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Comparative histopathological alterations in the digestive gland of marine bivalves exposed to Cu and Cd.

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SUMMARY

This study compares the histopathological alterations in the digestive gland cells of mussels, *Mytilus galloprovincialis* and clams, *Ruditapes phillipinarum* following exposure to copper and cadmium. The results show degenerative processes undergone in the digestive gland ranging from inflammatory responses to extreme vacuolation, particularly in Cd-exposed individuals. Unsatured neutral lipids tend to accumulate in pathologically enlarged lysosomes of the homogeneous-type or heterogeneous-type depending of the species and of metal. Lipofucsins containing granules were mainly found in Cu-exposed mussels and Cd-exposed clams. No granules were detected in Cd-mussels. The comparison of the methods indicate that paraffin sections are also a suitable material for the localization of lipofucsins.

INTRODUCTION

Bivalves can accumulate and tolerate very high levels of heavy metals, such Hg, Cd, Zn, Cu, Pb, etc., without apparent signs of toxicity (Engel and Flower, 1979; Janssen and Scoltz, 1979; Denton and Burdon-Jones, 1981; Bolognani-Fantin *et al.*, 1982; Mance, 1987; Enserink *et al.*, 1991). As such, they have evolved various detoxification strategies (Bryan, 1979; Coombs, 1980) in order to maintain low levels of heavy metals "free" cations in the cell (Viarengo, 1989). Partial regulator organisms incorporate metals bound to lipid peroxidation products into insoluble granules (George, 1982, 1983 a and b) and/or store metals in a soluble non-toxic form bound to cytosolic proteins (Noel-Lambot, 1976; George *et al.*, 1979: Viarengo

et al., 1984; Pavicic *et al.*, 1987) or to insoluble polymerized proteins in lysosomes and residual bodies (Viarengo *et al.*, 1985) mainly found in the digestive gland or kidney (Simkiss and Mason, 1983; Moore, 1985; Phillips and Rainbow, 1989). In some cases, the macrophage system plays a major role in the bioaccumulation and depuration of metals (George *et al.*, 1978; Yevich, 1980; Thompson *et al.*, 1985; Martoja *et al.*, 1988).

Different accumulation and excretion patterns (Brooks and Rumbsky, 1965; Janssen and Scholz, 1979; George, 1980; Eisler, 1981; Viarengo, 1985, 1989; Langston and Zhou, 1987; Langston *et al.*, 1989), physiological effects (Engel and Fowler, 1979; Akberali and Trueman, 1985; Kluytmans *et al.*, 1988; Redpath and Davenport, 1988) and histological alterations (Sunila, 1986; Herwig *et al.*, 1989) have been reported among different bivalve species (mussels, oysters, clams) environmentally- or experimentally-exposed to cadmium and copper.

From bioaccumultion and toxicological studies of heavy metals in bivalves, Denton *et al.* (1981) showed that *Saccostrea echinata* has a greater affinity for mercury than cadmium or lead. *Ruditapes decussatus* is highly specific to cadmium (Henry *et al.*, 1984). Vincente *et al.* (1988) investigated the effects of cadmium at the electronic level. However, no histochemical study has been made so far on the comparative effects of cadmium and copper in this genus.

This study compares, in mussels and clams, the histopathological alterations and lysosomal responses, testing alternative histochemical techniques for lipofucsins induced by exposure to cadmium and copper.

MATERIALS AND METHODS

Pre-acclimatized adult mussels, *Mytilus galloprovincialis* (Lamarck) and clams, *Ruditapes phillipinarum* (Adams and Reeve) (25 specimens in each tank) were exposed to either $CdCl_2$ or $CuSo_4$ (5ppm, 5 days, 20°C). Untreated specimens (25) were used as controls. The water and contaminants were changed daily and no food was added during the whole experiment. All assays were conducted in 15 litre tanks at constant temperature and salinity (20°C and 36%). The concentration of O_2 varied between 6.5 and 8.9 mg/l and the pH between 7.2 and 7.5. A high dose level of metal (5ppm) was chosen to impact the organism strongly, so that the histochemical methods could be tested for assaying pollution effects.

Buffered formaldehyde (pH 7.2)-fixed tissue from untreated and treated specimens was dehydrated and embedded in paraffin. Section of 5 μm were stained with Haematoxylin-eosin and Haematoxylin-V.O.F. (Gutierrez, 1967) to examine the histomorphological alterations.

The lipid content from the digestive gland cells was determined on cryostat sections (7 μm) with Oil Red O (ORO) (0.5% ORO in 60% isopropanol). Aqueous Nile Blue (1% Nile Blue Sulphate in 5% H_2SO_4 at pH 7.2) was applied to distinguish unsatured neutral lipids (in pink) from fatty acids (basophilia in blue). Different methods for lipofucsin were compared: Thionin (0.25% thionin at pH 3.0 in citric acid-phosphate buffer), modified Nile Blue (0.05% Nile Blue A in 1% H_2SO_4 at pH 0.9) and the Schmorl reaction on cryostat sections. Treatment with pepsin (5mg/ml) in

0.2 N HCl at pH 1.6 for 2 hrs at 37°C followed by Sudan Black B (SBB) was applied on paraffin sections. The methods were performed according to Barka and Anderson (1967); Culling *et al.* (1985); Pearse (1972, 1985) and Vacca (1985).

RESULTS

Degenerative processes in the digestive gland

The sections from untreated specimens reveal the normal histological patterns of the digestive gland; this is formed by duct and digestive diverticula consisting of columnar, acidophilic or secretory cells and pyramidal basophilic or generative cells. Differences appeared by comparing mussels with clams. In mussels (Fig. 1A), there is a slight separation of connective tissue and blood sinuses, whereas in clams (Fig. 1B) the ducts are more tightly surrounded by diverticula. Exposure to copper (Fig. 1C and 1D) induces an inflammatory response, and increased interducts and diverticula spaces, a moderate vacuolation of digestive cells, the occlusion of lumina and the formation of intracytoplasmic basophilic dark granules. Cadmium treatment produces in both species (Fig. 1E and 1F) an extreme vacuolation of digestive cells, a strengthening of the basament membrane and the appearance of basal membrane bound vesicles.

Lysosomal responses in the digestive gland

Lysosomes of the digestive gland of *Mytilus galloprovincialis* contain small droplets of neutral lipids (Sarasquete *et al.,* 1989a). Copper treatment enhances the accumulation of neutral lipids in enlarged homogeneous lysosomes located in the basal region of the digestive cells of copper exposed-specimens (Fig. 2A and 2C). These vacuoles stain pink with Nile Blue Sulphate, suggesting the presence of unsaturated neutral substances lipidic in nature (Fig. 2G and 2H). In Cu-exposed clams, few dark granules (basophilic lipofucsins) can be detected in the lumen with this stain. Cadmium treatment in mussels resulted in an increased size of homogeneous lysosomes, whereas in clams exposed to cadmium extremely enlarged secondary lysosomes of the heterogeneous-type predominate. These lysosomes exhibit a negative matrix to Oil Red O surrounded by lipids (Fig. 2B and 2D), but basophilic granula can be detected with Nile Blue Sulphate; it also distinguishes an increased amount of dark basophilic granules located both apically and in the lumen of digestive epithelium of Cd-exposed clams (Fig. 2H).

The technique on paraffin section (pepsin digestion-Sudan Black B) gave consistent results with the previous techniques tested in this study.

As Schmorl's method gives a positive reaction with lipofucsin, melanin, argentaffin granules and protein structures containing SH and other reducing groups, it is necessary to block these groups (with $HgCl_2$, N-ethylmaleimide, etc.) to evidence the lipidic nature of these substances (only lipofucsins).

Interspecific and metal related differences in the synthesis of lipofucsins

None of the methods tested detected dark granula in untreated specimens and Cd-exposed mussels. In Cu-exposed mussels, a large amount of lipofucsin granules was

observed, with variable staining intensity, size and location within the cells and among the digestive diverticula. However in Cu-clams, only very few positive granules could can be seen in the lumen. On the contrary, the epithelial cells of the digestive gland of Cd-exposed clams show enlarged lysosomes which are slightly stained in yellow, and lipofucsin (brown granula) which are located apically and in the lumen (Fig. 2A to 2H).

DISCUSSION

The generalized metal-induced histopathologies observed in this study for *Mytilus galloprovincialis* and *Ruditapes phillipinarum* corroborate previous reports (Sunila, 1986, 1987; Auffret, 1988; Lowe, 1988; Bright and Ellis, 1989; Lowe and Clark, 1989; Sarasquete *et al.,* 1989b) on the effects of toxic chemical exposure in other species of bivalves. The pathological enlargement of lysosomes, observed in the digestive gland cells of metal-exposed specimens, is indicative of a stress-induced fusion of vacuolar components and autophagy. It is also linked with atrophy of the digestive diverticular epithelium which is largely comprised of digestive cells (Moore, 1980, 1985).

The abnormal accumulation of lipids in lysosomes of digestive cells is a common response to toxic chemicals exposure (Lowe, 1988; Moore, 1988). However, it is not known whether the contaminants impair neurosecretory control associated with nutrient storage, and/or disturb lipid metabolism (Lowe and Clarke, 1989). Lysosomes of the heterogeneous type first described by Lowe (1988), have also been detected in this study. They only predominate in Cd-exposed clams, suggesting a more severe effect (Lowe and Clarke, 1989) of cadmium in digestive epithelium cells of cadmium-clams as compared to that of copper in clams and mussels. Lipofucsins, an end-product of free-radical peroxidative reactions, derive from autophagy of lipoproteic membranes (Pearse, 1972).

Fig. 1 - Histopathological alterations in the digestive gland cells of mussels, *Mytilus galloprovincialis* and clams, *Ruditapes phillipinarum* exposed (5 days, 5 ppm) to copper and cadmium.

A. Control mussels: normal aspect of the digestive gland: secretory cells acidophilic (sc) intercalated with generative cells basophilic (gc) in the diverticula (dd) and duct digestive (pd). Haematoxylin-V.O.F. X250.

B. Control clams. Haematoxylin-eosin. X250.

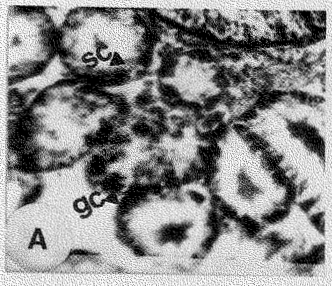
C. Cu-exposed mussels. Haemocytes infiltration around the primary duct (pd) and digestive diverticula (dd); accumulation of basophilic granules in the cells of the diverticulum (dd). Note the increase spaces between duct and diverticula and the occlusion of lumina (l) due to the vacuolation of digestive cells. Haematoxylin-eosin. X 250.

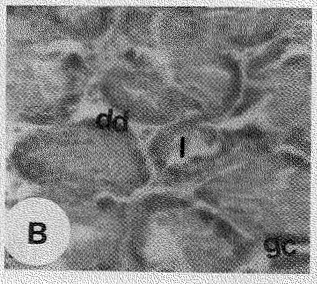
D. Cu-exposed clams: Advanced vacuolations (v) of digestive cells occluding lumina (l). Haematoxylin-eosin. X125.

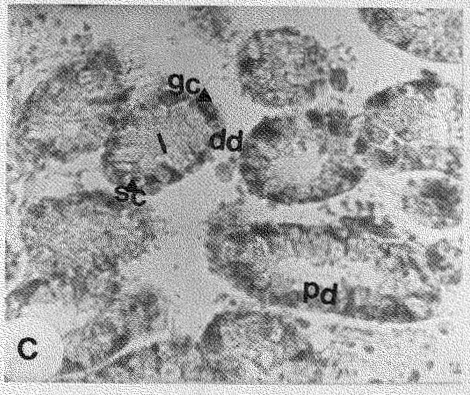
E. Cd-exposed mussel. More severe vacuolation (v) of cells in the duct (pd), note the reinforcement of the basement membrane (bm) of the ducts and the absence of granules in the digestive cells. Haematoxylin-eosin. X1250.

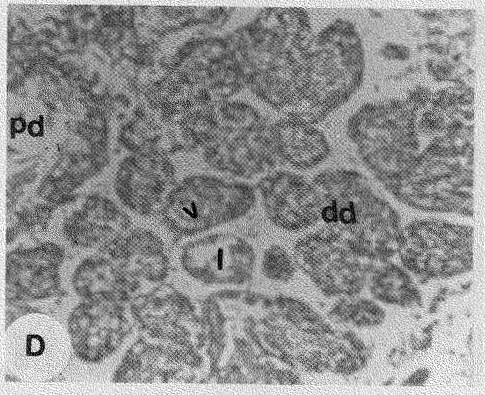
F. Cd-exposed clams: Extreme vacuolation in a necrotic digestive gland. Note the increased separation of connective tissue between ducts (pd) and diverticula (dd). Haematoxylin-eosin. X 625.

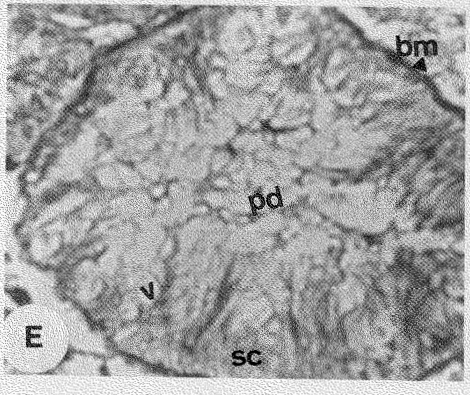
All the techniques tested gave consistent results. However, the Schmorl reaction used by Moore (1980) to evidence lipofucsin granules, is not specific for these substances. The specificity of this reaction depends on the absence of non-protein reducing groups and on its lipidic nature (Pearse, 1985). Proteins containing SH groups are present in the digestive gland of *Mytilus galloprovincialis* (Sarasquete *et al.,* 1989a). Thionin (pH 3.0) and the modified Nile Blue reactions, on cryostat sections, allowed various stages in the lipid peroxidations products, and their sites of formation and accumulation (yellow in early stages in larger II lysosomes, brown in highly oxidised lipofucsin in residual bodies) to be distinguished.

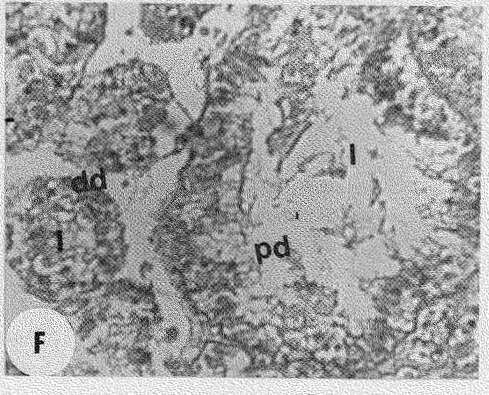












Moreover, paraffin sections could be used, as by increasing the age of the pigment, it becomes more insoluble in solvents (Barka and Anderson, 1967). The variable distribution, number and size of lipofucsin granules among cells and digestive diverticula can be attributed to the fact that more physiologically active cells (Samorajsji *et al.,* 1965) and diverticula undergoing disintegration (Janssen and Scholz, 1979) accumulate lipofucsins at a faster rate.

The combination of histochemical methods allows different detoxification mechanisms to be distinguished in the two species of marine bivalves studied for cadmium and copper, under these experimental conditions. Cadmium does not seem to be excreted through the release of residual bodies in the digestive gland of mussels, whereas this process seems to be active in clams, as lipofucsin granules are synthetised upon experimental exposure to this metal. Elimination of copper by release of residual bodies (containing lipofucsins) is not such an effective process in clams as it is in mussels, as suggested by the few lipofucsin granules detected in the digestive gland of copper-exposed clams. Another route of excretion may play a major role in the depuration of copper in this species. The macrophage system, as earlier mentioned in *Ostrea* (George *et al.,* 1978) and *Crassostrea* (Martoja *et al.,* 1988) could play a major role in this metal detoxification.

In summary, is of interest to emphasize that Thionin (pH 3.0) and the modified Nile Blue methods, on cryostat section, allow various stages of lipid peroxidation to be distinguished. Also an other technique described in this study: pepsin digestion followed by Sudan Black B, on paraffin sections, is specific for the highly oxidised, insoluble lipofucsins. It can, therefore, be applied in routine histopathology surveys as a stress index detecting the induction of lipidic peroxidation.

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Fig. 2 -Accumulation of lipids in pathologically enlarged lysosomes and synthesis of lipofucsins in the cells of the digestive gland of *Mytilus galloprovincialis* and *Ruditapes phillipinarum* exposed to copper or cadmium (5 days, 5 ppm).

A. Enhanced accumulation of lipids in enlarged homogeneous lysosomes (hol). Cu-exposed mussels. Oil Red O (ORO). X250.

B. Appearance of lysosomes of the heterogeneous type (he) in Cd-exposed clams. Oil Red O (ORO). X250.

C. Cu-exposed clams. ORO-Haematoxylin. X625.

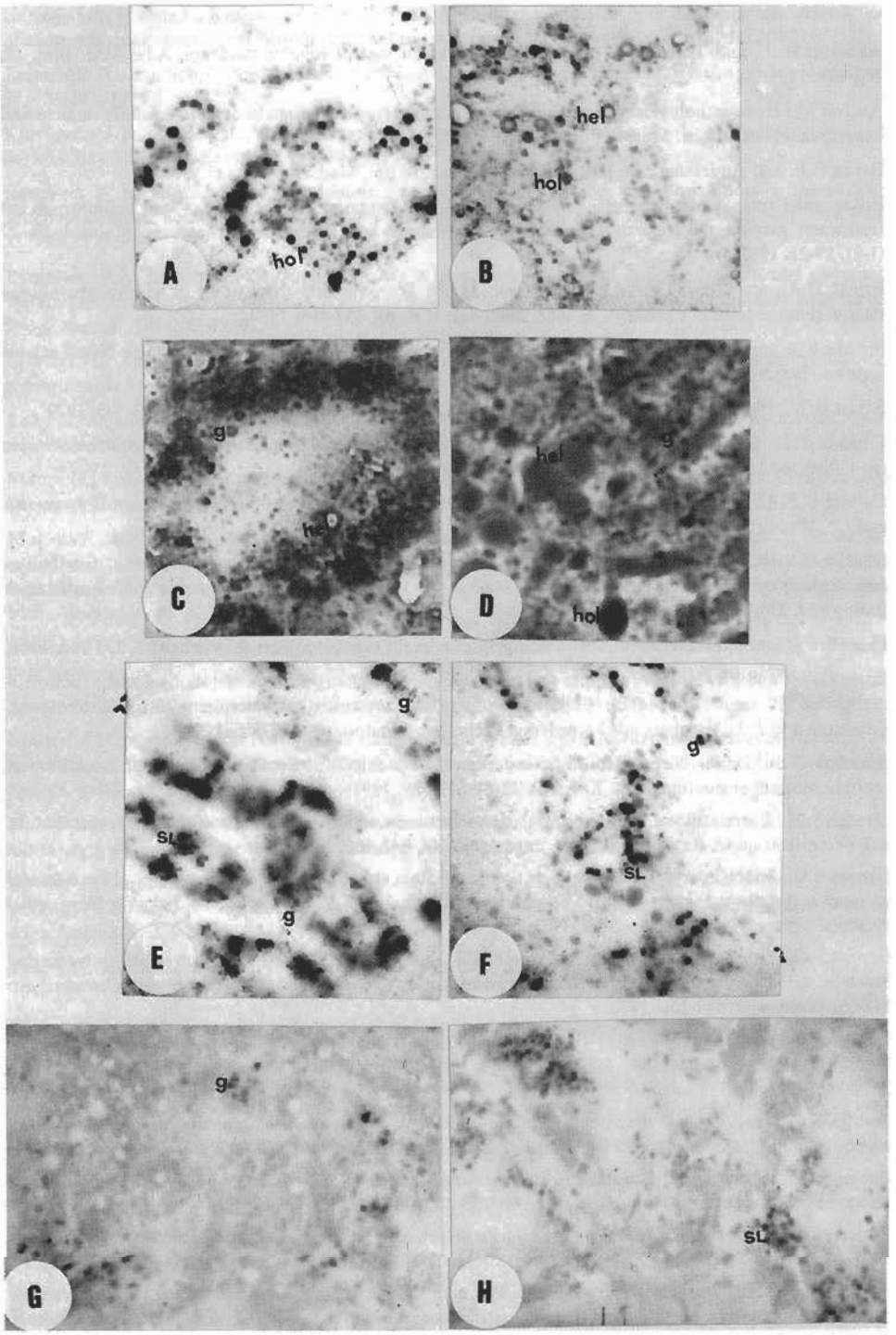
D. Cd-exposed clams: extreme enlargement and predominance of the lysosomes heterogeneous type (hel) with a negative matrix surrounded by lipids. ORO-Haematoxylin. X1250.

E. Large amount of lipofucsin granules (g) in Cu-exposed mussels. Thionin. X250.

F. Cd-exposed clams. Thionin. X250. sl: secondary lysosomes; g: lipofucsin granules.

G. Cu-exposed mussels. Aqueous Nile Blue. X250. g: lipofucsin granules.

H. Cd-exposed clams. Aqueous Nile Blue. X250. sl: secondary lysosomes.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