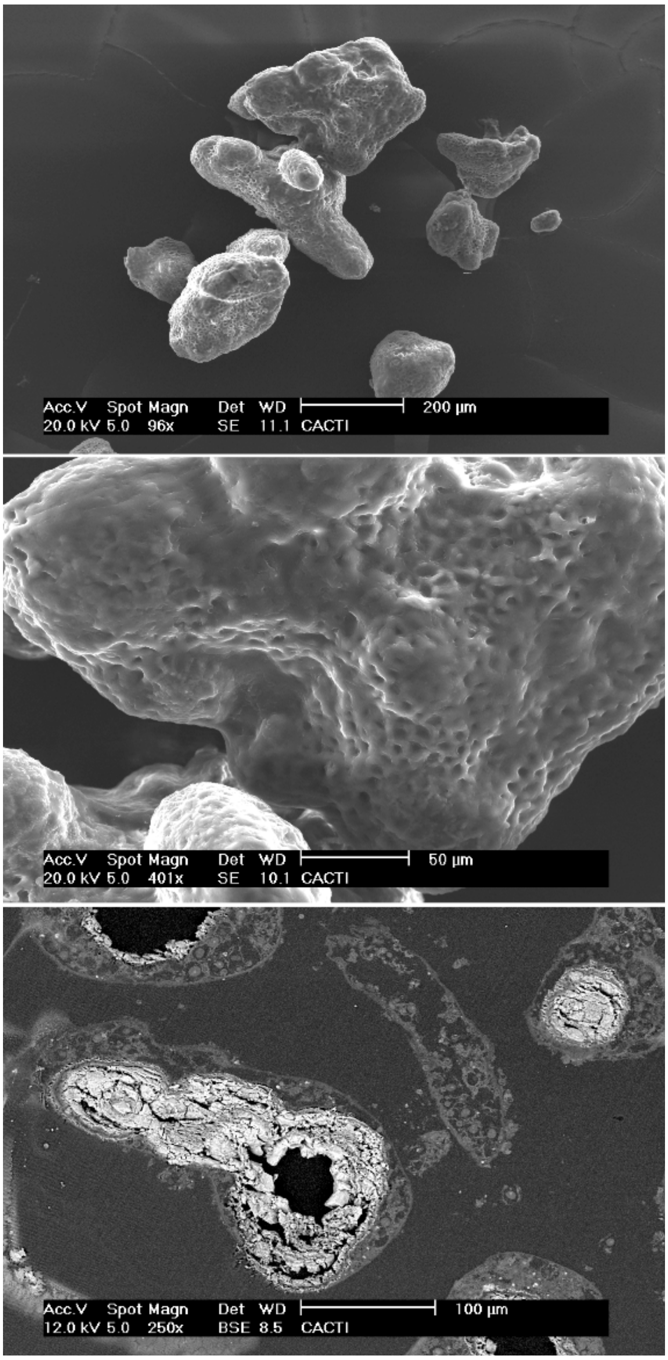

Fig. 2. Scanning electron microscopy images of isolated extracellular granules and a cross section of granules in the kidney lumen.

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

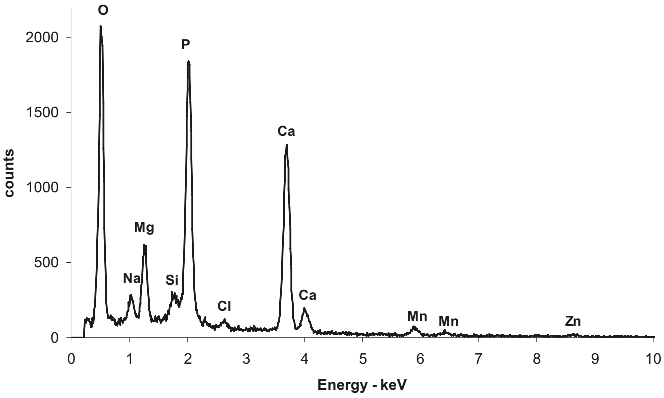

Fig. 3. Energy dispersive X-ray microanalysis of a cross section of a kidney granule showing its main elemental composition.

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.

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

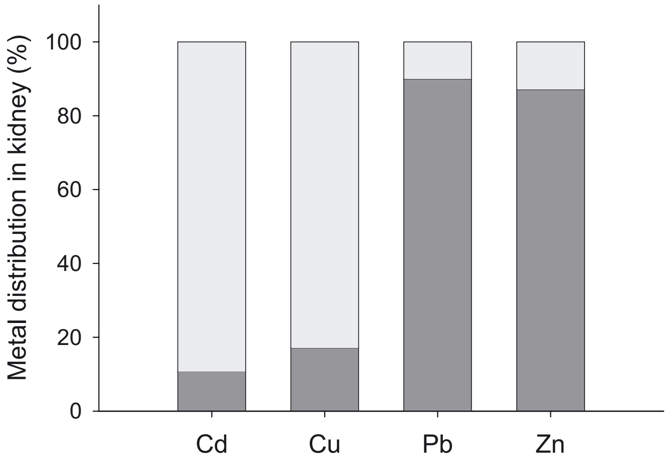

Fig. 4. Mean metal distribution in the kidney of *Dosinia exoleta* in the MRG (metal rich granules) fraction (dark grey) and the rest of the kidney (light grey). The majority (>80%) of Pb and Zn were found in the MRG fraction, while only <20% of Cu and Cd were found in this fraction.

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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