Insight of EDX Analysis and EFTEM: Are Spherocrystals Located in Strombidae Digestive Gland Implied in Detoxification of Trace Metals?

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ABSTRACT Digestive tubules of Strombidae are composed by three cell types: digestive cells, vacuolated cells, and crypt cells. The last one is characterized by the presence of intracellular granules identified as spherocrystals. Such structures are known to occur in basophilic cells of gastropod digestive gland, where they are supposed to be involved in the regulation of some minerals and in detoxification. In this study, energy-dispersive X-ray analysis (EDX) and energy filtered transmission electron microscopy (EFTEM) were used to determine the elemental content of spherocrystals in two Strombidae, *Strombus gigas* and *Strombus pugilis*. In freshly collected individuals of both species, the following elements were detected: Ca, Fe, Mg, P, and Zn. Aluminum and Mn were also detected in *S. gigas.* Their presence in spherocrystals indicates that, in Strombidae, spherocrystals are involved in the regulation of minerals and essential trace metals. In order to answer the question "are spherocrystals involved in nonessential trace metals scavenging?," artificial cadmium and lead exposure by both waterborne and dietary pathways was applied to *S. pugilis*. No evidence of cadmium ($Cd(NO_3)_2$) or lead ($Pb(NO_3)_2$) provided by food was found in spherocrystals. Cadmium provided in water ($Cd(NO_3)_2$ and $CdCl_2$) causes structural modifications of the digestive gland; however, this element was not trapped in spherocrystals. These results suggest that spherocrystals are not involved in detoxification of such nonessential trace metals. *Microsc. Res. Tech. 75:425–432, 2012.*

INTRODUCTION

Strombidae are marine benthic Gastropods, in which few species represent a Caribbean staple food and important trade commodity like *Strombus gigas* (Linnaeus, 1758) and *Strombus pugilis* (Linnaeus, 1758). Recently, according to ultrastructural analyses, apicomplexan-like parasites were detected in the digestive gland of various Strombidae (Baqueiro Cárdenas et al., 2007; Gros et al., 2009; Volland et al., 2010). Such analyses revealed that digestive tubules are represented by an epithelium composed by three cell types: digestive, vacuolated, and crypt cells. In this last cell type, spherical mineral inclusions, identified as spherocrystals, were detected in large number (Gros et al., 2009). Spherocrystals are small inclusions, mostly mineral, with a fairly low content of organic materials (Becker et al., 1974). They are amorphous to X-ray and electron diffraction (Masala et al., 2004; Mitchell et al., 1996; Taylor et al., 1989, 1990). Spherocrystals are present in various taxonomic group and they are particularly well described in arthropods (Ballan-Dufrançais, 2002). In Molluscs, spherocrystals have also been described, in particular in the basophilic cells (so-called crypt cells or calcium cells) of the common garden snail *Helix aspersa* digestive gland (Greaves et al., 1984; Howard et al., 1981) and in similar cells of the gray garden slug *Deroceras reticulatum* (Triebskorn and Koühler, 1996). *Aplysia punctata* also presents spherocrystals in its digestive gland cells (Taïeb and Vicente, 1999). Several studies have been published on spherocrystals in the digestive gland of *Littorina littorea* (Nott and Langston, 1989, 1993; Nott et al., 1993a). Gibbs et al. (1998) also reported the presence of phosphate granules in 40 species of Caenogastropods. Spherocrystals have also been described in Bivalves (George et al., 1980, 1982). Such structures are known to be involved in mineral and trace metal storage and in a not well understood detoxification process. Several studies focused on the spherocrystals capacity to incorporate metal ion in their structure (Corrêa Junior et al., 2000; Taylor et al., 1989, 1990; Walker et al., 1975). The digestive gland of Gastropods is supposed to be implied in detoxification pathways (Simkiss and Mason, 1988) but

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knowledge is scarce about the process involved. Spherocrystals are known to occur in basophilic cells of gastropod digestive gland, where they are supposed to be involved in the regulation of some minerals and in detoxification (Greaves et al., 1984; Howard et al., 1981; Lipovîsek Delakorda et al., 2009; Simkiss and Mason, 1988; Taïeb and Vicente, 1999; Wang and Rainbow, 2005). To our knowledge, there are no additional reports of such structures in Strombidae except those concerning *S. gigas* (Gros et al., 2009). In this study, energy-dispersive X-ray analysis (EDX) and energy filtered transmission electron microscopy (EFTEM) were used to determine the elemental content of spherocrystals in two strombids, *S. gigas* and *S. pugilis*. We analyzed spherocrystals from freshly collected and from artificially exposed conchs. Instead it is well known that some essential metals as Fe or Zn can be trapped and regulated by spherocrystals (Gibbs et al., 1998), the implication of spherocrystals in nonessential metals is not clear. For example, it is commonly admitted that cadmium is not sequestered in insoluble fraction of the cell (i.e., granules) but two studies, one on a marine mammal (Gallien et al., 2001) and one on a bivalve (George et al., 1980) reported its detection in spherocrystals. We used cadmium and lead, which are nonessential metals of high toxicity (Chandran et al., 2005; Dafre et al., 2004) which cause oxidative stress.

Actually, we tried to answer the question: "Are spherocrystals found in Strombidae digestive gland implied in a detoxification process of trace-metals?"

MATERIALS AND METHODS

Samples Collection and Histological Preparation

Individuals of *S. gigas* (average size of 25 cm) were collected during the authorized fishing period by professional fishermen on or near *Thalassia testudinum* sea grass beds in Le Gosier, Guadeloupe (FWI). *S. pugilis* (average size of 8 cm) samples were collected by hands on sand area in Saint François, Guadeloupe. Living materials were rapidly brought to the laboratory and digestive gland samples were dissected and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (0.1 M; 1100 mOsm; pH = 7.2) for 24 h at 4°C. Some samples were dehydrated through an ascending ethanol series and then infiltrated with Paraplast wax before embedding. Wax sections, 7 μm-thick, were obtained and stained with a modified Goldner trichrome method that included Alcian blue at pH 2.5 (Gabe, 1968). Freshly collected *S. gigas* and *S. pugilis* individuals were sacrificed and digestive gland samples were prepared for electronic microscopy as described later. Spherocrystals were localized. Some were analyzed by EDX (under SEM) and others by EFTEM.

Abbreviations

CC	crypt cells
EDX	energy-dispersive X-ray analysis
EELS	electron energy loss spectroscopy
EFTEM	energy filtered transmission electron microscopy
ESI	electron spectroscopic imaging
RER	rough endoplasmic reticulum
TEM	transmission electron microscopy
VC	vacuolated cells

Scanning Electron Microscopy

Fixed samples of *S. gigas* ($n = 3$) and *S. pugilis* ($n = 60$) were dehydrated through an ascending acetone series, critical point dried, and manually fractured before sputter coating and observation with a Hitachi® S-2500 scanning electron microscope at an acceleration voltage of 20 kV. Energy-dispersive X-ray analysis (EDX) was carried out using a pentafet detector (Oxford® Instrument), monitored by a Link Isis system, in the point mode.

Transmission Electron Microscopy (TEM)

Fixed samples of *S. gigas* ($n = 3$) and *S. pugilis* ($n = 3$), from both species, were dehydrated through an ascending ethanol series and embedded in epoxy resin accordingly to Volland et al. (2010). Semithin sections were stained with 0.5% toluidine blue in 1% borax for photonic microscopy observations. Noncontrasted ultrathin sections of *S. gigas* were analyzed by energy filtered transmission electron microscopy (EFTEM). Acquisitions in spectra mode (electron energy loss spectroscopy (EELS) were performed using a LEO 912 Omega transmission electron microscope (LEO Electron Optics GmbH, Oberkochen, Germany) at 120 kV. Observation on image mode (electron spectroscopic imaging (ESI)) was accomplished with the ESIvision program (version 3.0 Soft-Imaging Software, SIS, GmbH, 48153 Münster). For the elemental cartography, the subtractive method of the "three windows" was used.

Exposure via Food

Thirty individuals of *S. pugilis* were placed in a 400-L raceway filled with sand-filtered sea water (pH = 8; temperature = 26°C; salinity = 36‰; photoperiod = 12 h) which was continuously oxygenated using an air pump. Sea water was renewed twice a week and raceway was cleaned to avoid development of microalgae and biofilm which could be used as food source instead of artificial contaminated food. *S. pugilis* were fed with artificial food developed and provided by the CINVESTAV-IPN of Mérida (Nutrition and Aquaculture of Molluscs Laboratories). It has been controlled visually that food pellets were ingested by animals. This contaminated food appears as small pellets containing 100 mg kg^{-1} of $CdCl_2$ (= Cd at 61.3 mg kg^{-1}) and 500 mg kg^{-1} of $Pb(NO_3)_2$ (= Pb at 312.8 mg kg^{-1}) and were fed ad libitum to *S. pugilis*. Individuals were fed during 50 days. Three individuals were sacrificed after 12, 32, and 50 days of exposure. From each sacrificed individual, the digestive gland was dissected and prepared for EDX and histological analyses. Pellets contaminated by cadmium and lead were also processed for EDX analyses as controls.

Waterborne Exposure of *S. pugilis*

Before contaminations, individuals were starved for 3 days. Afterward, individuals were placed in 15-L plastic containers filled with sand-filtrated sea water which was oxygenated and renewed every 3 days. Short-term (3 days) and long-term (21 days) exposures were done using two cadmium salts ($Cd(NO_3)_2$ and

TABLE 1. Detail of durations and concentrations used for cadmium exposure of S. pugilis

	Control	0.05 mg l^{-1}	0.1 mg l^{-1}	0.5 mg l^{-1}	1 mg l^{-1}
$Cd(NO_3)_2$	3 d	NR	3 d	3 d	3 d
$CdCl_2$	3 and 21 d	3 d	3 and 21 d	3 and 21 d	3 d

D, days; NR, not realized.

$CdCl_2$) to ensure cadmium-ion availability. Pollutant concentrations and durations of exposures are resumed in Table 1.

After short-term exposure, in order to allow cadmium to concentrate in the digestive gland, animals were kept 3 days in unpolluted sea water. Then, indi-

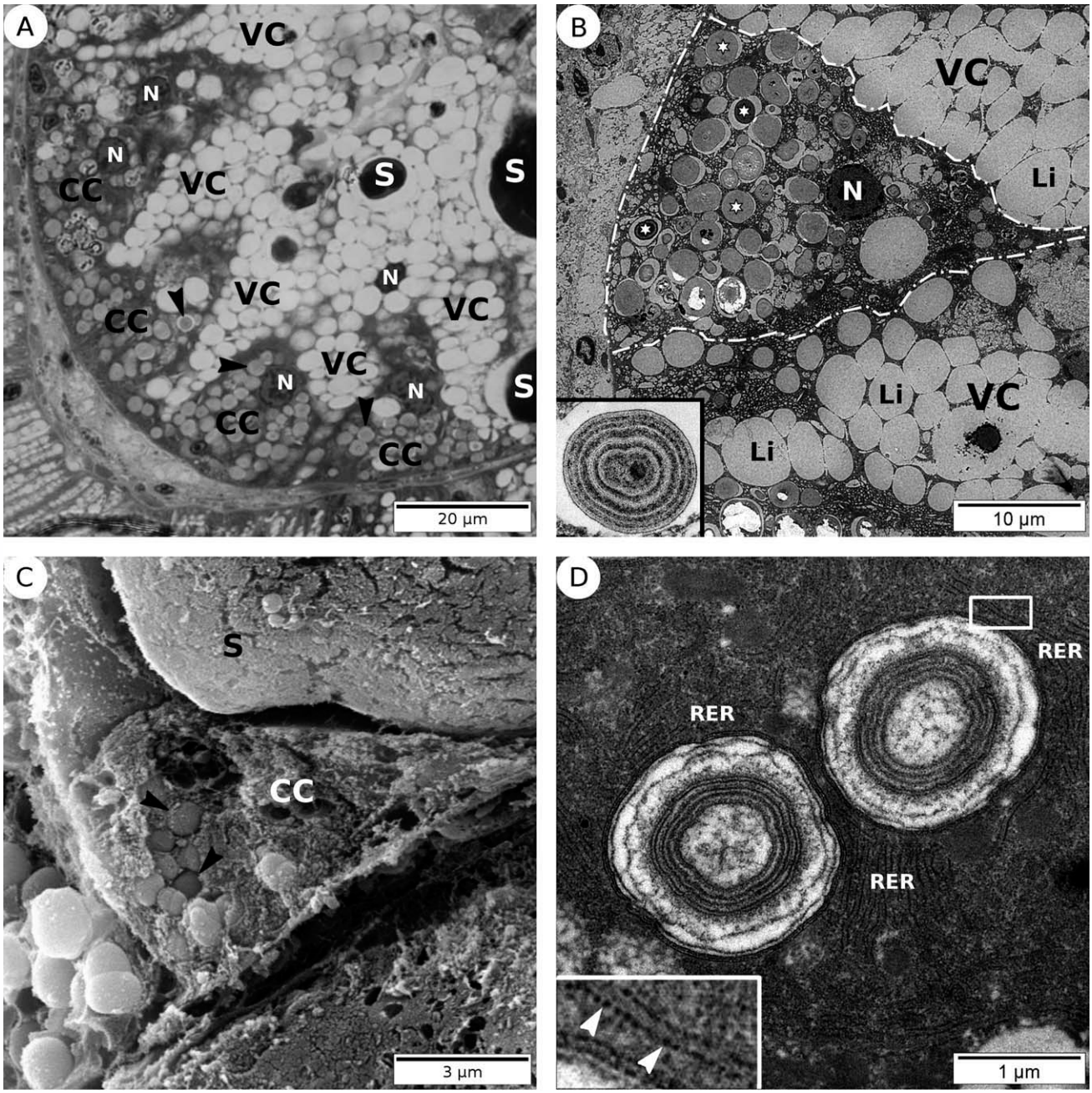

Fig. 1. Structure of crypt cells. (**A**) Semithin section of a digestive tubule of *S. gigas'* digestive gland showing crypt (CC) and vacuolated cells (VC). The crypt cells are full of spherical inclusions called spherocrystals (black arrow heads). (**B**) TEM observation of a *S. gigas* single pyramidal crypt cell (defined by the broken line) inserted in between two vacuolated cells full of lipid droplet (Li). Cytoplasm of the crypt cell is full of spherocrystals (stars). The detail of a spherocrystal is given in insert. (**C**) SEM observation of a fractured digestive gland of *S. pugilis* focusing on a single crypt cell with its numerous spherocrystals (black arrow heads). On the right of the cell, a symbiont (S) is present. (**D**) TEM detail of two spherocrystals in a crypt cell from *S. pugilis* digestive gland. The concentric layers are clearly visible, alternating electron-lucent and electron-dense. In the cytoplasm, spherocrystals are surrounded by an important rough endoplasmic reticulum (RER). Insert represents a higher magnification confirming the presence of ribosomes (white arrow heads) attached to the RER. N: nucleus.

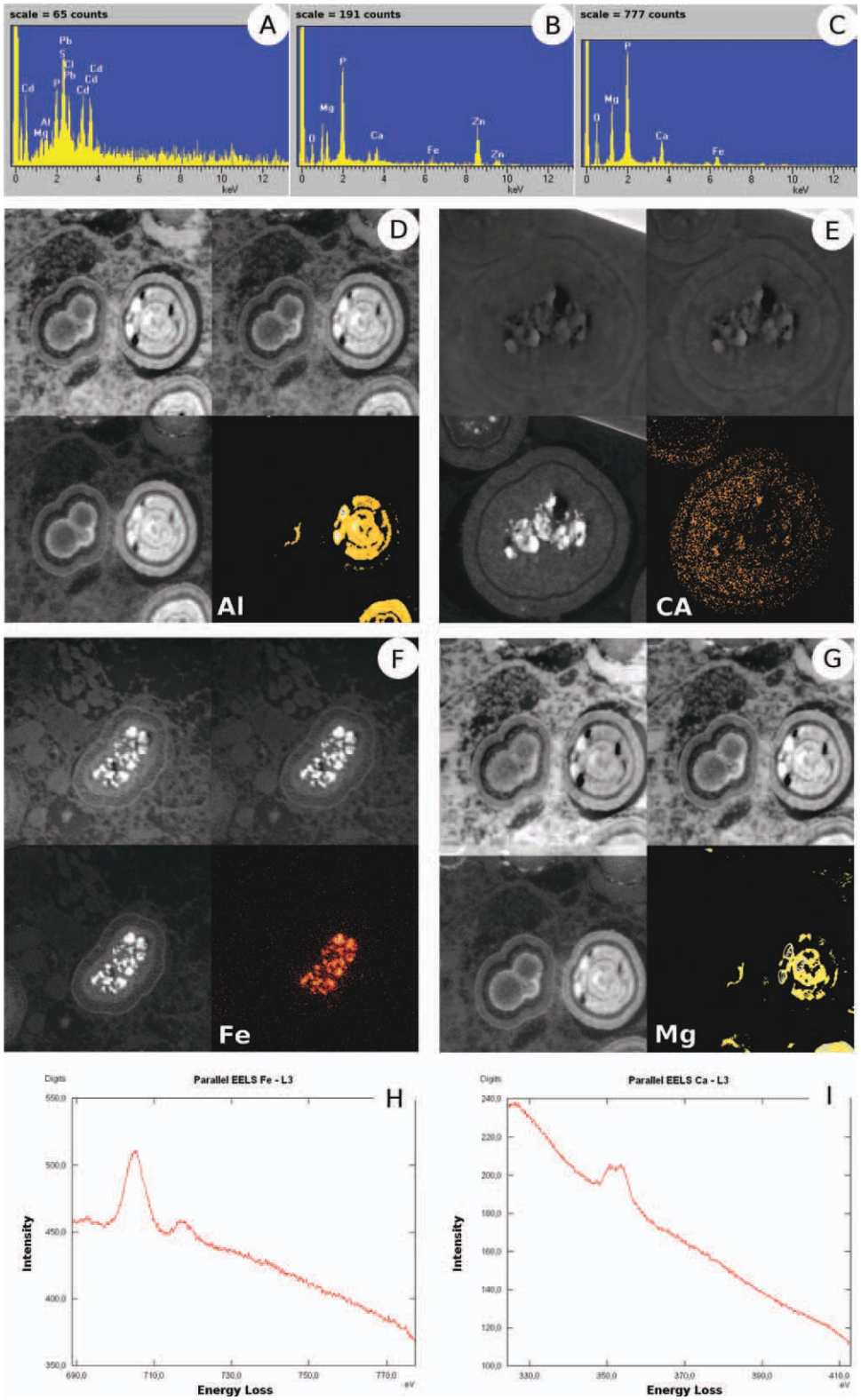

Fig. 2. EDX and EFTEM analyses. (**A**) EDX spectrum obtained from a pellet contaminated with $CdCl_2$ and $Pb(NO_3)_2$ where cadmium and lead peaks are visible. (**B, C**) Ca, Fe, Mg, P and Zn are detected on EDX spectra obtained from a spherocrystal of *S. pugilis* (B) and *S. gigas* (C) not contaminated. Peaks for Al and Mn have also been detected in some others spherocrystals of *S. gigas*. (**D–G**). Spherocrystals of uncontaminated *S. gigas* observed by Electron Spectroscopic Imaging (ESI) using the three windows method. The upper left image is taken just before the element peak, the upper right image correspond to the image taken at the maximum energy of the element. Down on the left is the high contrast image and on the right, the element localization is shown by colored pixels. Elements are localized in the whole structure (**E**), in the core (**F**) or in some layers of the spherocrystal (**D, G**). (**H, I**) Spectra obtained focusing on a spherocrystal after the back-ground subtraction for Fe (**H**) and Ca (**I**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

viduals were sacrificed and the digestive gland was individually processed for EDX analyses and histological observations. For each treatment, three individuals randomly collected were analyzed.

RESULTS

Analyses of Noncontaminated Individuals of *S. gigas* and *S. pugilis*

For both species, three cell types composed the digestive tubules: digestive cells, vacuolated cells, and crypt cells. This last cell type presented a pyramidal shape and was characterized by an important rough endoplasmic reticulum and a large number of small mineral inclusions identified as spherocrystals (Figs. 1A–1D). Such structures were structurally identical in both species. They were composed by several concentric layers alternating electron-dense and electron-lucent surrounding a matrix core (Figs. 1B and 1D). Spherocrystals are present uniformly throughout the cell. The number of these structures is variable depending on the cell considered. According to SEM observations, spherocrystals appear as small spherules from 1 to 3 μm in diameter (Fig. 1C). In both species, EDX analyses (Figs. 2B and 2C) revealed the presence of Ca, Fe, Mg, P, and Zn in spherocrystals. In *S. gigas,* spherocrystals also presented significant peaks for Al and Mn (Table 2). The elemental composition varies depending on the spherocrystal analyzed. In the same cell, two neighboring spherocrystals can present different spectra. Phosphorus is always present in spherocrystals. Calcium and iron are the more often detected elements.

TABLE 2. Elements detected by EDX analysis in spherocrystals of S. gigas and S. pugilis

	Al	Ca	Fe	Mg	Mn	P	Zn
S. gigas (10)	X	X	X	X	X	X	X
S. pugilis (15)		X	X	X		X	X

The number of spherocrystals analyzed is given in brackets.

Some elements detected with EDX were located on thin sections of *S. gigas* observed by EFTEM. A specific spectrum for Al, Ca, Fe, and Mg were found focusing on spherocrystals. Spectra of Fe and Ca are presented in Figs. 2H and 2I, respectively. The EFTEM microanalysis on image mode (ESI) allowed us to locate elements inside spherocrystals. Such cartography showed that elements are sequestered inside spherocrystals. Depending of analyzed structures, elements were located either inside the whole spherocrystal, in its core or only in some peripheral layers (Figs. 2D–2G).

Analyses of Contaminated *S. pugilis*

Lead and cadmium exposure via food did not cause any mortality or signs of health alteration in the individuals. However, waterborne exposures induced an important mucus secretion at 1 mg l^{-1}. Whatever the contamination pathway (food or water) or the pollutant used ($Cd(NO_3)_2$, $CdCl_2$ or $Pb(NO_3)_2$), the pollutant concentration and the duration of exposure, cadmium and lead were never detected in spherocrystals by EDX analyses. A pellet contaminated by cadmium and lead has been analyzed by EDX, showing peaks for both pollutants (Fig. 2A). At the same time, histological observations of individuals contaminated by sea water with $CdCl_2$ and $Cd(NO_3)_2$ showed a severe modification of the gland structure. Histological observations showed an increasing perturbation effect for cadmium concentrations of 0.05–1 mg l^{-1} leading to decrease in the number of digestive tubules. Semithin observations confirmed that cadmium exposition leads to complete disorganization of digestive tubule epithelium (Fig. 3).

DISCUSSION

Spherocrystals have already been reported in various tissues of diverse taxonomic groups. They have been noticed in mammalian (Gallien et al., 2001),

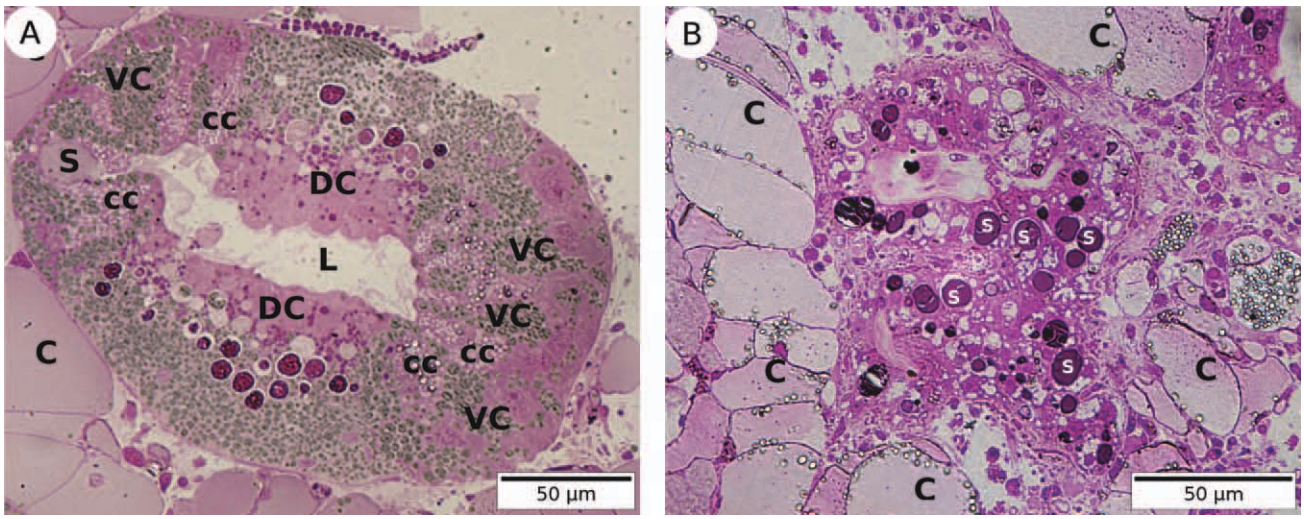

Fig. 3. Semithin sections of *S. pugilis* digestive gland focusing on a digestive tubule. (**A**) From non contaminated individual, digestive tubules are well formed. They are composed by digestive cells (DC), vacuolated cells (VC), and crypt cells (CC). A lumen (L) is also visible. (**B**) After a 3 days exposure with cadmium at 1 mg l^{-1} the digestive gland is totally disorganized and digestive tubules are not recognizable. C: connective tissue; S: symbionts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary. com.]

arthropods (Ballan-Dufrançais, 2002; Chavez-Crooker et al., 2003; Lipovîsek Delakorda et al., 2009; Pigino et al., 2006; Thomas et al., 1999) or in annelids (Jenkins et al., 2002; Mouneyrac et al., 2003). Taylor et al. (1988) have proposed that spherocrystals are deposits of pyrophosphate salt ($CaMgP_2O_7$) which acts as traps for metal ions. Moreover, the role of manganese have been emphasized in the structure and the formation of spherocrystals (Masala et al., 2002; Taylor et al., 1988). Elements detected by EDX in spherocrystals of other Molluscs families are Ca, Cl, Cu, Fe, K, Mg, Mn, Na, Ni, S, Ti, V, and Zn (Gibbs et al., 1998; Nott and Langston, 1989; Nott and Nicolaidou, 1996; Nott et al., 1993b). While EELS analyses are scarce, they are more precise than EDX due to a lower element detection threshold. To our knowledge, only four studies reported the use of EELS alone (Lipovîsek et al., 2002; Triebskorn and Köhler, 1996) or EELS coupled with EDX (Delakorda et al., 2008; Lipovîsek Delakorda et al., 2009) for spherocrystal analyses. EFTEM microanalysis allowed us to confirm the detection of some elements and to locate them on a spherocrystal. Trace metals can be trapped in the whole spherocrystal, just in the core or in some specific layers, so it is possible that different strategies may occur to store elements in these structures.

Regulation of Ions and Essential Trace Metals

In our case, EDX revealed the presence of Al, Ca, Fe, Mg, Mn, P, and Zn in freshly collected strombids. The presence of Ca, Mg, and P may be explained by the nature of the deposit ($CaMgP_2O_7$) and we can postulate that Mn could be involved in the structure and the formation of the spherocrystal as proposed by Masala et al. (2002). A direct correlation between the Ca:Mg ratio of the digestive gland and of the shell has been previously demonstrated during starvation in *H. aspersa* (Porcel et al., 1996). The three other elements detected in Strombidae's spherocrystals are Al, Fe, and Zn. Among these elements, Fe and Zn are essential metals in the sense that they are naturally present in living organisms and that they are necessary for some metabolic processes (Goyer, 1997). Their presence in spherocrystals indicates that in Strombidae, spherocrystals are involved in the regulation and/or the detoxification of such essentials trace metals. Moreover, the elimination of spherocrystals by feces, that represent an excretion pathway for trace metals, has been emphasized by different authors (Nott and Nicolaidou, 1990, 1996). Analyses of rejected spherocrystals have shown that they still present an important proportion of their trace metals content (e.g., 50% for Zn and 33% for Mn) after their passage in the digestive tract (Nott and Nicolaidou, 1993). Crypt cells of Strombidae have a large basal area in contact with the hemolymphe that probably allow an important uptake process and present an important protein synthesis (Gros et al., 2009). In this study, we highlight that crypt cells also contain a large number of spherocrystals that are able to store and/or to regulate minerals as Ca and Mg and essentials metals as Fe and Zn. The production of spherocrystals could be related to the high protein synthesis while the uptake process from the hemolymphe might represent the origin of trapped elements in spherocrystals. Thus, these observations suggest a regulatory function of crypt cells in the digestive gland of Strombidae.

Detoxification of Nonessential Trace Metals

Catalase assays (data not shown) and digestive gland structure showed an impact of cadmium exposure on the digestive gland. However, EDX spectra of analyzed spherocrystals have showed neither lead, nor cadmium, suggesting that such structures are not involved in detoxification of such elements. EDX analysis allowed the detection of these two elements in a contaminated pellet. The known concentrations in this positive control were 100 and 500 $\mu g\ g^{-1}$ for cadmium and lead, respectively. It has been demonstrated that *L. littorea,* after a cadmium exposure at 0.4 $mg\ l^{-1}$ showed 150 $\mu g\ g^{-1}$ of this element in the digestive gland (Nott and Langston, 1989). In scollops, tissue concentrations of Co, Mn, and Zn are 64–94 times higher than in water after 7 days of exposure (Metian et al., 2009a). Nevertheless, we have to consider the possibility that Cd and Pb could be present in spherocrystals but under the detection threshold of the EDX analysis. Note that the possibility of the loss of cadmium and lead by fixation or by solvent during the sample preparation has been rejected by several studies (Gallien et al., 2001; Ireland and Richards, 1977; Nott et al., 1993b). Finally, we also have to consider the possibility of a too short time of exposure not allowing the metals to be trapped on/in the spherocrystals or, conversely, a too high exposure concentrations susceptible to disturb the "normal" functioning of the digestive gland cells. To our knowledge, only three studies reported cadmium inside spherocrystals: one in kidney's spherocrystals in the white-side dolphin (Gallien et al., 2001) and two others in kidney's spherocrystals in scallops (Caramichael and Fowler, 1981; George et al., 1980). On regards to lead, we have only found two studies that report its detection in spherocrystals: in annelids (Ireland and Richards, 1977) and in polychaete worms (Mouneyrac et al., 2003). In a review on the localization of metals in pterygote insects, Ballan-Dufrançais (2002) also reported the detection of Cd and Pb in spherocrystals of ants. To our knowledge, lead has never been detected spherocrystals from Molluscan species. It has been proposed that spherocrystals scavenge class A metals (Ca, Co, Fe, Mg, Mn, Ni, K, Zn) more than class B metals (Ag, Cd, Cu, Hg) which are associated with metallothionein protein (Nott and Langston, 1989; Nott and Nicolaidou, 1989). Cadmium and lead are not sequestered in spherocrystals while they are known to be accumulated in the Molluscs digestive gland (Bustamante et al., 1998, 2000, 2008; Desouky, 2006; Dimitriadis et al., 2003; Metian et al., 2009b; Pernice et al., 2009). Our results indicate that these two elements probably used different detoxification pathway and it would be interesting to use similar analytical techniques to trace them in other cell compartments such as lysosomal residual bodies.

This study represents a preliminary investigation on the role of spherocrystals in Strombidae. EDX analysis is an appropriate technique to detect trace metals and other elements in such structures. These first results could be completed by a study focusing on essentials trace metals. A Cu/Zn exposure experiment is already

in course in our laboratory to precise the function of spherocrystals in essentials metals regulation.

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