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Influence of salinity on trace metal (Cu, Zn, Ag) accumulation at the molecular, cellular and organism level in the oyster *Crassostrea gigas* Thunberg

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Summary. The accumulation and physico-chemical forms of metals were determined by atomic absorption spectrometry in the whole soft tissues of oysters, by histological and microanalytical techniques in tissue sections, by gel permeation chromatography of soft tissue homogenates. Oysters were reared according to four types of experimental conditions: exposed to silver (20 µg Ag/l) or unexposed, in sea water (33‰) or brackish water (8‰). Copper, zinc and silver accumulation in oysters are inversely related to salinity. Amoebocytes, which play a key role in accumulating copper and zinc in natural sea water, are able to sequester an important part of added silver as Ag_2S. In brackish water an increase of the number of amoebocytes may be considered as responsible for the enhancement of Cu and Zn concentrations in the whole soft tissues. In such conditions, additional silver is concentrated in these cells rather than in the basement membranes which are target structures for Ag_2S accumulation in sea water. If the global fluctuations of metal concentrations in the soft tissues are ascribable mainly to changes at the histological level, the fate of metals in the soluble fraction must not be neglected since the speciation of metals influences their toxicity. The freshening of sea water induced a change in the distribution of cytosolic silver and zinc but in no case were the molecular masses of compounds associated with each metal perfectly identical. From these results, it is concluded that the accumulation of silver by oysters is not mediated by the same mechanisms as those for copper and zinc.

Key words: Copper – Silver – Zinc – Oyster – Salinity

Introduction

Silver accumulation and toxicity vary considerably even amongst nearly related species such as filter-feeding bivalves. Pectinidae and Ostreidae sequester much more silver in their soft tissues than Mytilidae (Metayer et al. 1990) whereas the scallop *Chlamys varia* exhibits a much greater susceptibility, (Martoja et al. 1989) than *Crassostrea gigas* (Martoja et al. 1988) and *Mytilus edulis* (Calabrese et al. 1984). However, the most striking feature concerns the difference between marine and freshwater organisms. The freshwater mussel *Dreissena polymorpha* and the gastropod *Viviparus viviparus* are not as susceptible to silver exposure as marine species (Martoja and Truchet 1991) and silver levels in their soft tissues remain comparatively low (unpublished data).

It is important to determine the influence of salinity on population responses to silver exposure since pollution due to this metal is often registered in estuaries (Bryan et al. 1983) and coastal areas (Calabrese et al. 1982; Cain and Luoma 1985) which are submitted to potential salinity changes. Salinity influences metal levels in organisms (Anon. 1980), sometimes as a consequence of changes in metal speciation; but, whatever the salinity, the strong affinity of Ag for Cl induces the formation of di-, tri- and tetra-chloroargentates (Bowen 1985; Martoja and Truchet 1991) since the occurrence of Cl^- is always sufficient. In order to determine the impact of this ecological factor on silver contamination, we have investigated metal accumulation in the oyster *Crassostrea gigas* which is able to tolerate a comparatively large range of salinities. In oysters which live in normal sea water, silver is partly stored in amoebocytes containing Cu and Zn (Thomson et al. 1985; Martoja et al. 1988). This suggests that Ag may compete with Cu and Zn for binding sites in these cells. Consequently, we have examined concurrently the storage of all three metals in oysters submitted to different salinities.

Materials and methods

Procedures of rearing and exposure. Sea water (salinity: 33‰; Cu: 1–2 $\mu g \cdot l^{-1}$; Zn: 3–17 $\mu g \cdot l^{-1}$) was obtained from the bay of Bourgneuf (France) and was filtered (0.4 µm) before use. Brackish water was obtained by diluting this sea water with freshwater (2–3 $\mu g\ Cu \cdot l^{-1}$; 1–2 $\mu g\ Zn \cdot l^{-1}$).

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Young oysters (6 months old; mean individual dry mass of soft tissues: 50 mg) were obtained from a hatchery in April 1988. They were acclimated in the laboratory for two weeks, then some of them gradually adapted to brackish water for 16 d in order finally to reach a salinity of 8‰. Acclimation to this salinity was continued for a further week. The temperature was controlled at $15 \pm 1°C$ during the acclimation and the experimental periods. Then young oysters were exposed for 21 d to silver, introduced in sea water or brackish water as nitrate diluted in nitric acid solution (20 $\mu g\ Ag \cdot l^{-1}$). The concentration of stock solution and the subsequent dilutions were chosen in order to retain the normal pH of sea water. In each case, 45 individuals were distributed in three containers leading to a density (wet mass of soft tissues) of 600 $mg \cdot l^{-1}$. They were fed lyophilized *Spirulina* once a day 1 h before renewing water and contaminant. After 7, 14 and 21 d of exposure, ten oysters were sampled (three or four from each container) to analyze trace metals in the whole soft tissues. At the end of the experiment, the remainder was divided into two groups, one for biochemical analysis and the other for histological study and microanalysis. The latter group was submitted to convenient fixations, whereas the others were frozen.

Biochemical techniques. Soft tissues of each individual were homogenized in 2 ml 0.02 M Tris (pH 8.6 with 150 mM NaCl). The homogenate was centrifuged at 30000 *g* for 1 h at 4° C. The supernatant was fractionated by gel chromatography using a column (870 × 16 mm) of Sepharose CL-6B (Pharmacia) equilibrated with the buffer used for elution (0.02 M Tris, 0.15 M NaCl, pH 8.6). A 2–2.7-ml aliquot of supernatant was applied to the column, eluted with buffer at 25 $cm \cdot h^{-1}$ and collected as 2.5 ml. Absorbance of the eluents was monitored at 280 nm. The column was calibrated for molecular mass estimations using standard markers (chymotrypsin, bovine serum albumin, aldolase, thyroglobulin). The molecular masses of the different Ag-binding components were derived using the calibration curve.

Total protein sulphydryl groups were determined with Ellman's reagent according to Tukendorf and Baszynski (1985). To 1 ml of the fractions, 0.45 ml SDS solution (4% sodium dodecyl sulfate in 0.1 M phosphate pH 8.0) was added. The colour was measured at 412 nm. For calculation of the sulphydryl group content the net absorbance was employed with a molar absorption coefficient of 13600 $M^{-1} \cdot cm^{-1}$. Absorption spectra and theirs derivatives were monitored with a Beckman DU-7 spectrophotometer. The whole procedure was carried out three times in each condition of salinity.

Metal determination by atomic absorption spectrophotometry (AAS). In fractions separated by gel chromatography, metal levels were determined by flame (Zn) or flameless (Ag, Cu) AAS using the Zeeman effect (Hitachi 180-80 and Z-7000). Measurements of Ag in acid-digested pellets from the biochemical study and in soft tissues of oysters were made using the analytical procedures described by Amiard et al. (1987) and validated by international intercalibration exercises (IAEA 1987, 1988).

Microscopic techniques. At the end of the experiment (21 d), samples of controls and of oysters contaminated in sea water and in brackish water were fixed in Carnoy's fluid. Microanalysis was performed on paraffin sections (7 μm) for S, Cu, Zn and Ag, using an electron microprobe (Cameca MS 46) equipped with wavelength-dispersing spectrometers.

Sections were stained by nuclear fast red-picroindigocarmine for general morphology of tissues and by periodic acid/Schiff reaction for polysaccharides. The amoebocytes were visualized with the von Kossa's method for mineral salts, the silver sulfide with the Schmorl's ferric ferricyanide reagent and its solubility in potassium cyanide (see Martoja et al. 1988).

Results

Bioaccumulation of metals in the whole soft tissues of oysters

Metal levels in soft tissues are presented in Table 1. Although oysters were allowed to acclimate to experimental conditions, the metal levels in controls increased during the experiments. Changes in rearing conditions, especially the change over from a plurispecific natural algal food to a monospecific lyophilized food, could induce a metabolic adaptation and a subsequent new equilibrium with the experimental medium. This equilibrium was not completed within the planned duration of acclimation.

Copper and zinc levels were higher in individuals reared in brackish water than in individuals living in normal sea water. A similar tendency was registered for silver in exposed oysters but the difference was significant after a 14-d exposure only. In contrast, in controls, silver levels were significantly higher in normal sea water than in brackish water (test *F* of Snedecor, $P < 0.05$).

Table 1. Metal concentrations in whole soft tissues of oysters

Metal examined in oyster tissues	Duration of exposure (d)	Concentration [$\mu g \cdot (g\ dry\ mass)^{-1}$] when exposed to Ag at			
		0 (controls) at salinity		20 $\mu g \cdot l^{-1}$ at salinity	
		8‰	33‰	8‰	33‰
Silver	7	2.1± 0.6	3.3± 1.8	243±153	318±186
	14	2.8± 1.6	7.4± 4.3	1012±426	292±118
	21	4.5± 1.8	8.8± 5.9	1089±715	761±544
Copper	7	136 ± 34	35 ± 9	134± 18	29± 8
	14	162 ± 41	60 ± 26	234± 40	41± 7
	21	263 ± 68	74 ± 12	305± 65	52± 9
Zinc	7	2749 ± 752	786 ±151	2886±465	733±160
	14	2992 ±1072	945 ±310	3776±612	995±151
	21	4383 ±1302	1356 ±321	4071±866	1280±162

Values are means ± confidence intervals at the 95% level

Table 2. Correlation (r) matrix between metal concentrations in whole soft tissues of individual oysters

Metal	Controls			Ag-exposed oysters		
	Ag	Cu	Zn	Ag	Cu	Zn
Ag		0.46*	0.54**		0.39*	0.35 NS
Cu	0.77**		0.89**	0.53**		0.92**
Zn	0.76**	0.71**		0.53**	0.82**	

Values above diagonals represent data for oysters living in normal sea water; values below diagonals, data for oysters reared in brackish water. NS: no significance; * significant at 95% level; ** significant at 99% level

It can be noted that individual variations of metal levels are generally enhanced in brackish water. In silver-exposed oysters, the individual variations of bioaccumulated silver increased with increasing concentrations.

The following features became apparent from the correlation matrix between metal concentrations in individual oysters (Table 2): (a) correlations between Cu and Zn were highly significant in all the experimental conditions; (b) coefficients of correlation between Ag and other metals were higher in brackish water than in sea water.

Biochemical data

Since the limit between soluble and insoluble forms varies according to the experimenter, we have first to make it clear that we consider as insoluble the silver fractions contained in pellets after centrifugation and in the void volume after gel chromatography ($m > 500$ kDa). According to this definition, the silver fraction which was associated with soluble compounds depended on the burden of this metal in the whole soft tissues (Fig. 1). In order to obtain a better estimation of the correlation between these two factors in normal conditions of salinity, data of the present study were mixed with previous results obtained in a similar way (Martoja et al. 1988; Berthet et al. 1990). Whatever the salinity, the proportion of cytosolic silver decreased

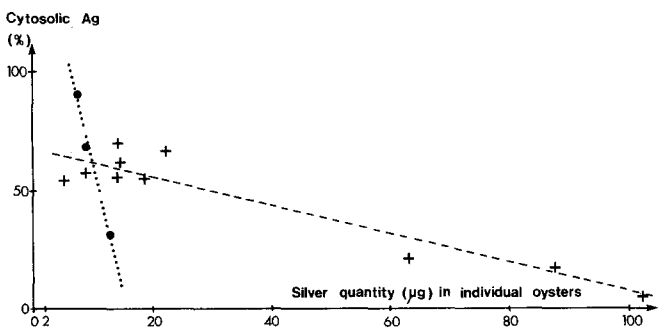

Fig. 1. Relationships between the cytolosic silver and the burden of this metal in the whole soft tissues of oysters. Salinity: (...) 8‰; (---) 33‰

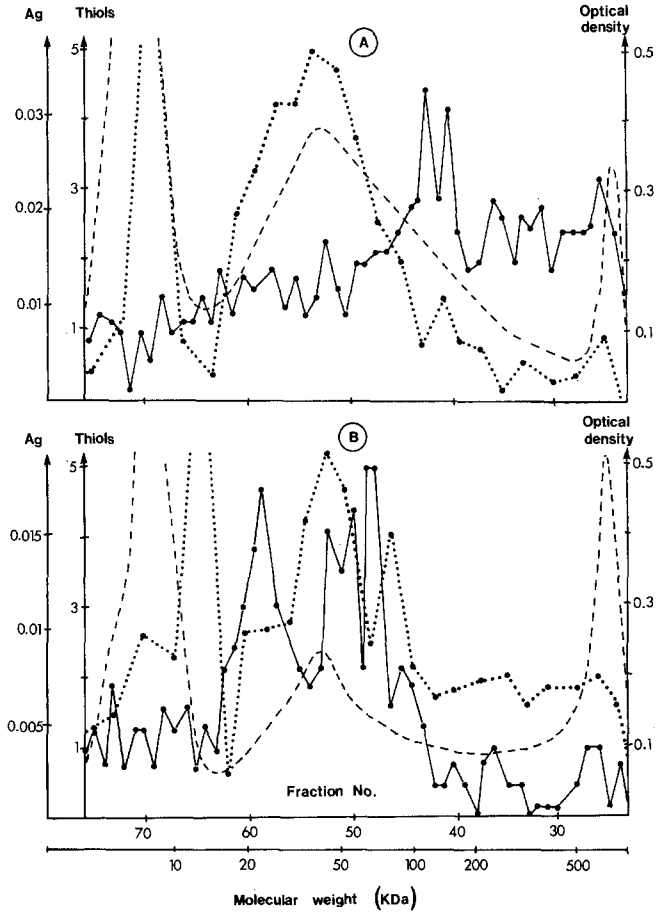

Fig. 2. Controls: concentration ($\mu mol \cdot l^{-1}$) of silver (——) and thiols (...), and optical density at 280 nm (---) in the different fractions separated by Sepharose CL-6B chromatography. Salinity: (**A**) 33‰; (**B**) 8‰. Fraction 25 corresponds to void volume, fraction 70 to total bed volume

with increasing body burden. This decrease was more important in brackish than in sea water.

In controls, the acclimation to brackish water induced a change in silver distribution among the cytosolic fractions (Fig. 2). In sea water (Fig. 2 A), the major part of the metal was associated with high-molecular-mass ligands, whereas the molecular mass of silver-binding compounds was lower in brackish water (Fig. 2 B). In this last case, silver-binding compounds were distributed in thiol-rich fractions. The freshening of sea water also induced a transfer of zinc to lower-molecular-mass ligands (Fig. 3). In sea water, zinc and silver were found at least partly in the same high-molecular-mass fractions; in brackish water, the zinc-binding fractions had a lower molecular mass (< 10 kDa) than silver-binding fractions (Table 3). The copper levels in chromatographic fractions were near the threshold of sensitivity and thus no clear features appeared.

In oysters exposed to silver in normal sea water (Fig. 4 A), this metal was associated mainly with compounds having a molecular mass lower than those of silver-binding ligands in controls (Table 3). A first class of compounds corresponding to a series of small peaks in fractions 39–49, was called α whereas the major peak

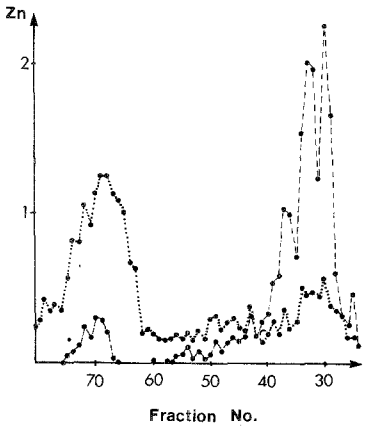

Fig 3. Controls. Influence of salinity on the distribution of zinc ($\mu mol \cdot l^{-1}$) in the cytosol of oysters. Fraction 25 corresponds to void volume, fraction 70 to total bed volume. Salinity: (...) 8‰; (---) 33‰

(fractions 55–60) was called β. In brackish water, the distribution of bioaccumulated silver in the cytosolic fraction exhibited important individual variations. In the first specimen, it was identical to the feature observed in sea water; in the second one, the relative importances of peaks α and β were reversed (Table 3). In the third case (Fig. 4 B), peaks α and β were mingled. Whatever the salinity, an important peak of thiols was determined around fraction 50, i.e. in the zone of peaks α.

In silver-exposed oysters, the freshening of sea water induced a transfer of zinc to medium- and low-molecular-mass fractions (Fig. 5). These medium-molecular-mass fractions (i.e. 40–50 kDa) also contained silver (Table 3). In contrast, in contaminated oysters living in sea water, the peaks of silver and zinc did not correspond to the same fractions (Table 3).

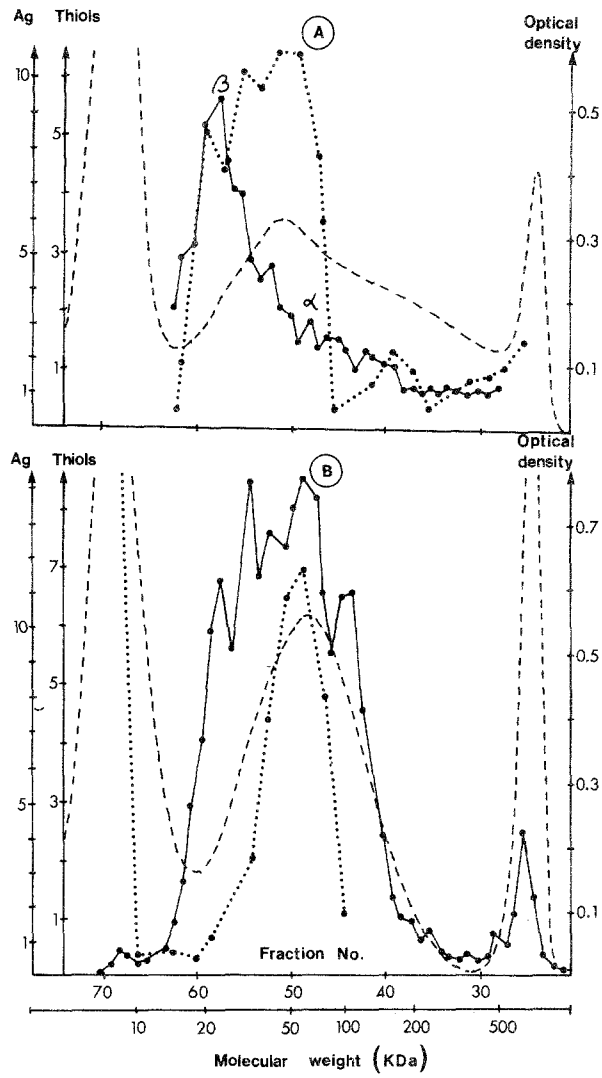

Fig. 4. Silver-exposed oysters. Details as in Fig. 2. Salinity: (**A**) 33‰; (**B**) 8‰ (Ag distribution in one individual; see description of others in the text)

Histological and microanalytical data

In oysters acclimated to brackish water, the number of amoebocytes was considerably increased (Fig. 6), but in the cells the intensities of the X-rays for Cu and Zn did not depend on salinity; in both brackish and sea water, intensities for Zn were much greater than for Cu. Just as for the oysters living in sea water, the contamination induced the precipitation of silver sulfide in the basement membranes and in the amoebocytes of brackish-water-adapted samples. However, a change occurred in

Table 3. Distribution of cytosolic silver and zinc in oysters

Rearing	Salinity	No. of Zn-containing fractions		No. of Ag-containing fractions	
Controls	33‰	29 to 34	68 to 72	24 to 44	
Controls	8‰	27 to 34	65 to 72		45 to 60
Ag-exposed	33‰	30 to 40	67 to 71	39 to 49 (α)	55 to 60 (β)
Ag-exposed	8‰	45 to 58	68 to 79	42 to 51* (α)	55 to 62* (β)
Ag-exposed	8‰			42 to 51* (α)	55 to 59* (β)
Ag-exposed	8‰			42 to 59*	

Double-underlined values: major peaks of metals; single-underlined: medium peaks; no underlined: weak peaks; * data for individual oysters; in all other cases, data are identical in replicates

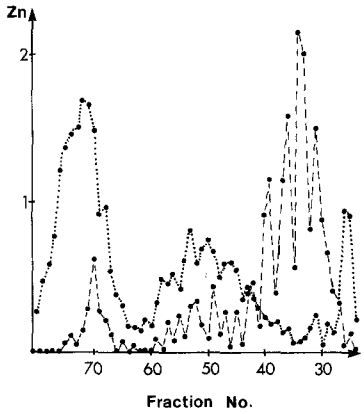

Fig. 5. Silver-exposed oysters: influence of salinity on the distribution of zinc ($\mu mol \cdot l^{-1}$) in the cytosol of oysters. Fraction 25 corresponds to void volume, fraction 70 to total bed volume. Salinity: (...) 8‰; (---) 33‰

Discussion and conclusion

Copper, zinc and silver accumulations in oysters are inversely related to salinity. In Chesapeake Bay, this relationship has been established by determining metal concentrations in *Crassostrea virginica* sampled from different stations of high and low salinity regimes (Phelps et al. 1985). Year-to-year comparison (1978–1981) between these data reveals that the negative correlation is always significant between Cu or Zn levels and salinity, whereas it is only occasionally significant in the case of silver. Wright and Zamuda (1987) have shown that the salinity effect was independent of cupric ion activity and, since the occurrence of Cl^- is always sufficient to induce the formation of di-, tri- and tetrachloro-argentates, the effects of salinity on the response of oysters to silver exposure also depend mainly on a biological phenomenon.

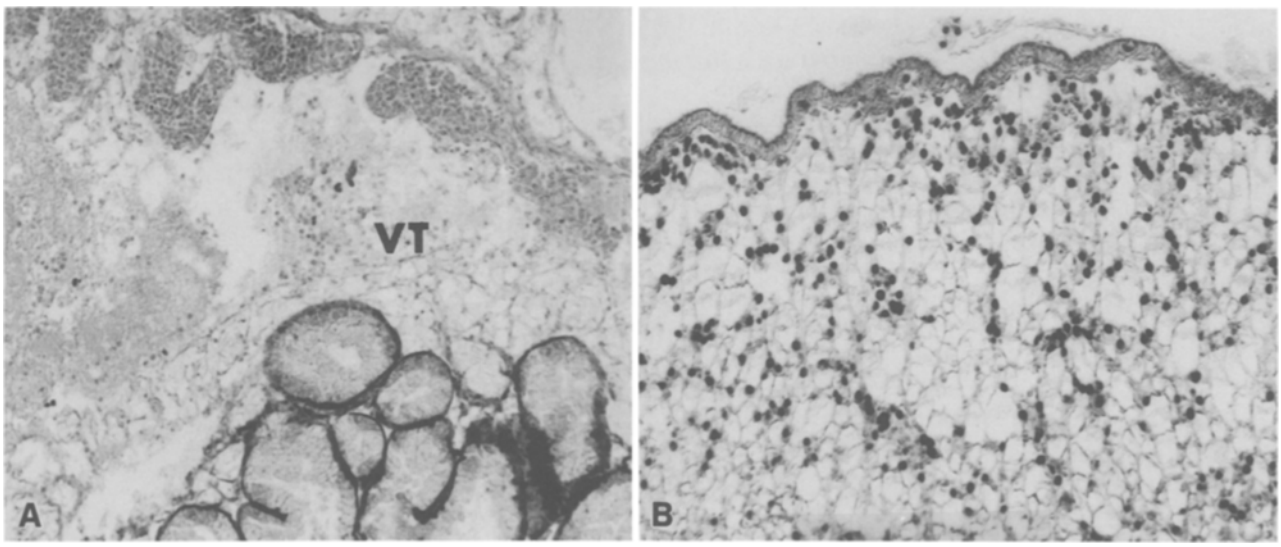

Fig. 6. Influence of salinity on the accumulation of silver sulfide. (**A**) In oysters exposed to silver in normal sea water, the deposit is located in the basement membranes of the digestive tubules (in black) whereas the vesiculous tissue (VT) is devoid of silver-containing amoebocytes. Magnification ×250. (**B**) In brackish water, the number of silver-loaded amoebocytes (in black) is very important in the vesiculous tissue. Magnification ×300

the distribution of the deposit among the histological structures: because the number of amoebocytes was increased in brackish water, the intracellular storage of silver was enhanced whilst the extracellular one was reduced since the sulfide of the basement membranes was observed almost solely in the digestive tubules (Fig. 7). In brackish water, amoebocytes form clusters beneath the internal pallial epithelium and the epithelium of the gills (Figs. 7 B and 8); an important extrusion of silver-sulfide-containing amoebocytes occurred through these epitheliums (Fig. 8 B). No organ showed any histological lesions. In contrast to the oysters that were contaminated in sea water, the storage of glycogen in the vesicular tissue was as important as in controls.

Global fluctuations of metal concentrations in the soft tissues of *C. gigas* are associated with changes at the biochemical and histological levels. The freshening of sea water induced a change in the distribution of cytosolic silver and zinc among the fractions separated by gel chromatography but the coincidence of the peaks corresponding to each metal was never perfect.

From previous studies mentioned by Thomson et al. (1985), amoebocytes play a key role in accumulating copper and zinc in oysters. According to these authors, the amoebocytes contain more than 90% of the body copper and zinc of *C. gigas*. In this species, an important part of the silver is sequestered as silver sulfide in amoebocytes but also in basement membranes (Martoja et al. 1988; Berthet et al. 1990). In the present study, we have shown that the proportion of insoluble silver in-

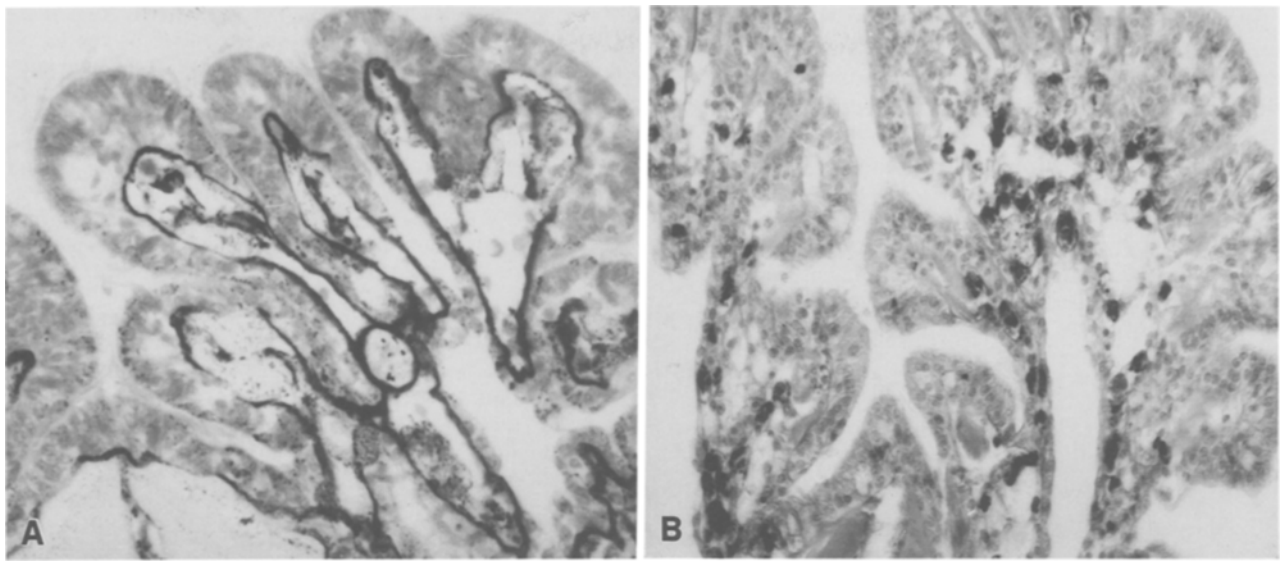

Fig. 7. Influence of salinity on the accumulation of silver sulfide in the gills. (**A**) In normal sea water, the deposit (in black) is located in the basement membranes. Magnification ×300. (**B**) In brackish water, the deposit (in black) is located in the amoebocytes. Magnification ×300

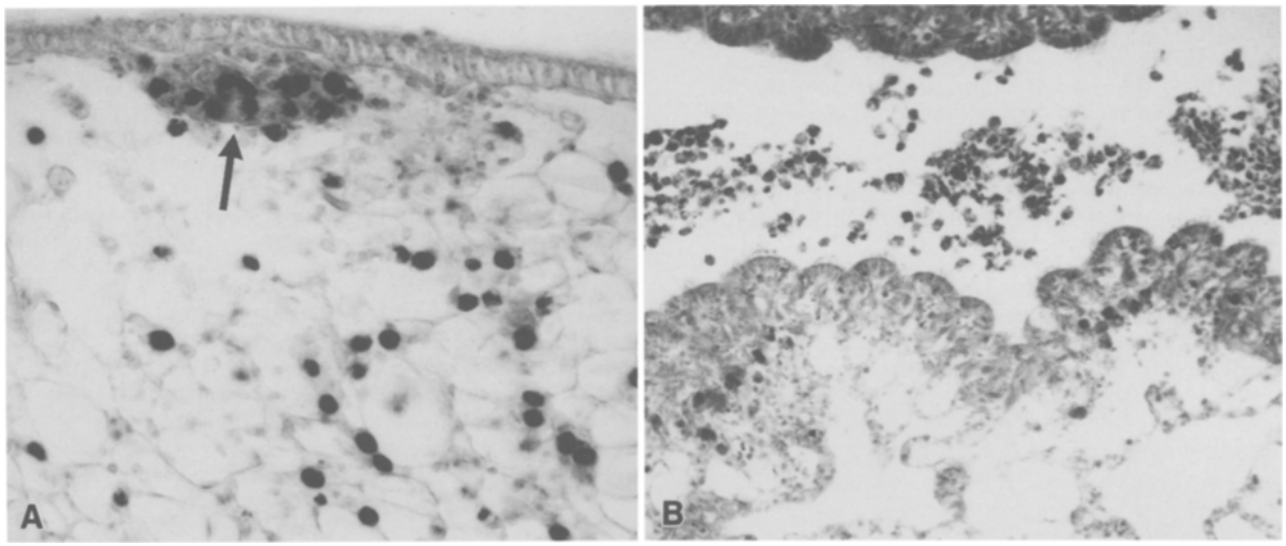

Fig. 8. Oysters exposed to silver in brackish water. (**A**) Cluster of silver-loaded amoebocytes beneath the internal palleal epithelium. Magnification ×500. (**B**) Clusters of silver-containing amoebocytes that have been extruded through the epithelium of the gills. Magnification ×120

creases with increasing body burden. The phenomenon was particularly well marked in brackish water.

The freshening of sea water induces an enhancement of these amoebocytes which may be considered as responsible for the increase of copper and zinc levels observed in the whole soft tissues, since these cells penetrate all of the body tissues (George et al. 1978; Martoja et al. 1988). The higher number of amoebocytes in brackish water favours the accumulation of silver experimentally added in these structures rather than in the basement membranes. In these conditions, the metabolism of silver parallels those of copper and zinc. In contrast, in normal sea water, basement membranes and amoebocytes play equivalent roles and thus silver metabolism differs, at least partly, from those of copper and zinc. These facts explain why correlations between silver and both the other metal levels in the whole soft tissues were more significant in brackish water than in sea water.

If the increase of silver concentrations in the whole soft tissues with decreasing salinities is ascribable mainly to the accumulation of silver sulfide in amoebocytes, the changes of silver binding in the soluble phase must not be neglected since the speciation of metals influences their toxicity. In the present study, we have shown that the effect of silver upon glycogen storage was cancelled in brackish water.

The purpose of the present investigation was also to determine whether accumulation of Ag by oysters is mediated by the same mechanisms as those for other trace elements such as Cu or Zn. The results clearly show that different storage mechanisms for Ag are present. A similar conclusion was drawn by George et al. (1986) from their investigations in *M. edulis*.

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