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Contribution of X-Ray Spectroscopy to Marine Ecotoxicology: Trace Metal Bioaccumulation and Detoxification in Marine Invertebrates

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1. Introduction

Among contaminants involved in pollution, metals are peculiar as they are natural components of the geosphere. Volcanic eruptions, soil leaching, anthropogenic inputs, constitute different sources of metal introduction in the aquatic environments, the sea being the final receptacle for toxic compounds (Ramade, 1992). Moreover, they are the only environmental pollutants that are nor made nor degraded by Men but that are transported and transformed in various products that affect living organisms. Metals are present in the sea within its different compartments: water column, sediments and in marine biota, where they are accumulated from water or food. Some metals are so essential to living organisms that when their availability is limited, the process of life development may be negatively affected. In fact, beyond a given threshold, all metals, whether essential or not, may have toxicological and ecotoxicological effects. Toxic effects may impair numerous biological and physiological processes, leading to animal death and threatening of species conservation. The energy costs associated with excreting and/or detoxifying the ingested/assimilated metals may decrease growth and reproduction with negative consequence on population density and on ecological intra and inter-specific relations.

Aquatic organisms accumulate metals to concentrations several folds higher than those in the surrounding medium (Bryan, 1979; Rainbow et al., 1990). However, metal bioaccumulation per se is not necessarily an indication of adverse effects (Campbell & Tessier, 1996) and knowledge of the physiological processes of accumulation and detoxification is needed to understand the significance of accumulated trace metal concentrations in marine animals collected from contaminated areas, especially in the frame of biomonitoring programs (Rainbow, 1993; 1997). Metal bioaccumulation in organisms is a natural physiological process or a consequence of an accidental surcharge. In both cases, common cellular structures involved in metal sequestration are requested (Jeantet et al., 1997). Cells have various biological ligands, such as metalloproteins, to which metals can bind, on the one hand, and their organites (ie lysosomes, endoplasmic reticulum) are involved in metal sequestration (Ballan-Dufrançais, 1975; Brown, 1982; Mason & Jenkins, 1995; Nott & Nicolaidou, 1989a; Simkiss & Taylor, 1995; Raimundo et al., 2008; Simkiss & Masson, 1983). Total metal body burden may reflect the presence of inert particles

mineralized (the so-called granules) within tissues and cells which are no longer involved in metabolism. Generally, metal bioaccumulation studies in marine invertebrates give quantitative data on the tissular partitioning of metals (organotropism) but few of them consider the physico-chemical form in which metals are present within tissues or cells. However, these informations would improve our knowledge on cellular mechanisms involved in metal sequestration, storage and excretion as those mechanisms neutralize the toxicity of metals and could then be considered as detoxification processes.

The presence of metals in granules has been evidenced in marine invertebrate cells by microanalytical techniques (Nott, 1991). These techniques have been currently used in the field of geology, mineralogy, petrology, paleontology, environmental atmospheric analysis, marine chemistry etc. Although their applications are increasing in material sciences, such as semiconductors, thin film, nanoparticles and surfaces (Van Cappellen, 2004), these techniques have been rarely used in the field of biological sciences even if it is currently used in medical science since 1995 and particularly in toxicology (George, 1993; Börjesson & Mattsson, 2004).

The aims of this chapter are to present the potential use of X-Ray spectroscopy as an analytical tool in biological systems and its possible applications (combined with ultrastructural studies) in the field of ecotoxicology, while highlighting its important contribution to understand how marine invertebrates cope with environmental metals.

2. Trace metal bioaccumulation in marine invertebrates

Metals are naturally present in seawater in very low concentrations (Bruland, 1983). Major metals (Sodium, Magnesium) are present in an order of mmol Kg^{-1}; minor metals (Lithium, Barium) in µmol to nmol Kg^{-1} and trace metals (Copper, Zinc, Mercury, Cadmium, Lead) in pmol Kg^{-1}. Metals are also present in sediments and in benthic biota (Bryan & Langston, 1992; Rainbow et al., 2011). Marine invertebrates are continuously exposed to variable concentrations of trace metals in seawater. There is a huge intra and inter-specific variability in metal accumulation among marine invertebrates. Metal accumulation occurs to different extent in individuals depending on the metal, its speciation, its bioavailability, and the considered species and biotic (body size, gender, molting, reproductive and developmental stage, and feeding habit…) and abiotic (temperature, salinity, pH…) factors (Marsden & Rainbow, 2004; Wang & Rainbow, 2008).

2.1 Background metal concentration

Metal concentrations in marine invertebrates collected from different natural areas show a considerable variation even in close phylogenetically related species (Table 1). As for essential metals, they are accumulated to fulfill metabolic requirement. Various proteins, enzymatic or not, require metals to be functional. The blood of marine invertebrates may contain respiratory pigments which bind to copper or iron. For example, annelids X-Ray microanalysis revealed the presence of Fe, Ca and small amounts of Zn. *Tubifex* hemoglobin also contained Cu and Pb (Rokosz & Vinogradov, 1982). The elemental mapping of several biological structures showed that different metals may also have a structural role as they can be constitutive of annelid jaws (Bryan & Gibbs, 1980; Gibbs & Bryan, 1980), crab cuticule (Schofield et al., 2009) or copepod exoskeleton (unpublished data). In fact, metals which are

essential for metabolic requirement can be theoretically estimated (Depledge, 1989; Pequegnat et al., 1969; White & Rainbow, 1985) and is necessary to identify these components when interpreting total essential metal concentration in invertebrate tissues or bodies or to identify a metal deficiency (Rainbow, 1993).

However, non essential metals such as silver or cadmium were also found in molluscs and crustaceans collected in non-polluted areas (Ballan-Dufrançais et al., 1982; Bustamante, 1998; Martoja et al., 1985; & Pétri, 1993). Their presence in animals could not be explained.

This illustrates the concept of metal background concentration which is considered as the typical metal concentration found in invertebrates collected from a habitat remote from anthropogenic inputs of metals (Rainbow, 1993). Evaluating background concentrations is necessary when invertebrates, collected from metal polluted areas, are used as biomonitors to know if metal concentrations have risen over a baseline in order to make realistic interpretations of the metal content in animals

2.2 Metal accumulation patterns

Aquatic invertebrates are able to cope with the pollution pressure of their environment due to the existence of different adaptive strategies which have been reviewed, for instance, by Mason and Jenkins (1995):

- they are able to limit the entrance of the contaminant into their body;
- they are able to balance uptake by an increased excretion, the total concentration in the organisms remaining constant;
- they are able to detoxify and store the metals which have entered the organism.

From and ecotoxicological point of view, and considering the fate and transport of metals in the marine environment, understanding metal uptake and excretion in invertebrates is important as these organisms may be exposed to very high levels of metals.

Experimental metal exposure of marine invertebrates, particularly well studied crustaceans, showed that these animals have developed different pattern of metal accumulation which vary within and between invertebrates depending on the metal and the species (Rainbow, 1998, 2002, 2007).

The first pattern, currently described in palaemonid decapod crustaceans, consists of a balance of the rate of zinc excretion to that of zinc uptake resulting in a regulation of this metal as its body concentration is maintained relatively constant (Rainbow, 1993, 1997; White & Rainbow, 1984). In many decapod species of crustaceans, it has been well established that essential metal concentrations remain steady over a large range of metal concentrations in their medium. This pattern has been described in numerous crustacean species not only for Zn but also for Cu (Amiard et al., 1987; Maranhaño et al., 1999; Rainbow, 1993, 1998; Sandler, 1984). Some exceptions have been mentioned such as the absence of Cu regulation in the freshwater shrimp *Macrobrachium malcomsonii* (Vijayram & Geraldine, 1996) or of Zn regulation in crabs *Uca annulipes* and *U. triangulis* (Uma & Prabhakara, 1989). In other classes of crustaceans (amphipods, copepods…), Zn and Cu regulation processes are generally absent (Amiard et al., 1987; Barka et al., 2010; Chen & Liu, 1987; Clason et al., 2003; Rainbow, 1988; Rainbow & White, 1989; Zauke et al., 1996) even if the amphipods *Allorchestes compressa* and *Hyatella azteca* appear to be able to control their internal metal content (Ahsanullah & Williams, 1991; Borgmann et al., 1993).

Taxon	Class	Species (geographical zone)	Cu	Zn	Ni	Cd	Hg	Reference
Crustaceans	Copepod	*Tigriopus brevicornis* (North Atlantic coast)	9	66	9.3	0.17	0.34	(Barka *et al.*, 2010)
		Calanus hyperboreus (Greenland Sea)	4.7 5.6	80 104	11.4	4.0 0.75	0.31	Pohl (1992) Ritterhoff & Zauke (1997b)
		Euchaeta barbata (Greenland Sea)	4.5	225	3.9	0.16	0.27	Ritterhoff & Zauke (1997b)
		Acartia clausi (Méditerranean)	55	1270		0.6		Zarifopoulos & Grimaris (1977)
		Metridia longa (Greenland Sea)	7.5	351	19.7	0.71	0,68	Ritterhoff & Zauke (1997b)
	Isopod	*Ceratoserolis trilobitoides* (Gould Bay) (Elephant island)	38 46	40 40				De Nicola *et al.*, 1993
		Idotea baltica males (Bay of Naples, Italy)		292		0.68		
	Barnacle	*Capitulum mitella* (Cape d'Aguilar, Honk Kong)	29.2					Phillips & Rainbow, 1988
		Amphibalanus amphitrite Algeciras Bay (Spain)	94-225	852-4170	2.7-26			Morillo *et al.*, 2008
	Amphipod	*Platorchestia platensis* Hoi Ha Wan, Honk Kong Cape d'Aguilar, Honk Kong		199 193		fl		Rainbow *et al.*, 1989
		Orchestia gammarellus Whithorn, Scotland Girvan, Scotland	132 66					Rainbow *et al.*, 1989
	Branchiopod	*Daphnia magna* (Lab culture, from Huo Qi Ying Bridge, China)	230					Fan *et al.*, 2011
	Decapod	*Systellaspis debilis* (Deep Sea North East Altlantic)				12		Ridout *et al.*, 1989
Molluscs	Bivalve	*Mizuhopecten yessoensis* (Peter the Great Bay, Japan) Digestive gland Kidney				142 11.6		Lukyanova *et al.*, 1993
		Mizuhopecten magellanicus (US Atlantic coast) Digestive gland Kidney				94 62.6		Uthi & Chou, 1987

Table 1. Mean metal concentration in marine invertebrates collected from natural marine areas (µg.g^{-1} dry weight of total body or organ when indicated).

In fact, metal accumulation can be modelled at a given exposure by a straight line, the slope of each line representing the accumulation rate (µg Metal g^{-1} d^{-1}) at that exposure. By plotting these rates against the exposure concentration, we can calculate the slope of this line which is the metal accumulation index (µg g^{-1} d^{-1} per µg L^{-1}, or L g^{-1} d^{-1}) (according to Rainbow & White, 1989). From this index, it is possible to calculate the expected accumulated concentration at any exposure concentration at any exposure time. Sometimes, in the case of the lack of significant change in animal body metal concentration over controls, the calculation of accumulation index helps to decide whether a true metal regulation is occurring or not (when the index is very low).

When metal exposure concentration is high, regulation breakdowns and the excretion rate fails to match the uptake rate, resulting in a net increase in body metal. Therefore, metals may remain in metabolically available form, without detoxification, thus exerting a toxic effect after metabolic requirement is fulfilled. Another possible route for metal is to remain in detoxified form such as mineralized granules. In both cases, metal excretion may occur in a different form, metabolically available and detoxified, depending on the considered component.

Amphipods have a low zinc uptake rate with no significant excretion, resulting in a weak accumulation of this metal (Rainbow & White, 1989). Conversely, barnacles (*Elminius modestus*) (Rainbow, 1987; Rainbow & White, 1989) strongly accumulate zinc, in detoxified granules, with no excretion. Net accumulation strategies were reported for crustaceans from polar regions with respect to Pb and Cu in Arctic zooplankton *Calanus hyperboreus*, *Calanus finmarchicus*, *Metridia longa*, *Themisto abyssorum* (Ritterhoff & Zauke, 1997a,d) and to Co, Cu, Ni, Pb, and Zn in the Antarctic copepods *Calanoides acutus* (Kahle & Zauke, 2002a) and *Metridia gerlachei* (Kahle & Zauke, 2002b).

3. Localization of metals in marine invertebrates

Invertebrates possess several mineral bioaccumulation structures in various organs. Bioaccumulation structures were first revealed empirically or with imperfect histochemical techniques (Ballan-Dufrançais, 2002). In 1815, Brugnatelli observed "shalky structures" in malpighian tubules (involved in mineral homeostasis) of silk worms using microchemical techniques. Ever since, literature reported the existence of metal-containing granules in invertebrates, whether these were exposed or not to trace metals. Numerous studies have shown that metal-containing granules occur in many different aquatic phyla (from protozoa to arthropoda) (Ballan-Dufrançais, 1975; Barka, 2007; Mason & Jenkins, 1995; Moore, 1979; Nassiri et al., 2000; Simkiss & Mason, 1983; Nott, 1991; Viarengo & Nott, 1993; Vogt & Quinitio, 1994).

3.1 Bioaccumulation structures in invertebrates: size, aspect, composition and role

Due to their chemical composition (Masala et al., 2002) and their different stages of development, metal granules are highly variable in form, composition, size, aspect and location (Brown, 1982; Mason & Jenkins, 1995; Roesijadi & Robinson, 1994). Sectioned granules show different types of internal structure. It may be homogeneous (Walker, 1977), diffuse (Mason et al., 1984), conglomerated (Isheii et al., 1986; Reid & Brand, 1989), crystalline (Masala et al., 2002) or arranged in concentric strata (Ballan-Dufrançais, 1975; Hopkin & Nott, 1979; Pigino et al., 2006). Their size is about less than a µm to several µm.

Depending on their elemental composition, cytochemical and chemical characteristics granules have been classified into three types (Brown, 1982 and references cited therein):

i. Fe-containing granules in which Fe may be present in ferritin (Brown, 1982; Hopkin, 1989; Viarengo & Nott, 1993). They are generally amorphous but crystalline structures have already been observed in copepods, , isopods, molluscs and amphipods (Barka, 2000; Jones et al., 1969; Quintana et al., 1987; Moore, 1979 respectively) (Fig. 1);

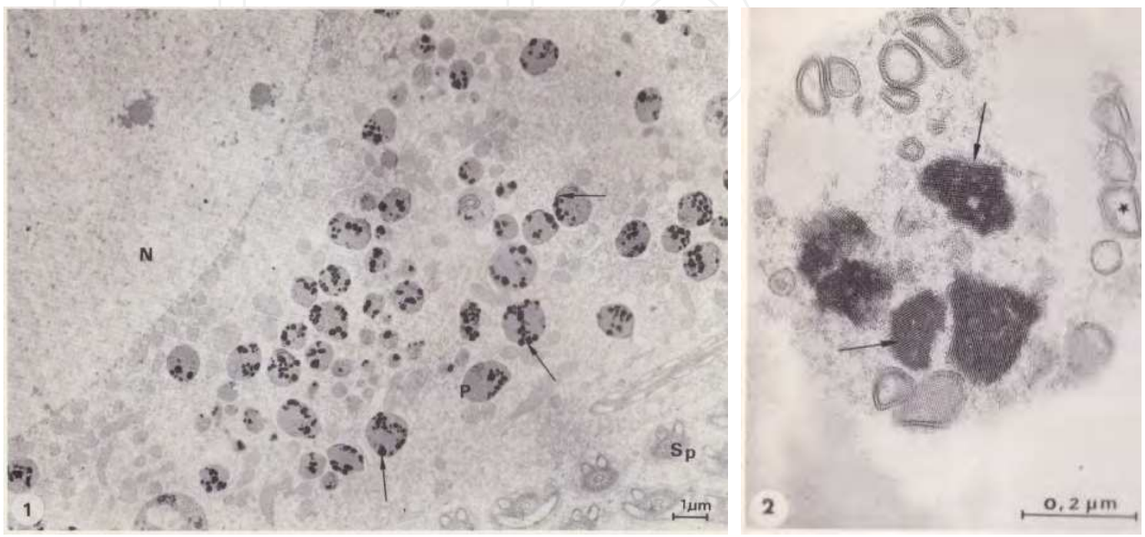

Fig. 1. (1) Ultrastructure of the ovotestis of *Planorbarius*, at the last stage of vitellogenesis.The platelets (P) of the oocyte contain electron-dense of ferritin (arrows), N, nucleus, Sp, cross section of the sperm tails. Unstained osmicated ultrathin sections. X 7800. (2) Detail of a snail platelet. Natural occurence of crytalline form of ferritin (arrows). Unstained non-osmicated section. X 50 000 (after Quintana et al., 1987).

ii. Cu-containing granules also contain S and small amounts of Ca, K, P, Cl and Fe. Cu-rich granules have been observed in the digestive tract of many crustaceans living in pristine areas (Al-Mohanna & Nott, 1987; Barka, 2007; Hopkin & Nott, 1979; Nassiri et al., 2000; Walker, 1977). Vogt and Quinitio (1994) found Cu-rich granules in hepatocytes of *Penaeus monodon*. Weeks (1992) and Nassiri et al. (2000) have also observed Cu-rich granules in the ventral caeca of the Amphipod *Orchestia gammarellus.* The same type of granule was found in the midgut of control and Cu-exposed marine isopods (Tupper et al., 2000) and in the digestive cells (sometimes in the apex) of copepods (Barka, 2007)(Fig.2a).

Ag was also found in Cu-S rich granules in control and Ag exposed crustaceans, (Barka 2007; Chou et al., 1998)(Fig.2b).. This may be explained by the fact that both metals, belonging to the group I.b of the transition elements, display many similar chemical properties as suggested by Hogstrand and Wood (1998) and Bury et al. (2003). Ag is probably naturally bioaccumulated which may indicate that these granules are not simply a sink for essential metals. Silver is often found in the aquatic environment in the 1-100 $ng.l^{-1}$ range and known to avidly bind to inorganic and organic ligand (Kramer et al., 2002). It is interesting to notice that larger Cu-containing granules were found in animals inhabiting Cu-contaminated environments (Barka, 2007; Brown, 1982). There is a great variability in the morphology

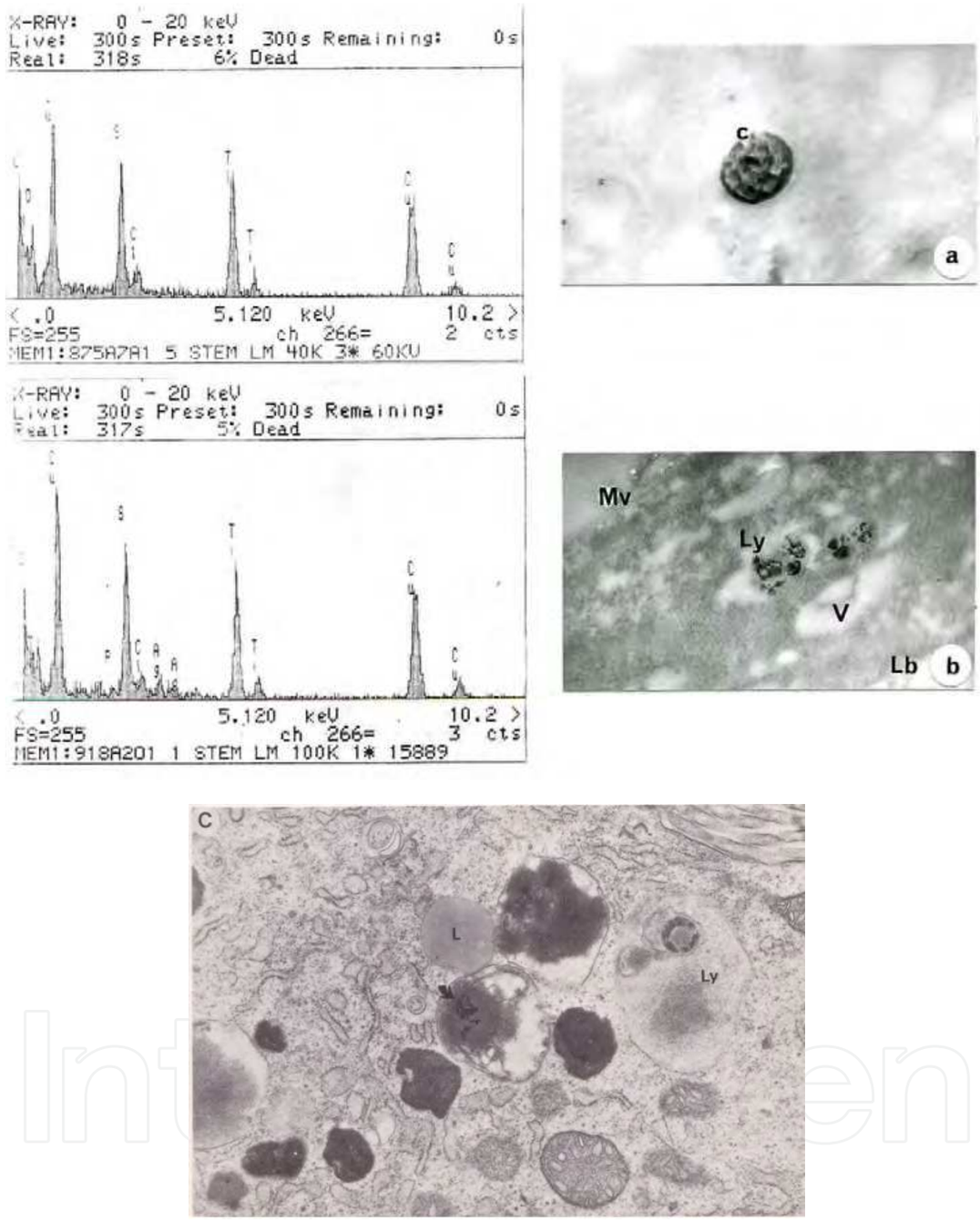

Fig. 2. (a,b) X-Ray microanalysis in the digestive epithelium of a copepod collected from an clean area. (a) Occurrence of Cu and S in a compact granule (c). x 29 000 (b) occurrence of Cu, P, S and Ag in the fine granular material of a lysosome (Ly), (Lb) basal lamina, (Mv) microvilli, (V) vacuole. x 19 000 (after Barka, 2007). (c) Bivalve (*Abra alba*) collected from Morlaix Bay, France. Ultrastructural aspect of a digestive cell, showing voluminous lysosomes (Ly) with vacuoles containing a flocculent material and electron dense particles (arrow). L, lipidic inclusion. Microanalysis revealed the presence of Si, Fe, Cu, P, Zn and S. X 26 000 (after Martoja et al, 1988).

of the different Cu-containing granules. However, they are all usually homogeneous, spherical structures with no concentric structuring;

iii. Ca-containing granules which may be of two types based on their composition:
   - Ca carbonates granules, of a high purity are found in arthropod and gastropod conjunctive tissues as they are involved in Ca storage. They play no role in heavy metal physiology - although Pb may substitute to Ca2+ (George, 1982). They are generally big (several µm) and have a typical aspect, with concentric strata. These particular structures, also called calcospherites or spherocrystals, have been found in the digestive cells of different terrestrial and aquatic arthropods (Ballan-Dufrançais, 2002; Barka, 2007; Corrêa et al., 2002; Defaye et al., 1985; Durfort, 1981) (Fig. 3);

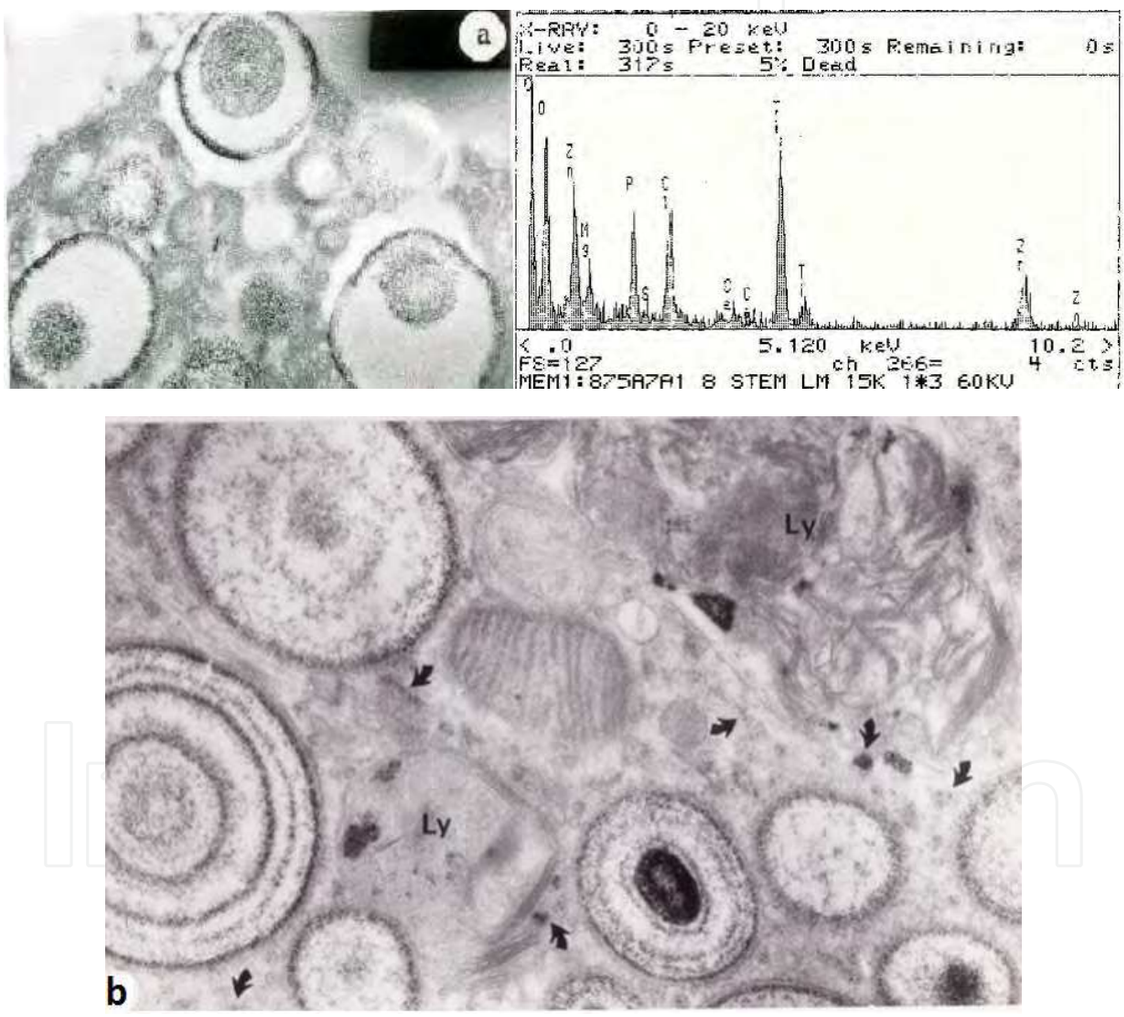

Fig. 3. (a) Occurrence of Zn, Mg, P, S and Ca in spherocristals (2–2.5 lm) in digestive epithelium of a benthic copepod collected from the North Atlantic french coast. Note the dark and light concentric strata. x ·14,000 (after Barka, 2007) (b) Coackroach (*Blatella*) exposed to CH_3HgCl through diet for 4 days. Ultrastructural aspect of ileum. Note the abundance of spherocristals. Microgranules of Hg, Zn and Cu (arrows) are present in lysosomes (Ly) and in the endoplasmic reticulum. x 40 000 (after Jeantet, 1981).

- Ca phosphate granules in which Ca is present together with Mg, but also other metals such as Al, Ag, Ba, Co, Fe, Mn, Pb, Sn et Zn; and possibly Cu, Cd, Cr, Hg and Ni (Roesijadi & Robinson, 1994). They are intra and inter-specifically highly variable in aspect and composition which depend on environmental metal concentration (George, 1982; Sullivan et al., 1988) (Fig. 4). They are generally small (less than 1 µm) although huge ones (10-15 µm) have been found in marine bivalves (Marsh & Sass, 1985).

The precise role of these particular granules still remains unclear but the literature reported two major functions: regulation of storage and release of Ca^{2+} and detoxification (Corrêa et al., 2002; Simkiss & Wilbur, 1989; Simmons et al., 1996). These granules may act as a calcium reservoir serving as a source of calcium for metabolic needs, maintaining Ca homeostasis, exosqueleton replacement after moulting as suggested for crabs (Becker et al., 1974) or building material of skeletal tissue in molluscs (Jacob et al., 2011). However, several authors proposed a metal detoxification role for Ca granules (Mason & Simkiss, 1982; Simkiss, 1981). Furthermore, calcium granules, whose formation being controlled by factors associated with ion fluxes as evidenced by Masala et al. (2002), could act as a passive sink for toxic metals during calcium precipitation.

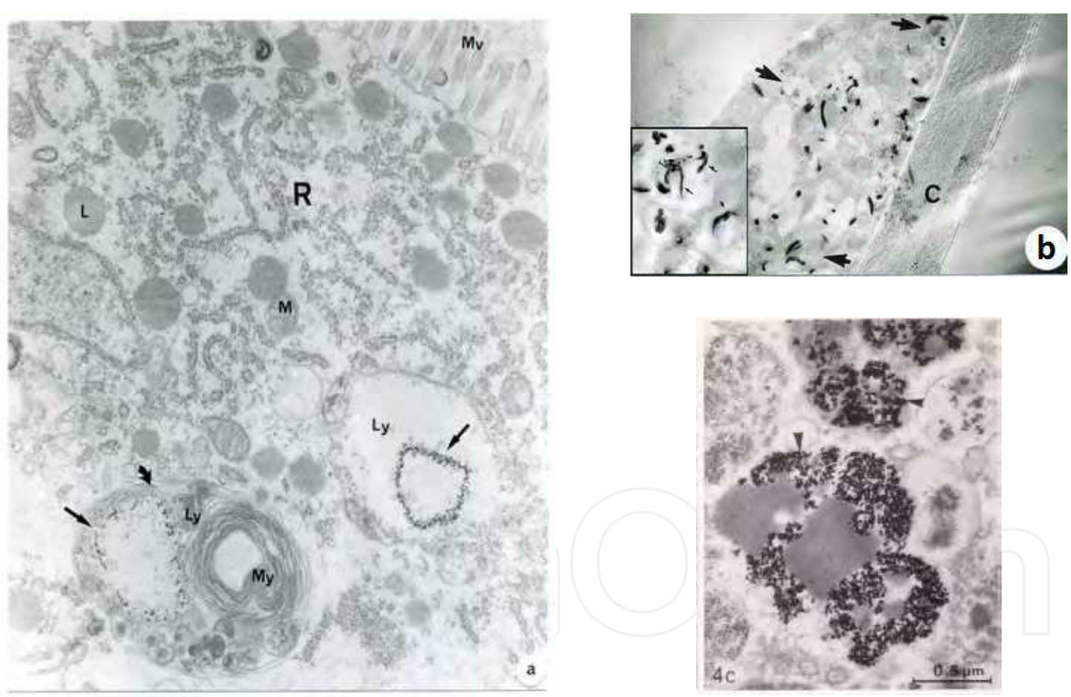

Fig. 4.(a) Ultrastructural aspect of a digestive epithelium cell of a benthic copepod exposed to Hg. Occurrence of dense granules (arrows) in lysosomes (Ly), L, lipids, Lu, lumen, M, mitochondria, Mv, microvilli, My, myelin. x 15 000. (b) Occurrence of Cu, S and Ag in needles (arrows) in lysosomes of the integument of Ag exposed copepods. x 19 000 and x 72 000 for detail (after Barka, 2007) (c) Scallop (*Pecten maximus*) collected from the Channel coast. The lysosomes of the digestive gland contain dense granules (arrows) in which numerous elements are detected (Cd, Ag, Mo, Br, Zn, Cu, Fe, Ca, S, Si, Al). x 48 600 (after Ballan-Dufrançais et al., 1985).

3.2 Localization of metals in tissues and cells

Depending on the species, metal granules have been observed in different tissues. In gastropod mollusks, granules have been found in different tissues such as the digestive gland (Mason & Nott, 1981), gills (Marigomez et al., 1990; Mason et al., 1984; Simkiss & Mason, 1983), gonads (Quintana et al., 1987) and conjunctive tissue (Bouquegneau & Martoja, 1982; Martoja et al., 1980; Martoja et al., , 1985;). In crustaceans, these structures have been found in the digestive tract (Bernard & Lane, 1961; Guary & Négrel, 1981; Hopkin & Nott, 1979, 1980; Vogt & Quinitio, 1994) and in the cuticular hypodermic parenchyme (Barka 2007; White, 1978 as cited in Mason & Jenkins, 1995). Actually, metal granules are present in almost every invertebrate tissues particularly in those involved in digestion and/or excretion (Mason & Jenkins, 1995).

At the cellular level, metal granules occur in the cytoplasm of cells and more specifically in the basal lamina, or cell apex and within specific organelles such as endoplasmic reticulum (ER) Golgi complex, Golgi vesicles and even in nucleus. Granules can also occur in the paracellular space or in the digestive lumen.

3.3 Origin and formation of metal mineralized structures

Metals entering cells may have different possible intracellular routes which have been summarized in Fig. 5.

X-Ray microanalysis associated with transmission electron microscopy showed that metal granules were found in vesicles originated from the lysosomal system. Granule elemental composition showed the presence of different minerals associated with C, S, O which have an organic origin. Cu-granules elemental composition showed the coexistence of Cu and S suggesting that sulphur may be the chelating agent. Heavy metals, especially class"b" metals, tend to form stable complexes with the sulphydryl residues of amino acids and polypeptides. Metallothionein, with its high cysteine content, and other metal-binding proteins, are sulphur donors to which Cu can bind. Degraded proteins are finally engulfed into lysosomes where they are digested. This pathway has been extensively described in the literature and explains the presence of metals in lysosomes (Mason & Jenkins, 1995; Viarengo & Nott, 1993). As some metals can induce metallothioneins, it is reasonable to think that the presence of Cu (associated with S), Hg or Cd in lysosomes results from the breakdown products of metal-thionein that might be turning over at high rates in metal exposed animal. However, Cu-granules may also be a consequence of respiratory pigment catabolism as suggested by Moore & Rainbow (1992) in amphipods although, Cu in lysosomal granules may have entered the organelle through an ATPase transport system localized on the lysosomal membrane as found in lobster hepatopancreatic cells (Chavez-Crooker et al., 2003).

In Ca phosphate granules, lipofuscin may be the organic matrix for metal incorporation within lysosomes as it has been shown for Cd and Zn which passively adsorb onto lipofuscin (George, 1983b). Over time, some lysosomes fit together and/or evolve into voluminous heterolysosomes in which cytoplasmic material is progressively mineralized. Heterolysosomes then get smaller, more condensed and filled with indigestible products and finally they mature into residual bodies (also called tertiary lysosomes) in which enzyme activities are exhausted. The great variability of granules may be due to these different development stages.

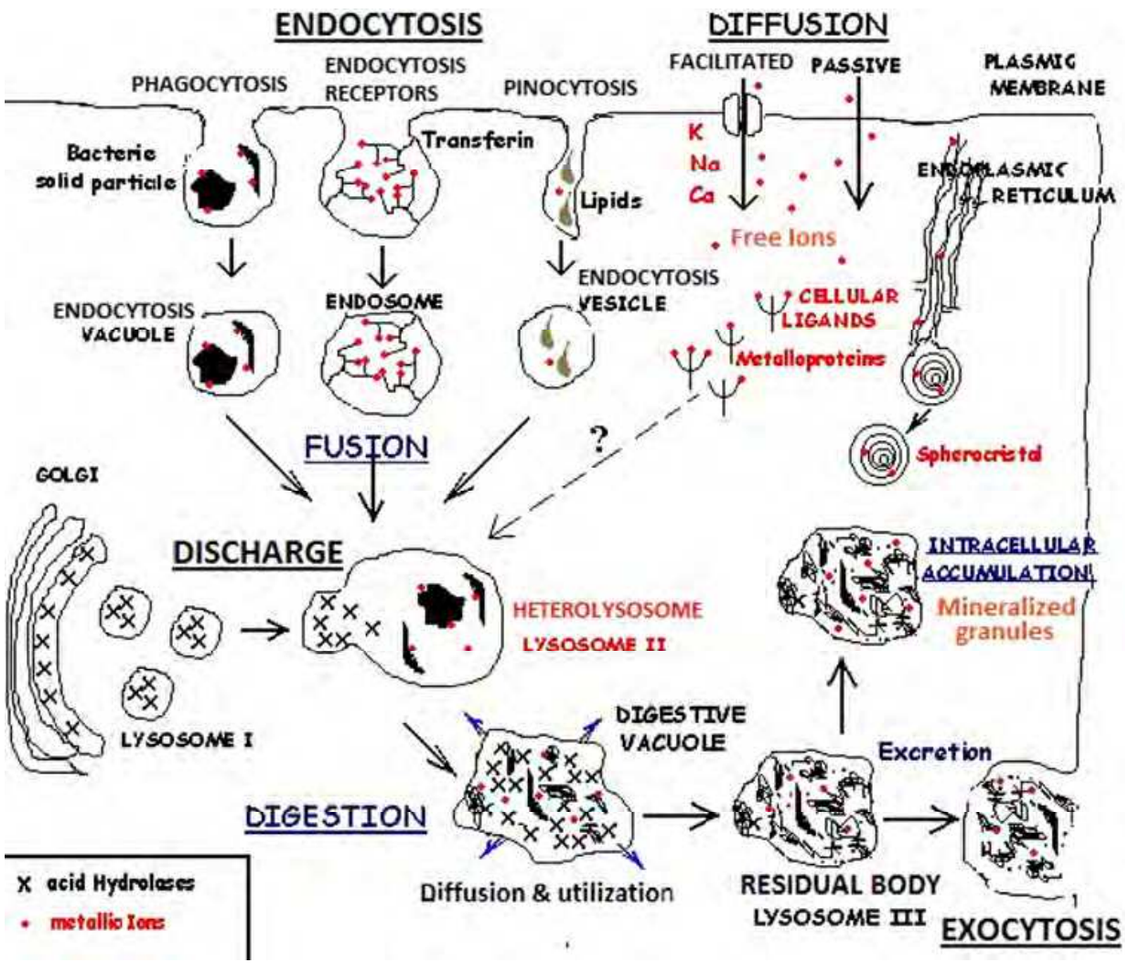

Fig. 5. Possible routes of metals in cells.

The structure of calcospherites granules is concentric and are thought to have a different origin. They may be derived from the endoplasmic reticulum (Ballan-Dufrançais, 2002; Cheung & Marshall, 1973; Corrêa et al., 2002). Studies based on cytochemical assays found a phosphatase activity associated to calcospherites, which strengthened the hypothesis that the reticulum is involved in the formation of these structures (Corrêa et al., 2002; Loret & Devos, 1995).

The mechanism of granule intra and extracellular growth was shown to be different from that leading to their initial formation. Metal accretion onto granules has been observed *in vivo* and *in vitro* (George, 1983a, b; Simkiss, 1981). Concentric strata have been interpreted as alternated period of accretion and resorption (Carmichael et al., 1979). In lysosomes, pH variations facilitate metal adsorption onto granule surfaces in sites where Ca dissociated during dissolution (Taylor et al., 1988) or following Mg^{2+} loss (Nott & Nicolaidou, 1989b).

Overall, the precise elemental composition of granules using microanalytical techniques would greatly improve our understanding of the origin and the formation process of metal granules and, indirectly, metal handling mechanisms in invertebrates.

3.4 Technical considerations: ultrastructure and microanalysis

3.4.1 Technique principle

Energy dispersive X-Ray Microanalysis in Transmission Electronic Microscopy (EDMA-TEM) consists of an analytical TEMs which is equipped with detectors for sample analysis. It works similarly to TEM: the sample is bombarded with an electron beam, emitting x-rays at wavelengths characteristic to the elements being analyzed. The elemental composition and abundance may be determined.

In the following section, technical considerations concerning Energy dispersive X-Ray Microanalysis connected with Transmission Electronic Microscopy are presented.

3.4.2 Sample preparation before analysis

The specimen-preparation technique influences the elemental data generated by granule microanalysis. Animal or tissue glutaraldehyde fixation was found to remove chemical elements such as Cd, as shown by the study of Nott and Langston (1989) who found Cd in granules in Cd-exposed molluscs when they used a cryo-preparation method instead of a chemical one. The original work of Vesk and Byrne (1999) on mussels, showed that chemical fixation and sectioning influenced elemental distribution and concentration of metals in granules. Furthermore, the conservation of tissues in a basic medium (sodium cacodylate) prior to dehydratation do not favour the preservation of cytoplasmic bioaccumulation structures (Ballan-Dufrançais, 1975).

Resin embedding composition must be known. In general, resins contain chloride and an important Cl peak appears among those of other elements in microanalysis spectra.

As for grids, on which embedded specimens are deposited, they should be made of a metal that is not suspected to be found in biological tissues. Titanium grids rather than copper or gold grids are preferable.

Sample section thickness must be chosen to fit the best resolution. TEM samples should be thin enough to be beam transparent and resolve morphological detail using the transmitted electron signal. X-Ray spatial resolution depends on thickness sections because the energy beam, commonly between 100kV - 400kV in the analytical TEM, should not be destructive to samples. However, X-Ray generation is low for thin samples as the ionization volume is small but when analyzing thicker samples, absorption corrections (material density and thickness) should be made to minimize electron scattering.

3.4.3 Advantages and inconvenient

X-ray microanalysis of thin specimens in the Transmission Electron Microscope (TEM) offers nano-scale information on the chemistry of materials. X-Ray microanalysis is a non destructive, sensitive, accurate and precise technique. It is qualitative and semi-quantitative. However, quantitative results can be obtained from the relative x-ray counts at the characteristic energy levels for the sample constituents. The spectra obtained are easy to interpret.

The inconvenient, besides the fact that the instrument is expensive and requires technical skills, is that X-ray signals (peaks) for some elements may interfere with those for non-

biological elements (grid, resin…), which make the interpretation difficult. The sensitivity threshold of the technique can also be considered as a limiting factor. When metal concentration is too low, it remains undetectable. Furthermore, the thickness of the non-osmicated specimen section does not allow a proper visualization of the elements located, in the tissue or cell. Generally, when TEM is used, even if metal granules are visualized at the ultrastructural level, these structures do not necessarily match microanalysis data. This is due to the fact that X-Ray microanalysis and TEM observation require different preparation for each specimen (non-osmicated/thin section versus osmicated/ultrathin section)

4. Ecotoxicological significance of metal bioaccumulation

Crustaceans exoskeleton, molluscs shell etc are likely to come into contact with the surrounding metals and to bind to them before these are absorbed. When ingested (via food or water), metals entering the gut or passing through permeable membranes (e.g. gills), are absorbed in soft tissues where they are stored or excreted. Metals accumulated in soft tissues are likely to be present in two phases: dissolved in the cytoplasm, mainly as complexes with metal-binding proteins, or incorporated in metal-rich granules (Mason & Jenkins, 1995). All metals have the potential to be deleterious to cellular mechanisms, even though some of them are essential to normal metabolic processes. The metal accumulation levels, and consequently the potential toxicity, cannot be predicted only on the basis of concentration in water or in tissues (Simon et al., 2011). It is the only bioavailable fraction that is potentially toxic and of ecotoxicological relevance (Rainbow 1998, 2002, 2006). Therefore, toxicity cannot be attributed to the global body/organ metal burden but depends on the physico-chemical form in which the metal is present in organisms. From an ecotoxicological perspective/point of view, partitioning of metals (soluble versus insoluble) is important as metals, within these two fractions, are involved in different metal handling mechanisms and may have different mobility from prey to predator species through trophic transfer.

4.1 Metal detoxification

Lysosomal systems are ubiquitous in all invertebrate cells, particularly well developed in digestive and excretory cells. Furthermore, the occurrence of increasing lysosomes in metal exposed animals, compared to controls, leads to think that these organelles are involved in metal cellular responses. Two major metal storage pathways have been reported in invertebrates, namely, cytosolic metalloproteins and/or the lysosomal system (Amiard et al., 2006; Marigomez et al., 2002; Mason & Jenkins, 1995; Rainbow, 2006; Viarengo & Nott, 1993). The complexation of metals by metallothioneins (MTs), non-enzymatic metalloproteins, is one mechanism used by the invertebrate cell to prevent the activation of toxic metals in the cytoplasm (Viarengo et al., 1987). Consequently, in addition to their role in homeostasis, MTs have often been considered as detoxification proteins, although this latter function is still open to debate (Cosson et al., 1991).

The great ability of invertebrates to accumulate metals in granules probably enables these animals to cope with the presence of (potentially dangerous levels of) metals in their surroundings and to be protected from their toxicity (Brown, 1982; Desouky, 2006; Masala et al., 2004; Mason & Jenkins, 1995; Mason & Nott, 1981; Simkiss & Mason, 1983; Stegeman et al., 1992). In some species, these concretions remain in the cell for a relatively long period of time before they are excreted (Fowler, 1987; Sullivan et al., 1988; Nott, 1991). Actually

granules were found in the digestive lumen of the crab *Callinectes sapidus* (Becker et al., 1974; Guary & Negrel, 1981) and of the copepod *Tigriopus brevicornis* (Barka, 2007) suggesting that metal detoxification occurs (if the release of granules into the lumen is followed by an excretion in the gut)(Fig. 6). In fact, the dissolution of mineralized granules may occur along the digestive tract as showed in a terrestrial arthropod by Krueger et al. (1987). The decrease of the pH of lumen contents from distal to proximal regions of the digestive tract appears to be a major effector of granule dissolution. These authors also studied the effect of pH on the dissolution of isolated granules *in vitro*. They showed that the release of calcium, phosphorus, and magnesium from granules increased exponentially as the pH of the bathing medium was decreased. Loss of structural integrity of the granules accompanied mineral release and also increased as pH of the bathing medium was lowered *in vitro*. This study suggests that, during gut transit, metals can be released from granules for both prey and predators.

The significant increase in the number of granules upon exposure to metals and the fact that they are generally insoluble and associated with digestive or excretory tissues (i.e., digestive gland, hepatopancreas, kidney, among others) and, consequently, can be excreted, may give further evidence for their role in detoxification (Desouky, 2006; Marigomez et al., 2002; Pullen & Rainbow, 1991; Simkiss, 1976; Simkiss & Taylor, 1989).

4.2 Biomagnification and trophic transfer

Many authors have suggested that the subcellular distribution of metals is critical for metal assimilation in predators (Amiard-Triquet et al., 1993; Nott & Nicolaidou, 1990; Reinfelder & Fisher, 1994; Wang & Rainbow, 2000). If we consider invertebrates as prey species, it is important to know how metals are distributed in animals because physicochemical characteristics of these contaminants (in prey species) control their mobility and thus their transfer to the next trophic level. Furthermore, accumulated metal in prey that is trophically available to one predator is not necessarily equally trophically available to another predator feeding on the same prey, given the variability between invertebrate digestive systems (Rainbow & Smith, 2010). The literature provides evidence that the assimilation of trace elements in predators is governed by the cytoplasmic distribution in prey (Ni et al., 2000; Nott & Nicolaidou, 1993; Reinfelder & Fisher, 1991, 1994; Wallace & Lopez, 1996 , 1997; Wallace et al., 1998;).

Metals, mainly present in the fraction operationally described as the soluble fraction, are probably more able to be transferred along a food chain than those in insoluble fractions. Consequently, detoxification processes based on metallothioneins presumably would not protect the consumer since these proteins can be degraded, in predator gut, during digestion. Incorporation of metals into detoxified granules may lead to a ''transfer of metal detoxification along marine food chains'' since such inorganic compounds may not be assimilated during gut passage in the predators. The fact that metals can strongly bind to pre-existing granules, should guaranty a minimal remobilization. Consequently, metal incorporation, in detoxified form, within granules should reduce metal toxicity to the next trophic level although Khan et al., (2010) showed that cadmium bound to metal rich granules from an amphipod caused oxidative damage in the gut of its predator. These findings suggest that granules, largely considered as having limited bioavailability, may as also be toxic to some extent.

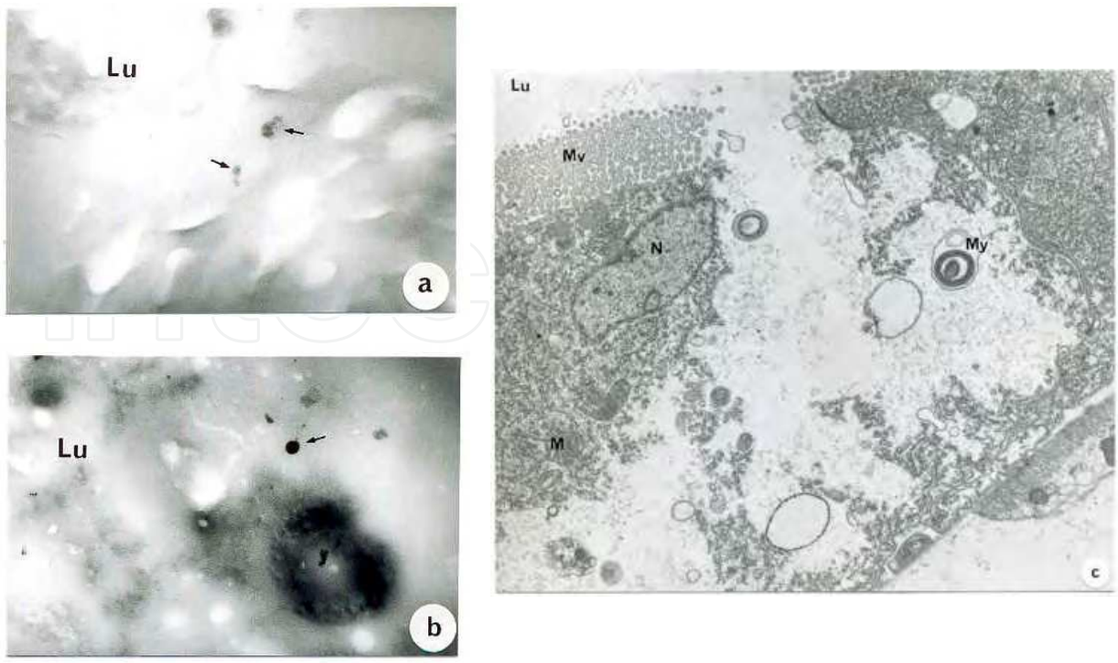

Fig. 6. Metal containing granules in the luminal tube (Lu) of a benthic copepod.
(a) Occurrence of Cu, P, S and Ag in granules (arrows). x ·7 200. (b) Occurrence of Fe, Cu, Zn, P, S and Ca in a granule (arrow). x ·29 000. (c) Ultrastructure of a digestive cell extruding its contents into the lumen. Note the presence of a spherocristal, a lysosome which membrane is electron-dense and another structure with myelin (My). M, mitochondria, Mv, microvilli, N, nucleus. x ·7 000 (after Barka, 2007).

Once in the gut, the reabsorption of the ions after the granule chemical and enzymatic dissolution may also occur, as suggested by Becker et al. (1974). Nott and Nicolaidou (1993), fed a carnivorous gastropod (*Nassarius reticulatus*) with another gastropod (*Littorina littorea*) hepatopancreas which contained intracellular phosphate granules. These granules passed through the predator gut. X-Ray microanalysis revealed that granules in the predator faecal pellets retained about 50% of the original zinc and 33% of the original manganese and showed that a metal bioreduction occurred along the molluscan food chain suggesting, however, that part of the metals were bioavailable for the predator. X-ray microanalysis revealed also that, in phosphate granules, an increase in the quantity of metal is associated with a reduction in the magnesium/calcium ratio. Metals displace magnesium from the phosphate granule to the carbonate granule (Nott & Nicolaidou, 1989b; Schönborn et al., 2001).

The ability of granules to dissolve or not in the predator lumen may have a consequence not only on the trophic transfer of metals but also on the subsequent handling of toxic metals in predators.

5. Conclusion

Various examples of trace metal bioaccumulation structures, mostly among marine invertebrates, were illustrated and discussed in this chapter. They revealed the potential of

X-Ray analysis in understanding cellular mechanisms underneath metal bioaccumulation, granule composition, origin, growth and trophic transfer as well as metal detoxification adaptive strategies. For its accuracy and precision, X-Ray microanalysis is a powerful technique which allows to resolve several questions about metal handling. In fact, the way marine animals cope with the presence of metals in their surrounding is still open to investigation. Hence, and in order to enable the monitoring of metals in the environment, granule microanalysis may be proposed as a new approach (Dimitriadis & Papadaki, 2004).

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This is a good book on upcoming areas of Ecotoxicology. The first chapter describes genotoxicity of heavy metals in plants. The second chapter offer views on chromatographic methodologies for the estimation of mycotoxin. Chapter three is on effects of xenobiotics on benthic assemblages in different habitats of Australia. Laboratory findings of genotoxins on small mammals are presented in chapter four. The fifth chapter describes bioindicators of soil quality and assessment of pesticides used in chemical seed treatments. European regulation REACH in marine ecotoxicology is described in chapter six. X-ray spectroscopic analysis for trace metal in invertebrates is presented in chapter seven. The last chapter is on alternative animal model for toxicity testing. In conclusion, this book is an excellent and well organized collection of up dated information on Ecotoxicology. The data presented in it might be a good starting point to develop research in the field of ECOTOXICOLOGY.

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