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Research Article
Evaluation of Cadmium and Lead Detoxification in the Sea Squirt *Cnemidocarpa amphora* (Kott, 1992) (Stolidobranchia, Styelidae)

Ibrahim Gaber

Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Postal Code: 21547, Egypt

Abstract

Background and Objective: The entries of trace elements into marine invertebrates are mainly in particulate form whereas the exits to the sea concern dissolved forms so that sea waters contain both dissolved and particular forms of the same element. The objective of this study is to detect how *Cnemidocarpa amphora* detoxifies cadmium and lead. **Materials and Methods:** This sea squirt is collected from Ras El-Tin beach of the Mediterranean Sea at Alexandria, Egypt from June-August, 2018-2019. Contamination of the sea squirt is carried out with $CdCl_2$ exposure (500 μg L^{-1}) for 7 and 21 days. **Results:** The first detoxification mechanism is immobilizing cadmium in the absorbent organs in a stable and inert state (phosphate). The second one is the most important which involves hemocytes with natural zinc storage in the synthesis of metallothioneins (MTs) which complexes cadmium. **Conclusion:** Safe our environment to save human lives. Sea squirts react against the chromate by incorporating it into the mucous secretions of the GI tract. The dissociation of a part of the compound releases assailable chromium and lead which cause general tissue contamination. Lead chromate has harmful effects on sea squirts than chromium and lead.

Key words: *Cnemidocarpa amphora*, $CdCl_2$, detoxification mechanism, absorbent organs, hemocytes, metallothioneins, tissue contamination

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Corresponding Author: Ibrahim Gaber, Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Postal Code: 21547, Egypt

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dumping of waste from zinc mining operations creates zinc-lead, copper and especially cadmium pollution[1-3]. It is, therefore, necessary to assess the risks that these contributions present on the sedentary fauna of the marine ecosystem[4-7]. Marine invertebrates are resistant to pollution by copper and assimilable zinc, due to the detoxification mechanisms which they have[8-10], but their behaviour towards cadmium is still poorly understood. This metal is considered to be the most potentially toxic to humans[11]. However, many marine crustaceans and molluscs support cadmium pollution without appearing to be affected, although the metal is assimilated and highly concentrated in the tissues[12] with its long biological half-life[13]. In fishes, cadmium accumulates in epithelial cells, gills, kidneys, digestive gland and hemocytes[14]. At the molecular level, unidentified ligands contained in the lysosomes of contaminated tissues can complex it *in vitro*[15] and metallothionein-related proteins have been isolated from experimentally contaminated animals[16-18]. In marine invertebrates the existence of such protein is controversial and the histological site of a possible accumulation of exogenous cadmium remains to be undiscovered[19]. Authors interested to study marine pollution and its effect on different species of sea squirts[20]. Carman *et al*.[21] and Radhalakshmi *et al*.[22] studied the water quality, nitrogen pollution and ascidian diversity concluded that ascidians are bioindicators of metals in a marine ecosystem. Radhalakshmi *et al*.[22] Agella *et al*.[23] studied the molecular and organism biomarkers of copper pollution in the ascidian *Pseudodistoma crucigaster* (Gaill, 1972). Villalobos *et al*.[24] studied the distribution and population dynamics of key ascidians in North Carolina harbours. Gallo *et al*.[25] tested the impact of metals on the reproductive mechanisms of the ascidian *Ciona intestinalis* (Linnaeus, 1767). Gallo *et al*.[26] studied the ocean acidification impact on ascidian *Ciona robusta* spermatozoa and concluded new evidence for stress resilience. Poynton[27] and Tzafriri-Milo *et al*.[28] studied the Potential use of invasive ascidians for biomonitoring heavy metal pollution. Regarding the effects of particulate pollutants, previous studies demonstrated the high-capacity suspensors to bio-capture particles or colloids not biodegradable from industrial effluents[29-31]. This is how mussels, on contact to phosphogypsum waste or titanium oxide manufacturing effluent, accumulate significant amounts of fluorine, iron hydroxide, titanium oxide and aluminum without interfering in their metabolism[32]. However, this bio-sensing phenomenon does not concern certainly on the particulate compounds that get into the digestive tract.

Thus, we do not know, at present, if *Cnemidocarpa amphora* (Kott 1992) has detoxification means effective enough to protect themselves from natural contamination. We also lack cytopathology data demonstrating the effects of the pollutant, while detoxification means are identified in *Patella vulgata* (Linnaeus, 1758) whose economic importance is less than that of the oyster. We have chosen lead pollutant as the lead chromate $PbCrO_4$, whose particle size is compatible with cell bio-sensing and which, in the event of dissociation of the molecule by the sea squirt itself or by microorganisms in the environment, would predict two toxic elements, lead and hexavalent chromium. For the sake of consistency, we preferred the sea squirt *Cnemidocarpa amphora*, species on which the study of bio-sensing particles is lacking the literature. The objective of this study is to detect how *Cnemidocarpa amphora* detoxifies cadmium and lead in its tissues.

MATERIALS AND METHODS

Study area: The exotic sea squirt *Cnemidocarpa amphora* is captured from Ras El-Tin beach 800 m of the beach, including 200 m of the Mediterranean Sea at Alexandria, Egypt for one year during June-August, 2018-2019.

Methodology: In the laboratory this sea squirt is harvested in glass aquaria, continuous aeration is provided and the seawater is changed every other day, *in situ* and subjected to contamination. Both control and treated squirts were subjected to histological and cytological examinations. The samples intended for the structure study were fixed by Carnoy's liquid (Sigma-Aldrich, Darmstadt, Germany) (absolute alcohol, chloroform, acetic acid, 6/3/1). After inclusion with paraffin, they were sectioned to 7 µm in thickness.

Histopathological examination: For histological and histopathological examination, the sections were stained with hemalin-picroindigocarmine (Sigma-Aldrich, Darmstadt, Germany). The mucus was stained by alcian blue, glycogen by the reaction to periodic acid-Schiff Stain, Kit (Mucin Stain), (ab150680) (Abcam, USA), phosphates by the Von Kossa method; SR groups by the Red Sulfhydryl Reagent, 2,2-dihydroxy-6,6-dinaphthyl disulfide (D.D.D.) (Mucin Stain) (ab150680) (Abcam, USA) and ferric ferricyanide[33].

Microanalysis: The microanalysis was carried out with the Cameca MS 46 microprobe in dispersion wavelength, on normal sections or subjected to various extraction tests and by secondary ionic emission microanalyzer. Samples for

ultrastructure study have been fixed with buffered 3% glutaraldehyde, i.e., sodium cacodylate 0.2 M, or by 0.2 M piperazine (PIPES) (Sigma-Aldrich, Darmstadt, Germany). The osmolality of the fixative was adjusted to 1050 mOs by adding 0.35 M sucrose. Some parts underwent post-fixation with 2% osmium tetroxide in the same buffer. After dehydration, inclusion was made in the Araldite epon. Microanalysis of ultra-thin sections of non-osmosis material, deposited on carbon titanium grids, was carried out on a Cameca MBX, type Camebax equipped with TEM (Hitachi High-Technologies, Japan) in WDS system, under a voltage of 45 KV, a sample current of 150 nA and a probe diameter of approximately 500 nm. The following crystals have been used: LIF: Fe (Kα)), Ni (Kα), Cu (Kα), Zn (Kα), Br (Kα), Pb (Lα); PET: S (Kα), Ca (Kα), Cr (Kα), Mn (Kα), Ag (Lα), Cd (Lα) and Tap: Al (Kα); P (Kα). The counts were made in 100 sec.

Organic material-contamination conditions: Contamination of the sea squirt *Cnemidocarpa amphora* has been carried. Preliminary experience, with varying doses of $CdCl_2$ for the duration of exposure of 7 days, led us to choose the dose of 500 μg L^{-1} of Cd and two exposure durations (7 and 21 days). For lower doses, the concentration of cadmium in tissues is insufficient for characterization at microprobe on paraffin sections, while the highest dose the strongest (1.25 mg L^{-1}) produces significant histological lesions.

Lead has been identified in the secondary ion emission analyzer on the spectra in low resolution, by its three isotopes major (206, 207 and 208). For chromium, the signal 52^+ of the spectra in low resolution in mass had to be treated in high resolution to eliminate the interferers MgC_2H_4 and especially CaCl.

Animal and human rights statement: Ethical clearance for this study was obtained from the Alexandria University, Egypt ethics committee. To improve bioscience research reporting, the ARRIVE guidelines for reporting animal research were applied.

RESULTS

No mortality was observed during the experiment. Some sea squirts have been studied after 7 days of contamination while others after 21 days. Contaminated squirts accumulated cadmium (266±85 μg g^{-1} per day weight after 21 days). Lead chromate forms particles smaller than 2.4 μm. therefore, compatible with biosensing by the digestive cells of the studied crustacean and mussels. Its solubility is, according to the chemical data, insignificant (55-65 μg L^{-1}); the product can release a maximum of 9 μg L^{-1} of Cr and 35 μg L^{-1} of Pb in distilled water and these values are certainly lower in seawater. The dissociation of the commercial sample used (Prolabo, ref. 26.516.361) was determined by atomic absorption spectrophotometry, after 1 and 24 hrs and 8 days of contact with seawater. For a sensitivity threshold of the method of 6 μg L^{-1}, no release of Cr has been observed. On the other hand, the water is immediately enriched by Pb, the concentration of which is 1 mg L^{-1} for a suspension of 55 g L^{-1} of lead chromate. In conclusion, the dissociation of the product is not detectable after being suspended in seawater; but one soluble lead compound (sulphate) present as an impurity, releases lead, the assimilation of which must be considered in the experiments of contamination. Intensities are shown (in counts/sec.) of the signals X of P, Zn, S, Cd and Ag hemocytes (crystals: KAP for P and S; LiF for Zn; PET for Cd and Ag) (Fig. 1).

Cadmium accumulation sites

Fore gut: The contamination does not cause any cytological alteration at the level of the digestive cells and basophil cells. Ultrastructure examination reveals no damage to gastric cells of contaminated squirts. The appearance of microvilli in the apical border and the various organelles contained in the hyaloplasm (nucleus, mitochondria, endoplasmic reticulum, dictyosomes) are identical to that of the control (Fig. 2a-d). In Fig. 2a the foregut cells contain lysosomes, in Fig. 2b lysosomes have a rich content of various elements (S, P, Ca, Cu, Zn, Al and Fe), Fig. 2c shows a digestive cell and Fig. 2d shows the digestive cell lysosomes contain many elements (S, P, Ca, Cu, Zn, Al, Fe and Br). At the apex of the cells, the lysosomes, the number of which is not greater than that of the controls, contain electron-dense opaque compounds. X microanalysis detects cadmium which is added to many other elements (S, P, Ca, Cu, Zn, Al and Fe) regularly concentrated by cell lysosomes as in the foregut of controls (Table 1). For each element, the counts were made in 100 s. on the characteristic line (I_p) and the background data measured on both sides of the peak (IBF). The values of the calculated intensities (I_c) are obtained by deducting the two measurements:

$$D^p \text{ intensity at peak } (I_c = T_p - T_{BF})$$

The statistical error on the measurement of the characteristic signal I_c is estimated as:

$$\alpha = \sqrt{T_p + T_{BF}}$$

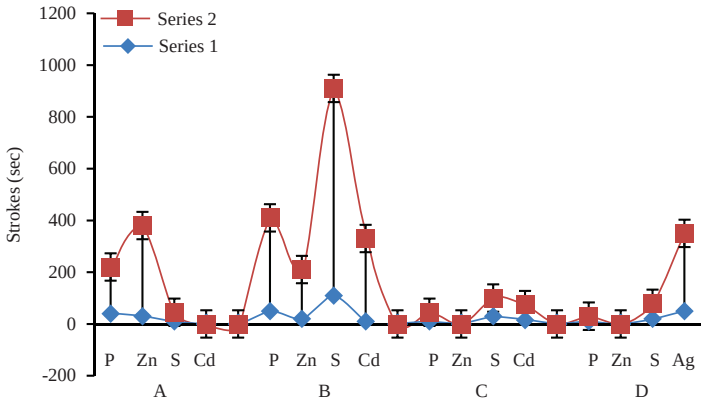

Fig. 1: Intensities (in counts/sec.) of the signals X of P, Zn, S, Cd and Ag hemocytes (crystals: KAP for P and S; LiF for Zn; PET for Cd and Ag)

A: Control sea squirts, B, C and D: Contaminated sea squirts (neighbouring sections of the same animal for all measures). B cup n 1 having undergone no treatment. Notice the high intensity, S signals (110 at 800 strokes/sec.), C section treated with HCl at pH 1 for 10 min, plus $CdC1_2$, D section treated with HCl at pH l for 10 min, then with $AgNo_3$

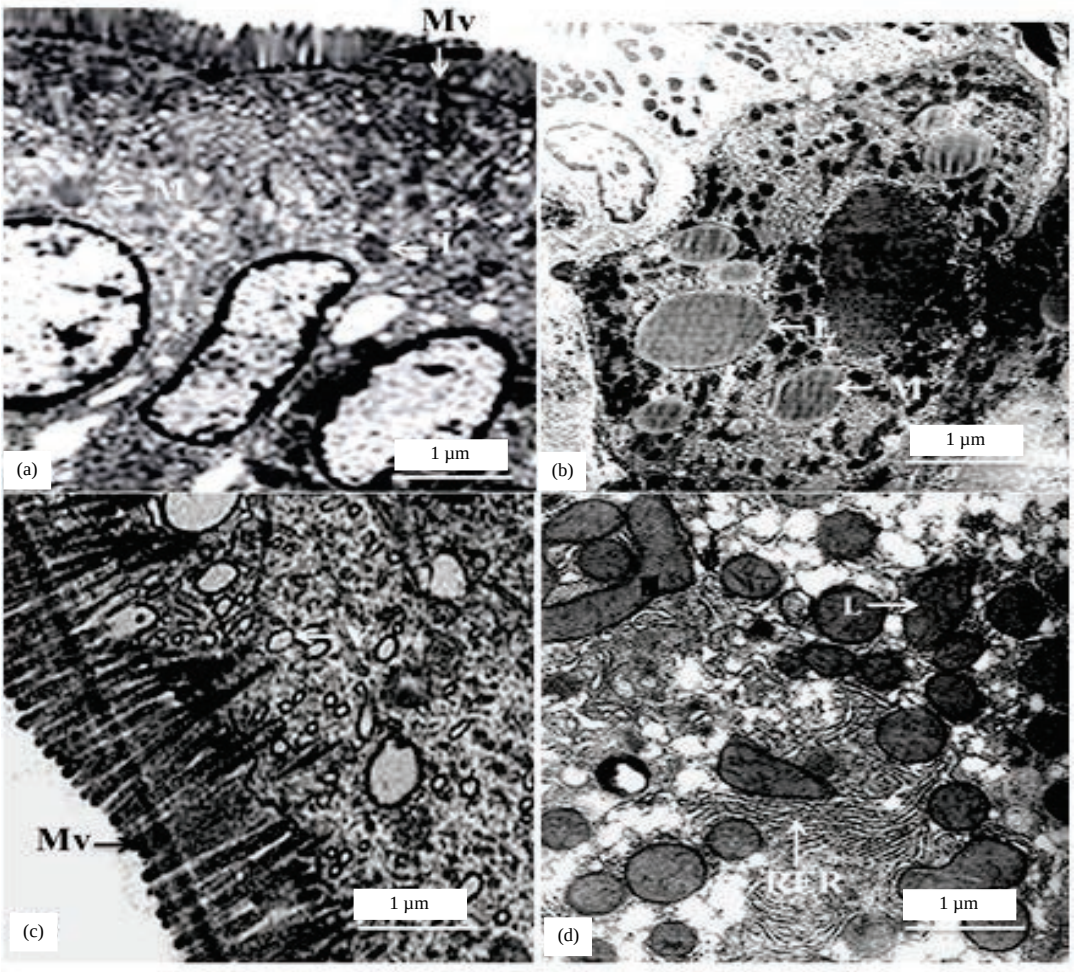

Fig. 2(a-d): Histopathological examination of Control sea squirts

(a) Fore gut cells contain lysosomes, (b) Lysosomes have a rich content of various elements (S, P, Ca, Cu, Zn, Al, Fe), (c) Digestive cell and (d) Digestive cell lysosomes contain many elements (S, P, Ca, Cu, Zn, Al, Fe, Br). L: Lysosomes, M: Mitochondria, Mv: Microvilli of the apical membrane, N: Core, RER: Rough endoplasmic reticulum, V: Vesicle

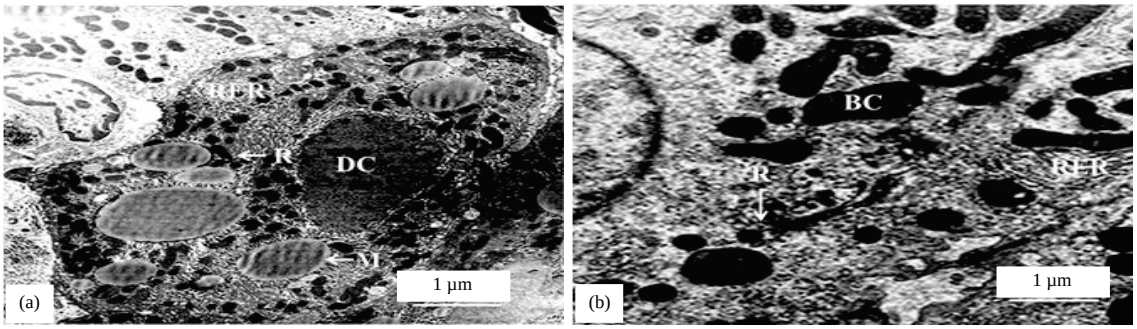

Fig. 3(a-b): Sea squirts contaminated by $CdC1_2$ (21 days)
(a) Ultrastructure aspect of intestinal epithelium digestive cells without lesions and (b) Cadmium is detected in lysosomal vesicles; the content is more or less opaque (arrows). CD: Digestive cells, CB: Basophilic cells, Ly: Lysosomes, M: Mitochondria, N: Core, RER: Rough endoplasmic reticulum, R: Ribosome

Table 1: Elementary composition of the particles contained in the lysosomes of the GI tract, the branchial epithelium and pyloric gland cells

Lysosomes and intra-cellular concretions	Control			Sea squirts intoxicated (7 days, 500 pph)			Sea squirts intoxicated (21 days, 500 pph)		
	GI tract	Branchial epithelium	Pyloric gland	GI tract	Branchial epithelium	Pyloric gland	GI tract	Branchial epithelium	Pyloric gland
Cd	ns	ns	ns	ns -200	ns	ns	ns-400	40-3100	ns-220
S	70-550	270	120-330	110-340	10-350	120-340	70-500	ns-800	20-510
P	450-1700	1400	610-1700	130-1800	600-7200	190-700	110-1700	140-51000	500-17000
Ca	220-540	1600	ns-120	20-140	ns-3500	90-220	120-310	130-800000	2400
Cu	ns-50	320	ns-140	ns-190	50-320	ns-300	40-300	120-7400	140-610
Zn	60-110	45	ns-80	ns-50	ns-900	ns-230	ns-130	ns-2200	70-150
Al	130-220	140	ns-70	ns-120	ns-300	ns-120	50-180	30-1200	ns-90
Fe	1200-1900	1600	ns-400	90-2100	ns-7000	70-1205	50-3000	30~8500	310-6400

Characteristic signals for each element (ns: Non-significant values). Analysis of ultra-thin sections was done by the electronic probe

The numerical values of the counts presented in the tables, obtained for a given structure and an element, were retained within a confidence interval of ±2 gold and are therefore highly significant. Nb. The presence of cadmium in lysosomes does not cause a significant change in values S and P signals. In particular, the very strong phosphorus signals recorded in the branchial chamber and pyloric gland of contaminated sea squirts are due to the calcium mineralization of lysosomes of concretions containing cadmium; therefore, a possible binding of cadmium to phosphorus is impossible to detect by the simple comparison of the values of the phosphorus signals contaminated sea squirts and control. It is in several strokes 100^{-1} sec.

Intestinal epithelium: The appearance of apical microvilli and hyaloplasmic organelles (nucleus, mitochondria, granular endoplasmic reticulum, dictyosomes) is similar to that of control (Fig. 2a-d and Fig. 3a-b). In controls, basophil cells do not contain structures rich in mineral elements. Intestinal cells, on the other hand, contain many lysosomal vacuoles of varied morphology and with a diameter of 0.6-1.7 µm. These lysosomal vacuoles are involved in intracellular digestion and are rich in opaque particles to electrons dense within a homogeneous stroma (Fig. 2a-d). Analysis reveals the presence of various elements (Al, P, S, Ca, Fe, Cu, Zn, Br, Table 1), at the level of the particles and the stroma of the various lysosomal vacuoles than controls. In contaminated sea squirts, cadmium is rarely detected in these vacuoles with heterogeneous content, but are preferably found in small lysosomal vesicles of a size close to that of the sections of mitochondria (0.5 µm, Fig. 3a-b and III), frequently located at the apex of the cells. There is no noticeable difference in the number of lysosomes containing cadmium between the two batches of squirts having undergone 7 or 21 days of contamination (Fig. 3a-b). The result of Fig. 3a shows an intestinal epithelium digestive cell without lesions and in Fig. 3b cadmium is detected in lysosomal vesicles where the content is more or less opaque (arrows). Due to the dispersion of lysosomes and their small size, cadmium is rarely detectable by microanalysis X of paraffin sections.

Branchial epithelium: After contamination, the ultrastructure aspect of the epithelial cells is not altered, as is that of mucus cells. Microvilli of the apical border and the organelles contained in the hyaloplasm (nucleus, mitochondria, smooth endoplasmic reticulum and granular ER, dictyosomes) have an

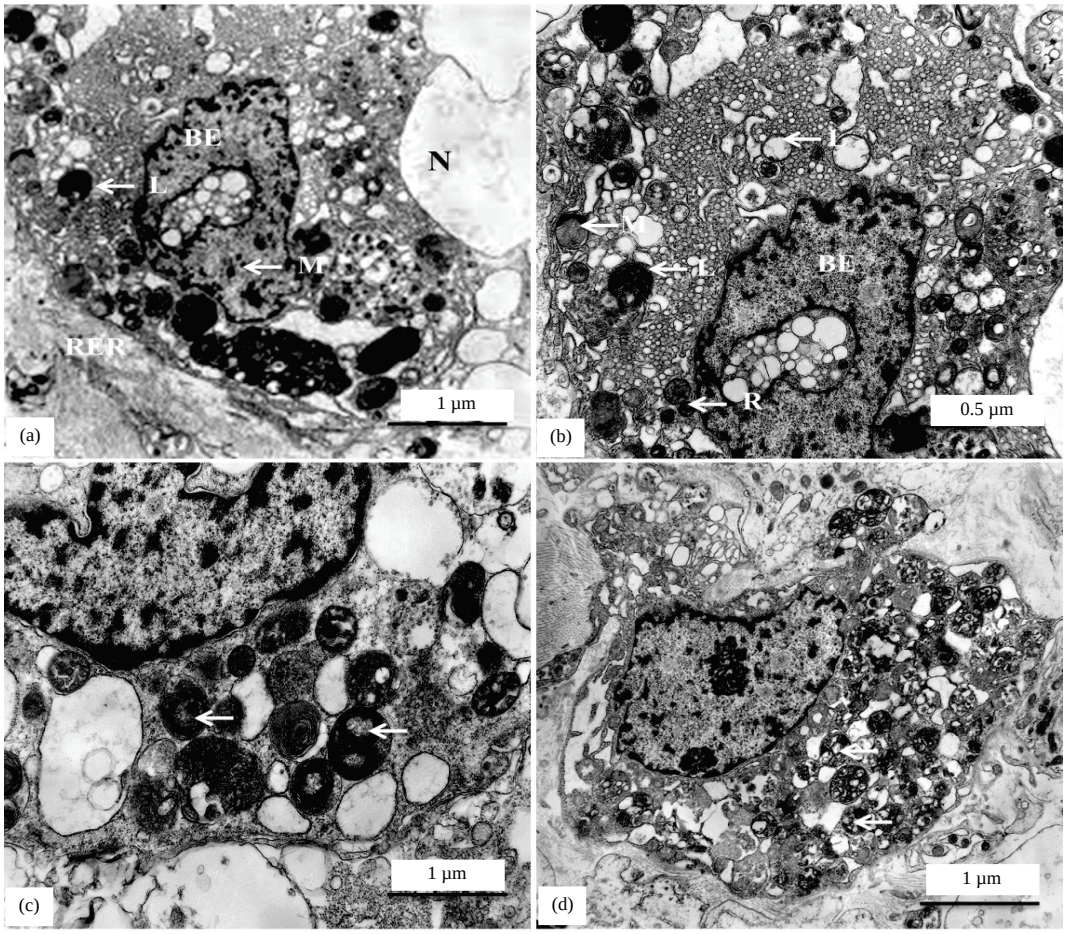

Fig. 4(a-d): Sea squirts contaminated by $CdC1_2$ and control cells of the branchial epithelium cadmium is detected in lysosomes
(a) Contaminations for 7 days, (b) 21 day represents contaminations, (c) A branchial cell and (d) Control sea squirt. In Contaminated sea squirt, 21 days cadmium is detected in lysosomes. BE: Branchial epithelium, L: Lysosomes, M: Mitochondria, MV: Microvilli, N: Core, R: Ribosomes, RER: Rough endoplasmic reticulum

identical appearance to that of the controls (Fig. 4a-d). The data of Fig. 4a shows contamination for 7 days, Fig. 4b for 21-day contamination, Fig. 4c shows a branchial cell and Fig. 4d shows a control sea squirt branchial cell. At the cellular apex, lysosomal vesicles are rare in the controls and contain the same elements as the lysosomes of digestive cells (see Table 1). After 21 days of contamination, we observed the presence of small apical lysosomes of 0.3 µm diameter, in which the cadmium is detectable (Fig. 4a-d Table 1). These lysosomes are sometimes very richly mineralized and contain large amounts of phosphorus and calcium. Cadmium was not detected by microanalysis of X paraffin sections.

Hemocytes: Unlike the foregut, intestine and bronchial epithelium, cadmium is always identifiable by analysis of paraffin sections, in the hemocytes of contaminated squirts and therefore, the study of these cells has not been addressed at the ultrastructure level. Cadmium has been detected in hemocytes of the branchial chamber, digestive tract epithelia and vesicular connective tissue. The signals X of Cd are of very variable intensity. They coexist with important P, Zn and S signals, sometimes with weak Cu signals. The presence of P and Zn indicates that Cd is concentrated in zinc phosphate hemocytes. Phosphate storage is interesting for the topographic location of these hemocytes since Zn is substitutable for Ag and thus hemocytes react positively to Von Kossa's method (Fig. 5a-d). Control sea squirts are shown in Fig. 5a-b and sea squirts contaminated by $CdC1_2$ are shown in Fig. 5c-d. In Fig. 5a the hemocytes are localized in the digestive tract epithelium, Fig. 5b the hemocytes do not reduce the ferric ferricyanide. Fig. 5c. location of hemocytes in the foregut wall and Fig. 5d hemocytes reduce the ferric ferricyanide, due to their metallothionein. While Cd contamination does not cause any change in intensity for the

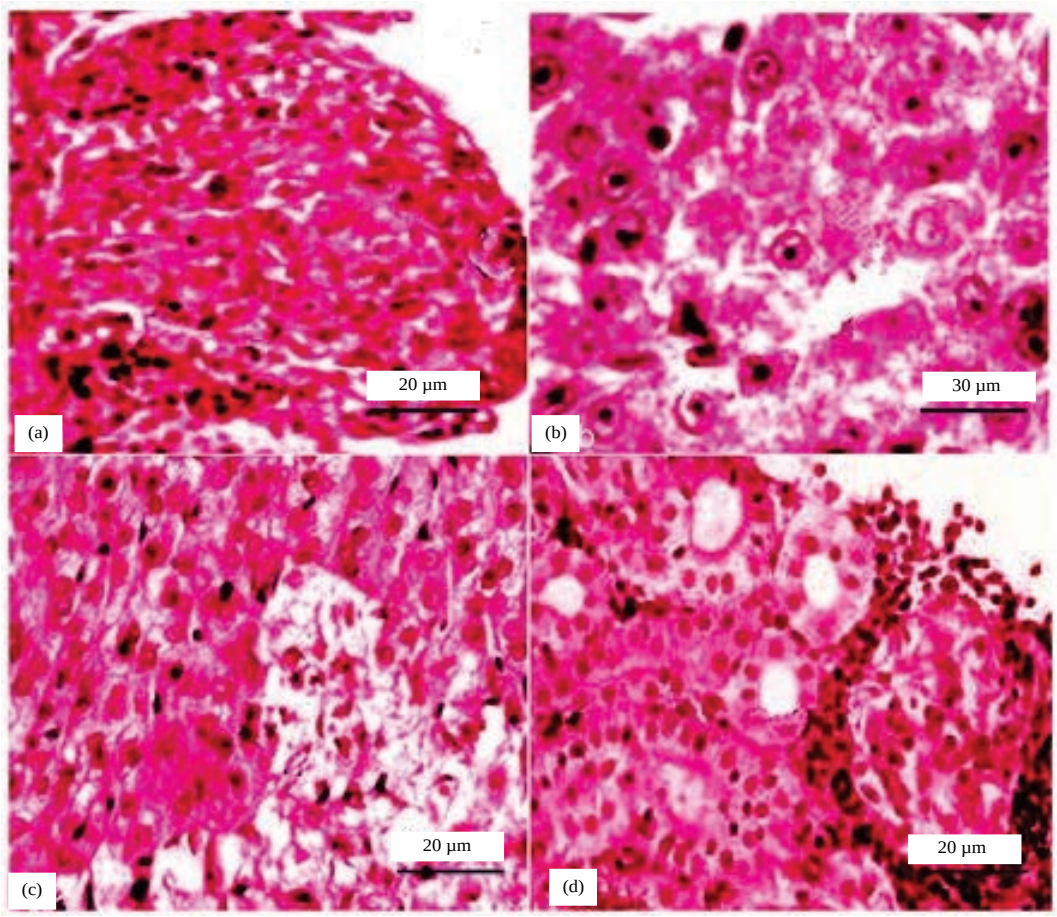

Fig. 5(a-d): (a-b) Histopathological analysis of indicator sea squirts and (c-d) Sea squirts contaminated by $CdC1_2$
(a) Hemocytes are localized in the digestive tract epithelium via the reaction by Von Kossa, (b) In this control, the hemocytes do not reduce the ferric ferricyanide, (c) Location of hemocytes in the foregut wall by the reaction of Von Kossa and (d) In these contaminated sea squirts, hemocytes reduce the ferric ferricyanide, due to their metallothionein

signals X of P and Zn, the increase in the intensity of the signals S is very strong and correlated to that of Cd signals (Fig. 4a). The Cd coordinate is therefore suffering. Although 7 µm section analysis is not suitable for a precise calculation of the ratio signal S/Signal cd: it appears that this one (5.92±0.64) is superior to that of a reference sample of cds (1.75±0.14) and fairly close to the theoretical ratio (5.76) characteristic of a bond between 1 Cd and 3 S. The possibility of sulphide, a salt frequent in marine sessile invertebrates, is therefore excluded, which is confirmed by the pH sensitivity of the bond between Cd and his coordinator. Thus, at pH 6 (acetate buffer), the emissivity X of Cd is conserved, while it is weaker at pH 3.5 (buffer acetate) and zero at pH 2 (citrate buffer). In all three cases, emission S is unchanged. Acid treatment with HCl at pH 1 decreases it by extracting a fraction of the ligate. Ethylenediaminetetraacetic acid (EDTA) 10-2 M and $AgNo_3$ also release Cd from its ligand. After an acid treatment (HCl at pH 1) which removes both Cd and Zn phosphate, the residual sulfur ligand retains its ability to complex again Cd or Ag, when the demineralized sections are treated with $CdC1_2$ or $AgNo_3$ (Fig. 4a). Finally, the digestion of the coordinate by proteolytic enzymes, pronase and trypsin, is not possible only after the elimination of Cd by EDTA. These results approximate those obtained *in vitro* on metallothionein of fishes, lead us to the hypothesis of a bond between cadmium and a protein of this type, of which the high sulfur content is attributable to cysteine residues. The choice of methods for characterizing the group SH is unfortunately restricted. Indeed, the coordinate is extracted by the prolonged treatment at 60°C in the Domain-driven design method and the method R.S.R. is too insensitive. Only ferricyanide reduction methods, which have good sensitivity, which acts cold, in a short time and at a pH low enough (2.4-3) to dissociate an important number of Cd-SR bond, give strongly positive results on hemocytes from contaminated squirts only (Fig. 5a-d). The involvement of SR groups in the

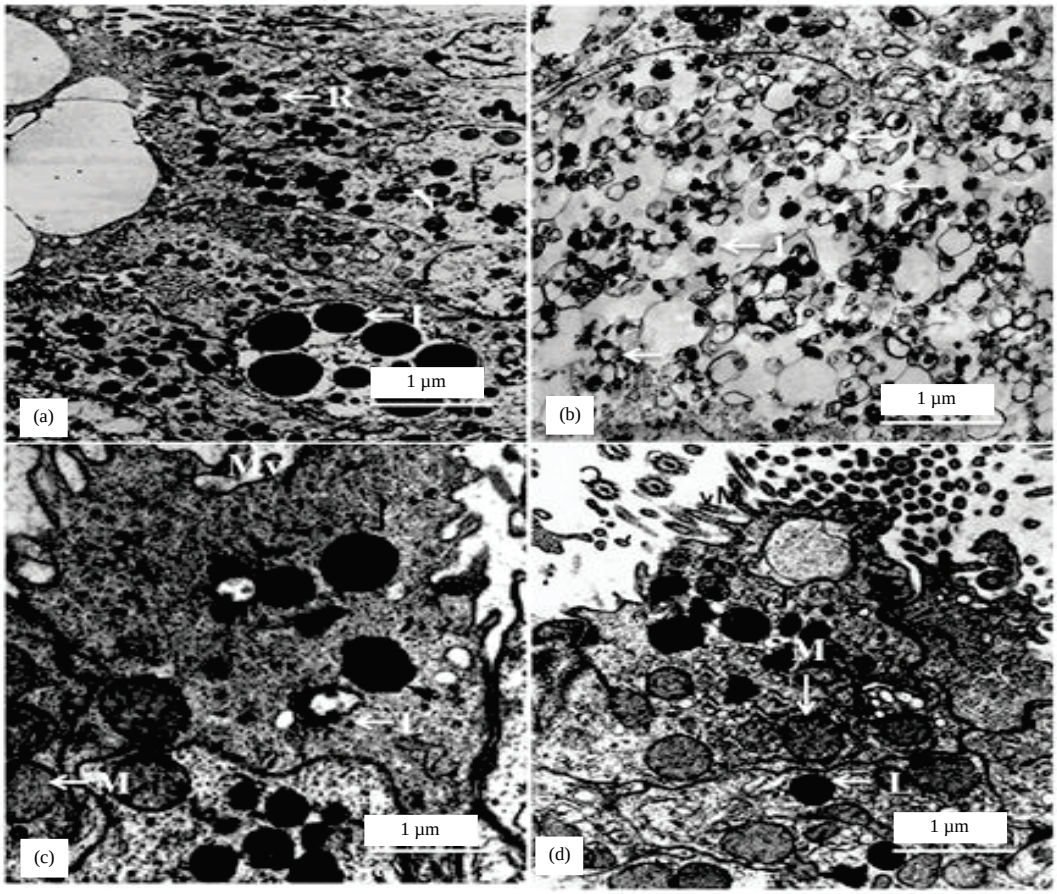

Fig. 6(a-d): TEM in a part of the pyloric gland of *Cnemidocarpa amphora*. Pyloric gland
(a) Control sea squirts pyloric gland epithelium, (b) Control sea squirt pyloric gland cells contain lysosomes (Ly) and concretions (arrows), (c) $CdC1_2$ contaminated sea squirt pyloric gland epithelium and (d) $CdC1_2$ contaminated sea squirt, in the apex of pyloric gland cells; Cadmium is detected in lysosomes with slightly opaque content. L: Lysosomes, Mv: Microvilli, N: Core

reaction is confirmed by their elective blocking by N-ethylmaleimide, which is not effective, as for the mammalian TM, only after elimination of Cd by EDTA. Cadmium concentrated in hemocytes is therefore linked to a protein which, like metallothionein, owes its complexing capacities to its cysteine residues.

Pyloric gland: Renal epithelium cells in treated squirts have an ultrastructure aspect close to that of the control and no pathological alteration is noticed at the level of the apical membrane systems and hyaloplasmic organelles (Fig. 6a-d). The data of Fig. 6a shows Pyloric gland epithelium, Fig. 6b shows Pyloric gland cells contain lysosomes and concretions, Fig. 6c shows Pyloric gland epithelium and Fig. 6d shows in the apex of pyloric gland cells, cadmium is detected in lysosomes with slightly opaque content. At the apex of pyloric gland cells of control, the lysosomes are numerous and of varied morphology; they contain the same elements as those of the foregut and branchial epithelium lysosomes and are sometimes very richly mineralized, then taking the aspect of concretions. It is sometimes in these concretions, but more frequently in less opaque lysosomes than cadmium is detected after 21 days of contamination (Fig. 6a-d and Table 1). As in the case of the branchial epithelium is, cadmium cannot be identified by X microanalysis paraffin sections. Mantle cells contain many rich lysosomes elements (S, P, Ca, Cu, Zn, Al and Fe), but which never contain cadmium.

Effects of cadmium on metabolism and reproductive activity: It is well known that, in fishes, cadmium activates glycogenolysis and, therefore, opposes glycogen storage. This effect is probably responsible for the impossibility of storing the glycogen that insects have intoxicated by Cd or the elevation of the content in blood glucose that the limpets living in polluted environments undergo by Cd. The effect of the pollutant on the metabolism of sugars could therefore be

Table 2: Estimation of the glycogen content (from 0-+++) of the vesicular cells of control sea squirts, experimentally contaminated and harvested in the laboratory

	Glycogen			
Number of animals	0	+	++	+++
Control and contaminated sea squirts with $CdC1_2$ 500) lg L^{-1} 21 days				
10			3	7
19	3	5	5	6
20	2	8	6	4
14	1	5	5	3
14			4	10
17	2	5	7	3

0-+++ represent vesicular cells classification, 0-+ cells with a low load in glycogen and ++ and +++ cells with a high load in glycogen

Table 3: Sexual state of sea squirts, contaminated with $CdC1_2$ 500) lg L^{-1} 21 days

	Sexual state							
Number of	Male					Female		
animals	1	2	3	4	5	Small oocytes	Medium oocytes	Large oocytes
Control and contaminated sea squirts with $CdC1_2$ 500) lg L^{-1} 21 days								
11			4				7	
24		2	4	2	7	6	3	
12		3	5				3	1
21		2	7	2	4	2	2	3
16			8		3	2	2	2
17			5	2	2		3	5

quite general and should be taken into consideration in a study on the squirts, a species that stores very large amounts of glycogen. The study of carbohydrate metabolism cannot be separated from that of the genital cycle. In animal species including the oocytes vitellus and glycogen, as is the case of the squirt, oocytes must synthesize their glycogen and import from other organs, the carbohydrate precursors of yolk. Of this fact, when the synthesis of these precursors is blocked, the oocytes cannot develop. For this purpose, indirect cadmium on genital activity, opposes the action and direct metal to male germ cells. However, this direct action that causes testicular necrosis of fishes is not general since it does not affect the testicles in insects. In the case of sea squirts, no study reports about the indirect effects of cadmium on the ovary and direct effects of the metal on the testicle.

Accumulation of glycogen: The accumulation was estimated by the glycogen load of the connective tissue cells which ensure the storage of the polymer and its recirculation in the form of glucose. The method used is cytochemical: after reaction with periodic acid-Schiff, the glycogen colours all the more intensely as its concentration is high; a comparative assessment of glycogen loads is; therefore, possible and vesicular cells could thus be classified into 4 categories according to their load (0, +, ++ and +++). The results (Table 2, lines 1 and 2) show that the 9 control sea squirts have cells with a high charge in glycogen (++ and +++). On the 18 contaminated sea squirts, on the other hand, 4 have weakly charged cells (+), 3 have no cytochemical glycogen detectable.

State of the gonads: Gonad lesions were investigated by examining sections stained with hemalin- picroindigocarmine and sections treated with periodic acid- Schiff. Individuals' sexual status has been determined by reference to the scale of the stages of gonad development (Table 3, lines 1 and 2). The ovary: The six control squirts are all at the medium-sized oocyte stage; glycogen and yolk load are normal for this stage, i.e., very weak. Two of the 7 treated squirts are similar to controls, both for their genital maturation and the glycogen load of their vesicular cells. The other five contaminated squirts show retarded oocyte growth; three of them have a glycogen load of the vesicular cells weaker, one is devoid of cytochemical detectable glycogen. Contamination causes, in an appreciable number of squirts, a decrease, or even a suppression of glycogen reserves. Ovaries undergo a delay in oocyte maturation, due to the stage of maturation of the squirt put in experience if vitellogenesis would have been normal. But the deplorable state of most of them leads us to consider a gamete emission earlier than in controls (Fig. 7a-d). The data of Fig. 7a is a CS in the ovary. The ovarian follicle contains healthy oocytes on which performed the distribution

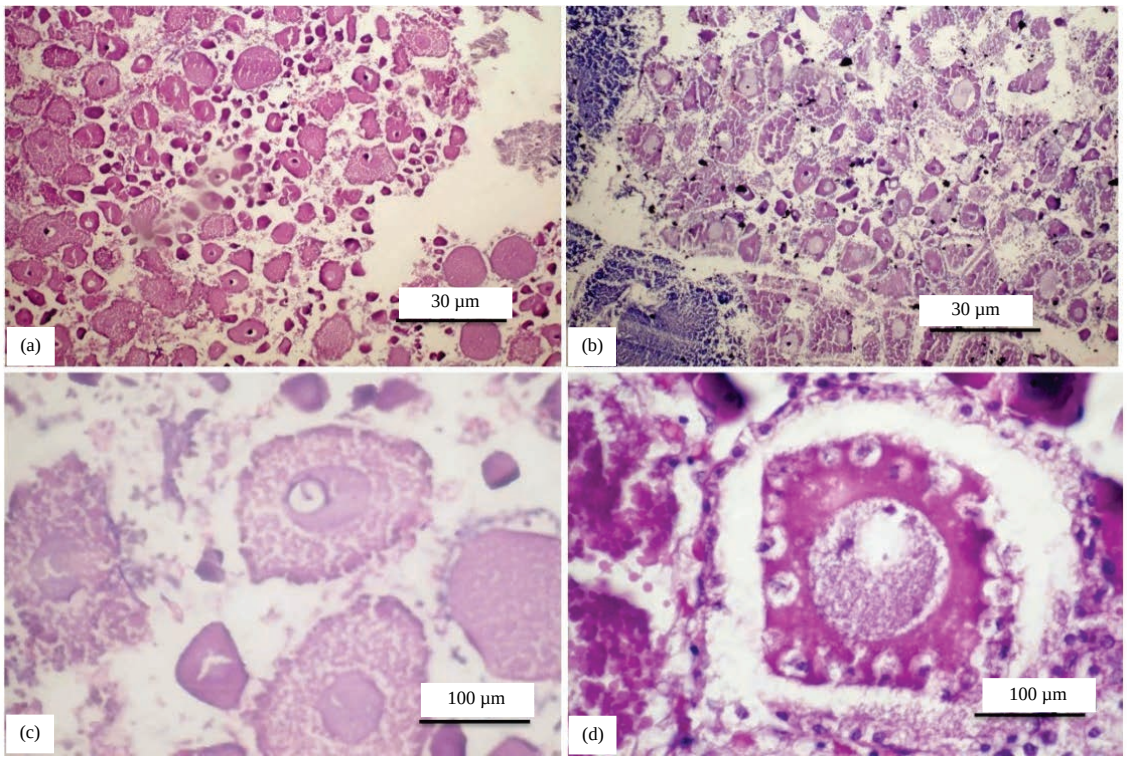

Fig. 7(a-d): CS in a part of the ovary of *Cnemidocarpa amphora*

(a) Ovarian follicle contains healthy oocytes on which performed the distribution of Ca, Cr and Pb, (b) Ovarian follicle contains vitellogenic oocytes, (c) Ovarian follicle contains lysed oocytes and (d) Ovarian follicle contains advanced vitellogenic oocytes

of Ca, Cr and Pb (Fig. 7b). The ovarian follicle contains vitellogenic oocytes. The data of Fig. 7c the ovarian follicle contains lysed oocytes. The data of Fig. 7d the ovarian follicle contains advanced vitellogenic oocytes. Experiences, however, were not performed on a sufficient number of samples so that these results can be considered final. Male stages are 1-early gametogenesis, 2-well-developed follicles; immature gametes, 3-average and maximum states of gonad repletion, 4-abundant wall gametes, 5-start of gonad depletion, 6 almost complete depletion. The testis: the three controls are all at stage 3 of very abundant and mature gametes, with vesicular cells high in glycogen. The testes of treated squirts do not show necrosis after 7 days. On the other hand, the stages of development of the gonads are very varied. Three squirts have a testis at the same stage as that of the control; another has a delay in gametogenesis. Within 7 others, testicular depletion is partial (stage 4) or complete (stage 5) and in the latter case, the vesicular cells are poor or without glycogen. The testis suffers from necrosis in some males after 21 days of contamination (Fig. 8a-d). The data of Fig. 8a shows CS in the testis. In the testicular follicle in spermatogenesis on which performed the distribution of Ca, Cr and Pb. In Fig. 8b testicular advanced spermiogenesis, Fig. 8c the testicular follicle undergoes necrosis, Fig. 8d damaged testicular follicle.

DISCUSSION

This study finds that chromium and lead pass through the body under a different chemical form from Pb chromate. The muscular masses and the mantle epithelium emit Cr^{+} signals strong enough to provide distribution images of the element, the testis is mainly contaminated with Cr, while the signals Pb are extremely weak. The ovary is loaded with Cr and Pb whose cytological localizations are uniform. The two elements are therefore concentrated and excreted independently one on each other and in different chemical states (phosphate for lead).

Zinc, iron, manganese and copper are some of the essential elements required by organisms, in small quantities, while mercury, lead and cadmium, as they do not have a biological function, are normally toxic to biota, even in small

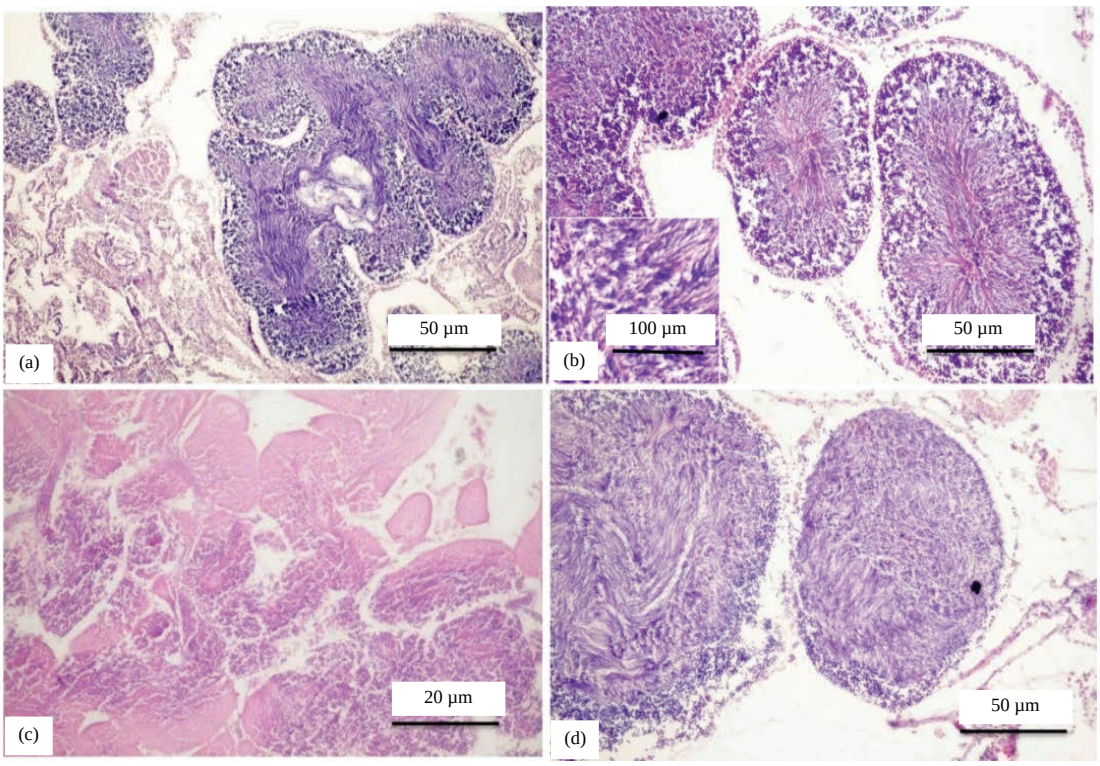

Fig. 8(a-d): CS in a part of the testis of *Cnemidocarpa amphora*

(a) Testicular follicle in spermatogenesis on which performed the distribution of Ca, Cr and Pb, (b) Testicular advanced spermiogenesis, (c) Testicular follicle undergoes necrosis and (d) Damaged testicular follicle

concentrations[34-36]. However, anthropogenic activities have contributed considerably to increase these elements in the marine ecosystem, including industrial activities, agriculture and mining[37,38]. Once in an ecosystem, these elements can be associated with particulate material in suspension and eventually be deposited in the bottom sediments, forming relatively stable reservoirs. However, changes in physical and chemical conditions of the ecosystem can cause the mobilization of metals deposited in the sediments and the release of more bioavailable forms for the column of water, thus favouring the assimilation of these by biota, being able to reach very high levels in these organisms[39], through processes of bioaccumulation, bioconcentration and biomagnification. The term bioaccumulation refers to the assimilation and retention of chemicals present in the ecosystem through the respiratory, dermal, or digestive tract, be dissolved and associated with sediment or other organisms[40]. Bioconcentration, in turn, refers exclusively to the accumulation from the aqueous phase and, biomagnification to the transfer of contaminants from a trophic level to another which tend to exhibit increasing concentrations as that pass to higher levels. All types of metals have potential toxicity and are therefore capable of causing adverse effects to biota when present in concentrations[41], in such a way that these effects, types and intensity, can be used in biomonitoring studies and thus offer responses regarding the availability and concentrations of these agents in the environment. In crustaceans, trace elements can cause a series of physiological changes, including changes in osmotic and ionic balance, decreased oxygen consumption, changes in hemolymph pattern, as well as histological damage[42]. In fish, these elements can inhibit the activity of enzymes, such as acetylcholinesterase, cause mutagenic, genotoxic and cytotoxic effects, as well as induce the formation of reactive oxygen species, which can lead to oxidative stress[43,44]. Accumulated levels, as well as differential assimilation in tissues, depend on intrinsic factors to the species, such as diet, metabolic rate, purification capacity, as well as environmental characteristics, which can increase or even reduce the availability of these elements, including the type of sediment, salinity, temperature, pH, organic carbon content, are among others factors[45,46]. Through the food chain, these elements can be transferred to the and, as in other organisms, cause damage to health, including, in the human species,

dysfunctions in the nervous, renal, gastrointestinal and reproductive capabilities[47]. This study can be applied in aquaculture like shrimp and mussel farms to test whether invertebrates in captivity have detoxification mechanisms. This study recommends if invertebrates in captivity lack these mechanisms, the farming firms have to clean up the aquaria from trace elements to evade human diseases. It is worse to tell that still, many companies throw the industrial disposal in marine ecosystems and this represents a strong limitation to apply this study.

CONCLUSION

Two types of detoxification processes should be attributed to the excellent resistance of *Cnemidocarpa amphora*. In absorbent epithelia, the cadmium is immobilized in a stable chemical state (phosphate). In hemocytes, the metal is completed with metallothionein. Zinc hemocytes represent the site of synthesis and storage of cadmium complexion protein. The non-detoxified cadmium exerts an effect on the reproductive system and the glycogen cycle.

SIGNIFICANCE STATEMENT

This study concludes that pollutants can be absorbed by the four-way method, which is: food, gills, water intake and the body surface. Absorbent epithelia detoxify and transform $CdCl_2$ to metabolically inert and hemocytes are the sites of storage of cadmium complexion protein. This study will help the researcher to uncover the critical areas of detoxification in the marine ecosystem that many researchers were not able to explore. Thus, a new theory on pollution monitoring capability may be arrived at.

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