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Histochemical distribution and accumulation of trace metals in the heart of Green and Normal *Crassostrea angulata* specimens from different Southwest Spanish coasts

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SUMMARY

Histochemical distribution and quantification of trace metals (copper -Cu-, zinc -Zn- and iron -Fe-) were studied in oyster, *Crassostrea angulata* heart (auricle and ventricle) from different southwest Spanish coasts. In green *Crassostrea angulata* specimens (green coloration in gills and visceral mass), numerous brown cells (blackish-brown) were observed in the wall of the auricles and dispersed by connective tissue, where inflammatory lesions related with granular amoebocytes were observed. However, only a few brown cells (translucent light-brown) were detected in auricles of normal oysters. Histochemically, Fe, Cu and Zn granules were observed in the wall of the heart (auricles), in brown cells, as well as in the granular amoebocytes, which are increased in green *Crassostrea* specimens. Auricle brown cells reacted weakly with PAS and Alcian Blue techniques. These cells contain proteins, as well as cysteine and cystine groups; these residues (-SH and S-S) being very abundant in the heart of green specimens, specially in brown cells and in granular amoebocytes. Lipofuscin granules were detected in these brown cells and in granular amoebocytes. On the other hand, in the heart (normal and green specimens), Zn levels were higher than Fe and Cu concentrations and heavy metals analyzed were lower in heart of normal than in green specimens. In green specimens, Cu and Zn levels were higher in auricles than in ventricles.

INTRODUCTION

Oysters are bivalve molluscs of great commercial value, which have been widely used to evaluate the contamination in coastal ecosystems, because they are sedentary, abundant, of relative longevity and easily collected. In general, oysters can accumulate copper in higher levels than mussels and other bivalves, and also zinc levels in oysters are overwhelmingly higher than those in other species and the other metals in oysters (Establier and Gutierrez, 1970; Establier, 1977;

George *et al.*, 1978; Ikuta, 1988 ; López-Artiguez *et al.*, 1989; Schuhmacher and Domingo, 1996: Han *et al.*, 1996). The high copper content in the oysters could be explained both by the high copper contamination in a determinate area, and the great capacity of the oyster to accumulate this particular element (López-Artiguez *et al.*, 1989).

Green-colored oysters have been observed for more than 100 years, with algae being implicated as a cause in *Ostrea edulis* in France and copper contamination in *Crassostrea virginica* in the United States (Farley, 1988). Green-oysters or "green-sick " are found in particular estuaries which contain increased concentrations of copper (Galtsoff, 1964; Establier, 1977; George *et al.*, 1978). The green colour is due to copper contained in the amoeboid lymphocytes present in both the haemolymph and the tissues (Ruddel, 1971).

In molluscs, haemocyanin, a Cu-enriched protein, is the main protein in the blood and is able to bind Fe, Zn, Cd, etc., and different cell types (auricle brown cells and/or basophilic haemocytes), with the sole common characteristic of exhibiting a well developed rough endoplasmic reticulum and haemocyanin-like protein crystals, have been suggested as haemocyanin producers (Marigomez *et al.*, 1995). A histological study on *Crassostrea gigas* and *Crassostrea virginica* has suggested that granular basophils contain high concentrations of both zinc and copper, and there is a correlation between the number of basophil amoebocytes and the concentration of some metals in the tissues (Ruddel and Rains, 1975).

In oysters, there is direct evidence that the copper and zinc are immobilized in membrane-limited vesicles within the oyster amoebocytes. The metals, however, are localized within different cell types, granular acidophils for copper and granular basophils for zinc, and are associated with different chemical compounds within the vesicles (George *et al.*, 1978).

In bivalve molluscs, it is well established that internal defense mechanisms is primarily cellular (Bayne, 1983), involving phagocytosis and/or encapsulation (Cheng, 1988). In Ostreids, there is a direct connection between heart and kidney; both organs play a major role in bioaccumulation, excretion and detoxification of heavy metals (Galtsoff, 1964; Camichael *et al.*, 1980). On the other hand, cells containing brownish vesicles are observed in the connective tissue, muscle bundles of the auricle and lining the pericardial wall of oysters. Many functions have been attributed to auricle brown cells (Stein and Mackin, 1955; Cheng and Burton, 1965, 1966). Stein and Mackin (1955) and Kato (1960) were of the opinion that the vesicles of bivalves brown cells, were crystalloid and of lipoid composition. In *Crassostrea virginica*, auricle brown cells show lysosomal activity and are the first line of defense for detoxification and degradation of heavy metals (Zaroogian and Yevich, 1993)

The present paper studies some histochemical characteristics of the auricle brown cells, as well as the accumulation and distribution of copper, zinc and iron in the heart (auricles, ventricle) of green and normal *Crassostrea angulata* specimens from different zones (clean and copper contaminated stations) of South Atlantic coasts of Spain.

MATERIALS AND METHODS

Crassostrea angulata specimens were collected along different zones of the South Atlantic Spanish coast . Normal specimens were collected from a clean station, relatively free of industrial and domestic wastes (station 1), and green specimens were collected from polluted sites (station 2) where an elevated concentration of copper is present. The heart of the oysters was isolated and auricles and ventricle were separated. An aliquot of the samples was used for quantification of heavy metals (Cu, Zn and Fe) by flame atomic absorption spectrophotometry according to Establier (1969 a, b). Significant differences between heavy metals, tissues (heart, auricle, ventricle) and specimens (normal and green) were determinated by one-way analysis of variance and Tukey-Kramer multiple comparison test. Other samples were fixed in 0.1M formaldehyde-phosphate buffer at pH 7.2 and embedded in paraffin. Sections (6-7 μm) were stained with histomorphological and histochemical methods.

Histological sections were stained with histomorphological techniques such as Haematoxylin-Eosin and Haematoxylin-V.O.F (Gutierrez, 1967,

1990), as well as with Azan trichromic and Silver impregnation techniques (Kiernan, 1990) for differentiating connective fibers (collagen, reticulin). Histochemical techniques for carbohydrates and proteins (PAS and diastase-PAS, Alcian Blue pH 0.5, 1 and 2.5, and bromophenol blue reactions), cysteine and cystine residues (ferric ferricyanide and thioglycollate reduction techniques), lipids (calcium-formalin fixed samples and Sudan Black reaction), as well as techniques for sulphydryl groups and/or lipofuscins location (Schmorl technique), performed in this paper, were taken from monographs by Martoja and Martoja-Pierson (1970), and Pearse (1985). On the other hand, histochemical heavy metal techniques such as Perls reaction (Fe, ferric iron); Turnbull blue (Fe, ferrous iron); rubeanic acid-dithiooxamide (Cu, copper) and dithizone method (Zn, zinc) were used according to Martoja and Martoja-Pierson (1970) and Pearse (1985).

RESULTS

Histological characteristics of the heart

The heart of *Crassostrea angulata* is located in the pericardium, a thin-walled chamber between the visceral mass and adductor muscle (Fig. 1).The wall of pericardium is formed of connective tissue similar to that in the mantle, well supplied with blood vessels, blood sinuses and branches of the cardiac nerve. The epithelium lining of the side facing the heart consists of small flattened cells and a few scattered eosinophilic and mucous cells; on the opposite side, facing the shell, the pericardium wall is covered with large columnar epithelial cells with oval nuclei and many eosinophilic and mucous cells. Basal membrane on the upper side of the wall is well developed.

The auricles are darkened by pigment cells - brown cells - in their walls and the degree of pigmentation varies from light brown to almost black. The vesicles within the brown cells are a translucent brown and vary in size and number (Fig. 2). The wall of the ventricle is formed by a framework of muscle fibers and connective tissue forming an irregular trabecular structure, with amoebocytes in the spaces between fibers and in the connective tissue. The outer surface of the ventricle is covered with epithelium of a single layer of flat and thin cells with conspicuous nuclei.The walls of the auricles, thinner and lighter than those of the ventricle, also form a trabecular framework supported by connective tissue. On the outside, the auricles are covered with tall columnar epithelium which contains many glandular and darkened pigment cells. The fibers of the heart muscle are surrounded by delicate connective tissue. In general, the muscle tissue has a spongy appearence (Fig. 2). In ventricle, and specially in auricles, numerous hyaline and granular amoebocytes are present between the connective tissue cells and along the muscle fibers.

In green *Crassostrea angulata* specimens (green coloration in gills and visceral mass), numerous brown cells (blackish-brown) in the wall of the auricles and dispersed by connective tissue were observed. Collagen and reticulin fibers were observed throughout the heart. Symptoms of fibrosis (reticulin and collagen), as well as inflammatory responses or haemocytic infiltration by granular amoebocytes were observed in the connective tissue. However, only a few brown cells (translucent light brown) were detected in the auricle of normal oysters (Fig. 2C). Iron, copper and zinc granules were observed in the epithelium of the heart (Fig. 2G, 2H and 2I), within brown cells, as well as in the granular amoebocytes (acidophilic and/or basophilic), especially in green *Crassostrea* specimens.

Auricle brown cells of green *Crassostrea angulata* specimens were weakly stained with PAS and Alcian Blue (pH 2.5); these reactions were more intense on the scarce brown auricle brown cells present in normal specimens. Lipids, proteins, numerous cysteine (Fig. 2E) and cystine residues, as well as lipofuscin granules (Fig. 2F) were observed in auricle brown cells. Moreover, sulphide and disulphide residues were more abundant in the heart (wall, brown cells and granular haemocytes) of green than in normal specimens (Fig. 2E).

Quantification of trace metals

In the heart of green and normal *Crassostrea angulata* specimens, Zn levels were higher than Cu and Fe concentrations. In general, auricle and ventricle heavy metal levels (Zn, Cu and Fe) were

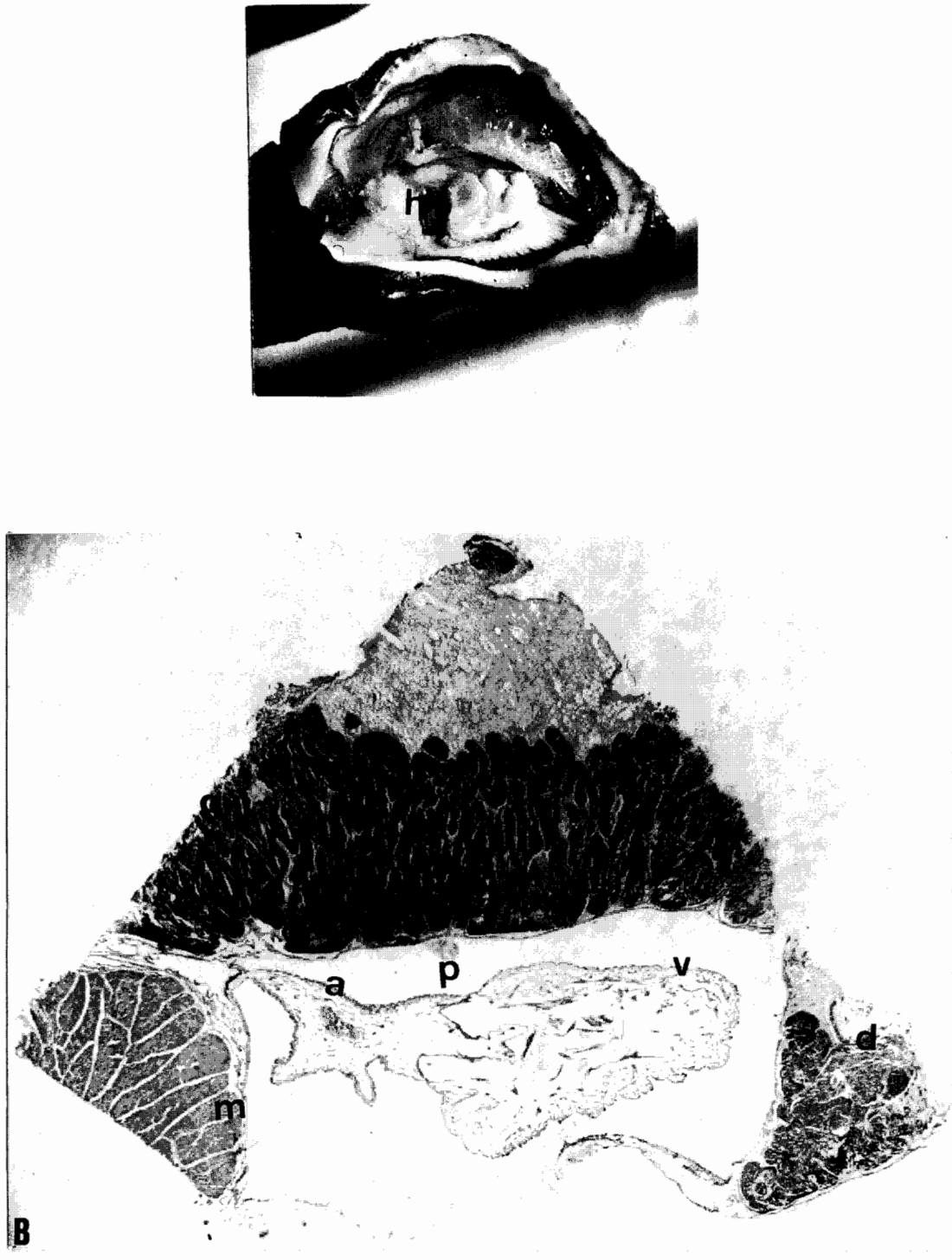


Fig. 1 - Normal *Crassostrea angulata* fresh specimen (A) and histological section showing the anatomic location of the heart (B). Haematoxylin-eosin. X40.

h: heart; **a:** auricle; **d**: digestive gland; **g**: gonad; **m**: adductor muscle; **p**: pericardium; **v:** ventricle.

higher in green than in normal oysters (P<0.001) (Fig.3)

In normal specimens, auricles and ventricles showed similar copper and zinc levels (P>0.05) and the iron levels were higher in auricles (P<0.001). In green specimens, auricles showed higher Zn and Cu levels than ventricles (P<0.001), and the Fe concentrations were higher in ventricles (P<0.001) (Table 1 and Fig. 3).

DISCUSSION

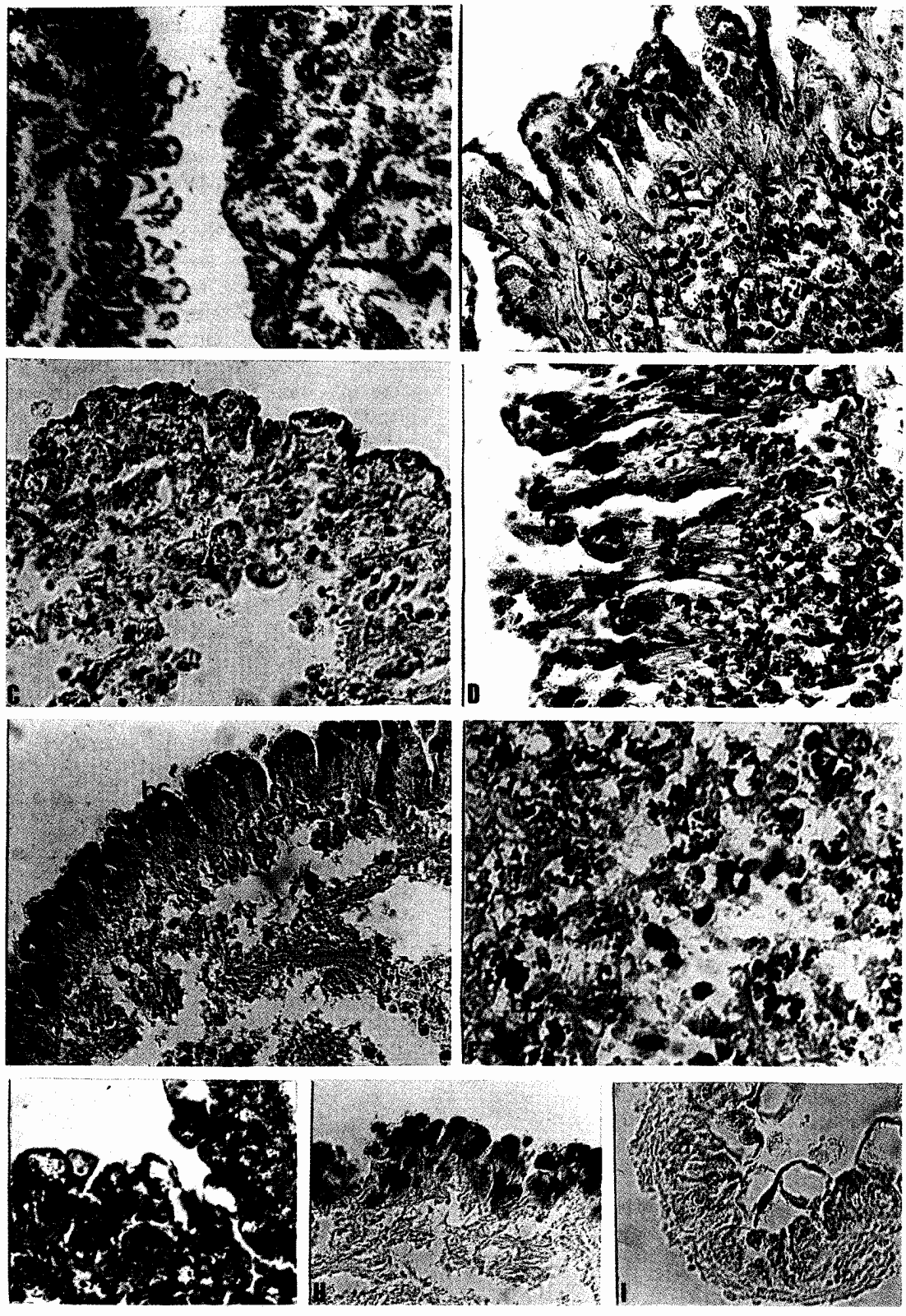
In oyster, *Crassostrea* specimens, auricle brown cells possess the requisites for detoxification and have the potential for using them as a biomarker of exposure to contaminants and stress. The vesicles within brown cells have been identified as lysosomes, and auricle brown cells contained proteins, acid phosphatase, glutathione reductase and lysozymes. In oysters, these cells are the first line of defense for detoxification and degradation of heavy metals (Zaroogian and Yevich, 1993). In *Crassostrea angulata*, auricle brown cells contain proteins with cysteine and cystine residues, as well as lipofuscin granules. According to different authors (in Martoja and Martoja-Pierson, 1970) lipofuscin granules are positive to PAS, ferric-ferricyanide, Schmorl and lipid reactions, as was observed in this study.

Auricle brown cells and granular amoebocytes containing Fe, Cu and Zn granules were increased in the heart of green *Crassostrea angulata* specimens. In the heart of these specimens, symptoms of fibrosis (reticulin and collagen), as well as a granular haemocytic infiltration of connective tissue were observed. Inflammatory lesions, containing massive amounts of heavy metal - presumably copper - occuring as internal granules in the involved haemocytes were observed by Farley (1988) in mantle and digestive gland of *Crassostrea virginica* specimens contaminated by copper. Similar inflammatory responses by amoebocytes, as well as fibrosis in different tissues, were observed by Zaroogian and Yevich (1993) in *Crassostrea virginica* from contaminated stations.

Table I
One-way analysis of variance. Tukey-Kramer multiple comparison test. Significant differences between heavy metals (Cu, Fe, Zn), organs (heart, auricle, ventricle) and specimens (normal and green).

	Normal specimens								
		Cu			**Fe**			**Zn**	
	Md	q	P	Md	q	P	Md	q	P
Auricle-Ventricle	-18.6	2.1	ns	87.8	10.6	***	-86.9	1.9	ns
Auricle-Heart	-24.7	2.9	ns	39.6	4.8	*	-133	3.04	ns
Ventricle-Heart	-6,04	0.7	ns	-48.2	5.8	**	-46.4	1.06	ns
	Green specimens								
Auricle-Ventricle	59.4	6.9	***	-271	32.9	***	367.7	8.3	***
Auricle-Heart	-105.4	12.3	***	214	26.1	***	-272.8	6.2	**
Ventricle-Heart	-164.9	19.3	***	485	59.04	***	-640	14.5	***

Md: Mean differences: P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***).


C
D

In different bivalve molluscs, elimination of copper can be carried out by release of residual bodies - containing lipofuscins - and/or through the macrophage system (George *et al.*, 1978; Martoja *et al.*, 1988; Sarasquete *et al.*, 1992: Marigomez *et al.*, 1995). Metals can also be trapped by cytosolic cysteine-rich proteins - metallothioneins - (Viarengo, 1989; Marigomez *et al.*, 1995). In oysters, the wall of the auricles is covered with tall columnar epithelium which contains many glandular and dark pigment cells; this epithelium constitutes a part of the excretory system (Galtsoff, 1964). In the oysters, ultrafiltration of the hemolymph takes place through the auricle wall (Meyhofer *et al.*, 1985). According to Zaroogian and Yevich (1993), as the hemolymph flows through the auricle in oysters from relatively contaminated stations, it comes in contact with the brown cells where soluble foreign material is imbibed and passes into the lysosomes (vesicles) of the brown cells and detoxified. In oyster from highly contaminated stations, the brown cells become overburdened and abound in lysosomes, they are sloughed into the hemolymph. Here they are probably lysed, and the detoxified material is eventually excreted through the auricle wall into the pericardial cavity. From here, detoxified material makes its way via the pericardial funnel into the kidney where it is filtered by the epithelial cells of the kidney tubules and finally excreted through the urogenital pore.

Green coloration, in oysters, has been attributed to copper contained in the amoebocytes, which may indiscriminately phagocytose particulate metals and metals bound to proteins as any other foreign material, but additionally it appears that they are involved in metal regulation since soluble metals are also taken up through pinocytotic vesicles (George *et al.*, 1978). In *Crassostrea angulata*, auricle brown cells and granular amoebocytes were positive to Fe, Cu and Zn histochemical techniques and these cells contain proteins, cysteine, cystine residues, as well as lipofuscin granules. On the other hand, the increase of proteins containing cysteine and cystine residues in the heart of green *Crassostrea* specimens, could be related with metallothioneins, the synthesis of which rapidly increases in response to the accumulation of heavy metals in the cells (Viarengo, 1989).

In *Ostrea* and *Crassostrea* specimens from different Spanish coasts, Establier and Gutierrez (1970) observed higher iron, copper and zinc levels in the heart than in other tissues (gills, visceral gland, muscle, etc) . In the heart of *Crassostrea angulata* (normal and green specimens), Zn levels were greater than Fe and Cu levels, such as was observed (Establier, 1969 a, b; Establier and Gutiérrez, 1970; Ikuta, 1988) in soft tissues of different bivalves. Auricle brown cells and heavy metals were strongly increased in the heart of green oysters. In general, these metals (iron, copper, zinc) were located in the wall of the auricle, dispersed through connective tissue (granular haemocytes) and within brown cells.

The excessive accumulation of copper by oysters produces a strong green coloration, mainly in the mantle and gills, and confers to oysters an abnormal and unpleasant coppery flavour (Galtsoff, 1964; Ruddel, 1971; Establier, 1977; George *et al.*, 1978). In this study, copper levels in water from a clean station (1) varied between 2.47 and 8.7 µg/L; copper levels in soft tissues of *Crassostrea angulata* specimens from this clean station ranged between 15.48 and 35.46 mgCu/100g d.w. Oysters at station 2, where green *Crassostrea* specimens were collected, had a copper concentrations in water ranging between 3.95 and 8.5 µg/L and copper levels in soft tissues from these green specimens varied between 83.36 and 232.7 mgCu/100 g d.w. (personal communi-

Fig. 2 - Histological section of heart of normal and green *Crassostrea angulata* specimens from station 1 and station 2.

A.- Auricle and ventricle of green specimens. Azan-trichromic. X100; **B.**- Auricle of green specimen. Azan-trichromic. X400; **C.**- Auricle of normal specimen. Haematoxylin-V.O.F. X400; **D.**- Auricle of green specimen. Haematoxylin-V.O.F. X400; **E.**- Auricle of green specimen. Ferric-Ferrycianide technique. X250; **F.**- Auricle of green specimen. Schmorl reaction. X400; **G.**- Auricle of green specimen. Dithizone method (Zn)/Haematoxylin counterstaining. X250; **H.**- Auricle of green specimen. Perl's reaction (Fe^{++}). X250; **I.**- Auricle of green specimen. Rubeanic acid technique (Cu). X400.

a: auricle; **bc**: brown cells; **f**: connective fibers; **h**: haemocytes; **v**: ventricle.

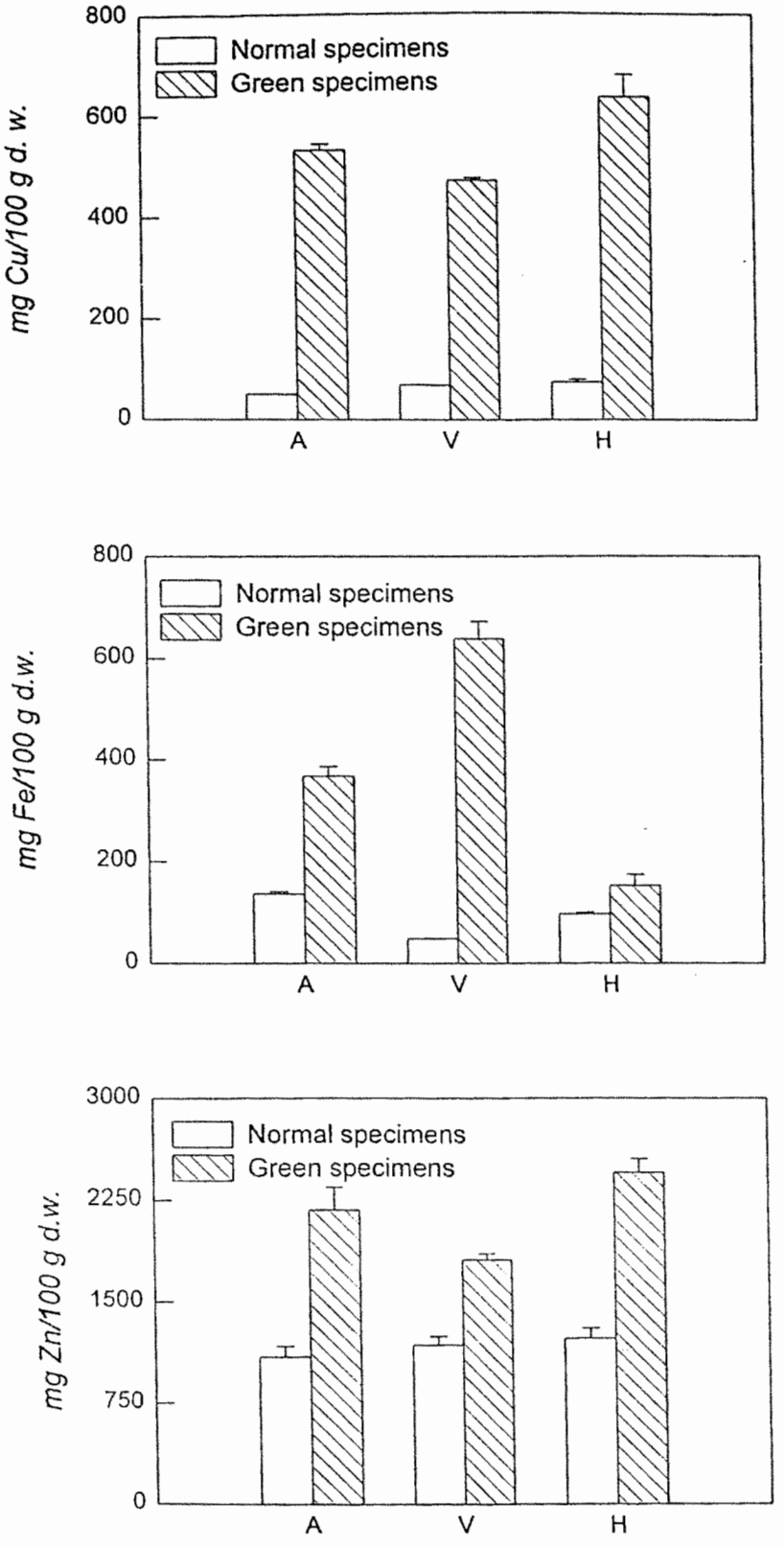


Fig. 3 - Quantification (atomic absorption spectrophotometry-AAS-) of copper (Cu), iron (Fe) and zinc (Zn) in the heart of normal and green *Crassostrea angulata* specimens.
A: auricle; **H**: heart; **V**: ventricle

cation). Moreover, copper levels in water from clean and copper contaminated stations were not correlated with copper accumulation by the heart. Similar remarks were made by Establier (1969b) and by Cordon and Cabrera (1987), in relation to different metals, such as the copper and zinc concentrations (dissolved and particulate) in water, and heavy metal incorporation by *Crassostrea angulata* tissues. However, according to Han *et al.* (1996), the copper concentration in oysters, *Crassostrea gigas* were significantly correlated with the concentration of particulate copper; this suggest that the food pathway from surrounding water may dominate the accumulation of copper by oysters (Han and Hung, 1990). On the other hand, the total copper concentration in sediments from different environments, similar to Spanish stations studied in this paper (station 1 and 2) differed greatly ; copper levels in sediments (>50 μgCu/g) can indicate high levels of copper pollution (Cordon and Cabrera, 1987). The copper levels in sediments are so much higher than those in the overlying water that even a minute fraction may represent an important source for uptake, specially in benthic filter and burrowing organisms (Han *et al.*, 1996), such as *Crassostrea* specimens, since copper concentrations in purple clams, *Huatula diphos* and hard clams, *Meretrix lusoria* are not affected by copper concentration in sediment (Han *et al.*, 1996).

In summary, zinc, copper and iron levels, as well as auricle brown cells and granular amoebocytes were higher in the heart of green than in normal *Crassostrea angulata* specimens. Auricle brown cells and granular amoebocytes accumulate iron, zinc and copper granules and contain proteins rich in cysteine and cystine residues, as well as lipofuscin granules. This paper will be completed with ultrastructural and immunocytochemical studies, as well as with determinations of lysosomal markers of cellular stress, such as acid phosphatase, β-glucuronidase, N-acetyl β-hexosaminidase, gluthatione reductase, etc.

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