

Sublethal and histopathological effects of trace levels of tributyltin fluoride on adult oysters *Crassostrea gigas*

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Abstract

Shell malformations and histological effects of environmental concentrations of TBTF (2, 13.1 and 64.8 ng/l) were assessed on adult oysters *Crassostrea gigas* during a 1-month experiment, including both an exposure and a depuration phase. The results showed that the digestive gland is the primary target organ. Recoverable modifications were observed at the lowest concentration. Limited necrosis was observed at the highest concentration and a longer exposure could have led to extensive and irreversible tissue lesions. Shell malformations (chambering) were observed during the depuration phase. Finally the authors suggest that safe TBT levels in mariculture waters should be lower than 2 ng/l.

Keywords : Tributyltin, *Crassostrea gigas*, histopathological effects.

Titre français à venir.

Résumé

Les malformations des coquilles et les effets histologiques du TBTF à des concentrations rencontrées dans l'environnement (2, 13.1 et 64.8 ng/l) ont été évalués lors d'une expérimentation ayant duré 1 mois et comportant une phase d'exposition et une phase en eau non contaminée. Les résultats montrent que les diverticules digestifs sont les organes cibles primaires. Des modifications réversibles ont été observées à la plus faible concentration. Des nécroses partielles ont été observées à la plus forte concentration et une plus longue exposition aurait pu occasionner des lésions tissulaires étendues et irréversibles. Des malformations des coquilles (chambrage) ont été observées pendant la phase en eau non contaminée. Finalement, les auteurs suggèrent que les teneurs en TBT admissibles dans les eaux conchylicoles devraient être inférieures à 2 ng/l.

Mots-clés : Tributylétain, *Crassostrea gigas*, effets histopathologiques.

Tributyltin (TBT) is widely used as antifoulant in paints for protection of hulls and other immersed surfaces. Consequently, increasing quantities of TBT have been introduced into marine coastal waters. In France, as early as 1981, TBT contamination of oyster culture areas has been strongly suspected of having deleterious effects on reproduction and growth of Pacific oysters *Crassostrea gigas*. It has now been shown clearly that tributyltins are highly toxic for marine molluscs:

— larval development of *C. gigas* is affected when seawater contamination is higher than 20 ng/l (His and Robert, 1983, 1985);

— anomalies of calcification (thickening) have been experimentally produced at levels as low as 20 ng/l (Gendron, 1985) and field correlations observed between TBT contamination and frequency of *C. gigas* shell anomalies (Alzieu *et al.*, 1989);

— decline in populations of the gastropod *Nucella lapillus* associated with imposex has been observed at

TBT concentrations lower than 1 ng/l (Gibbs and Bryan, 1986).

In contrast, few data are available on histopathological effects of TBT on molluscs. Tissue lesions have been described only in the freshwater fishes *Salmo gairdneri* and *Tilapia rendalli* (Chliamovitch and Kuhn, 1977).

We developed a 3 week-long laboratory experiment designed to evaluate histopathological effects of environmental TBT levels on adult *C. gigas* and the recovery potential after oysters are returned to clean water.

MATERIAL AND METHODS

General procedures

Adults *C. gigas* were collected in Marennes-Oléron Bay, which is the main farming site of cup-shaped oysters in France. Once specimens were taken from the beds, the oysters were initially stored for over 15 days in land-based tanks and later allowed to acclimate for 4 days in the laboratory environment. They were then divided into four groups of 150 individuals homogeneous in size: individual mean weight for each group ranged from 52 to 64 g. Each group was held separately in a 200 l tank in a flow-through seawater system at ambient temperature (18°C). The flow of filtered (10 µm) natural seawater (salinity $29-30 \times 10^{-3}$) was 40 l/h and food (diatoms) as well as oxygen were artificially supplied.

TBT fluoride (Fluka) dissolved in ethanol was delivered by a peristaltic pump. All glassware and teflon tubing were previously acid washed and kept in the dark during the assay to prevent any TBT photolysis. TBT concentrations in the water of each tank were checked every 2 days using the analytical method described by Michel (1987) (hydride generation/Atomic Absorption Spectrometry). During the experiment, the mean concentrations in experimental tanks were 64.8 (SD=5); 13.1 (SD=2) and 2 ng/l (SD=0.5); the control tank was below the detection limit (1 ng/l). TBT degradation products, dibutyltin or monobutyltin, were below the detection limit (0.5 ng/l).

Animals were kept in contaminated tanks during 18 days (contamination phase) and then held for 21 days in clean water (deuration phase). Every 3 days, five oysters were sampled from the low and medium concentration groups, in order to analyse the TBT content in their tissues. At days 0, 3, 10, 13 corresponding to the contamination phase, and 6, 14 and 20 of the deuration phase, five live individuals from each group were sampled and fixed for histological examinations. Mortalities were recorded daily. Dead and sacrificed animals were checked for calcification anomalies of the shells (chambers).

Histological procedure

After breaking the ligament, the flat valve was lifted and the adductor muscle was cut, taking care not to damage the underlying body. Whole animals were immersed for 24 hours in pH 7.4 Carson's fixative [NaH₂PO₄: 23.8 g; NaOH: 5 g; distilled water: 900 ml; 40% formalin, filtered, 100 ml, according to Pearse (1980)]. Two cross sections were then made, in order to permit examination of the stomach, intestine, digestive diverticula, gills, mantle, gonad, and kidney. Tissues were left for another 24 hours in a fixative, dehydrated with ethanol, cleared with xylene and embedded in paraffin. Sections (3 µm) were cut, and stained with Ehrlich's hematoxylin and eosin (EHE) for general examination and with periodic acid-Schiff (PAS) for glycogen and mucopolysaccharide evaluation.

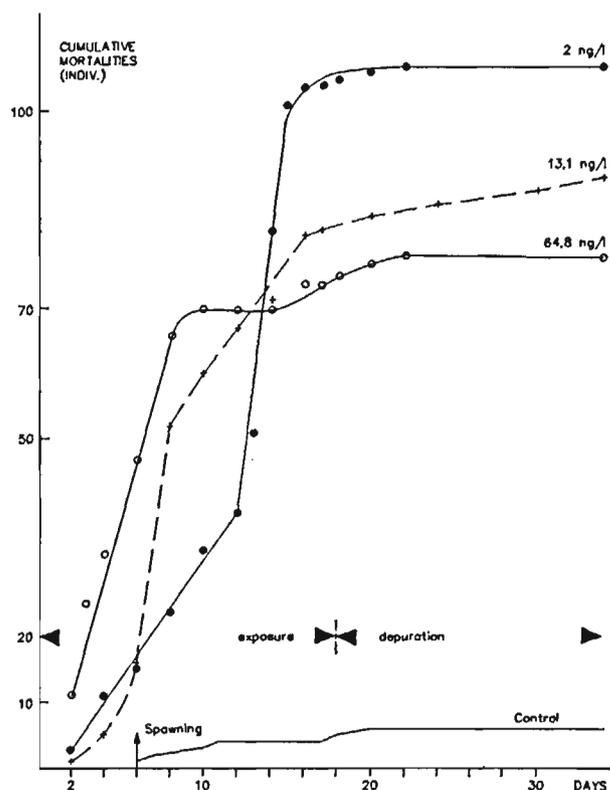


Figure 1. — Cumulative mortalities during the experiment.

RESULTS

Mortalities

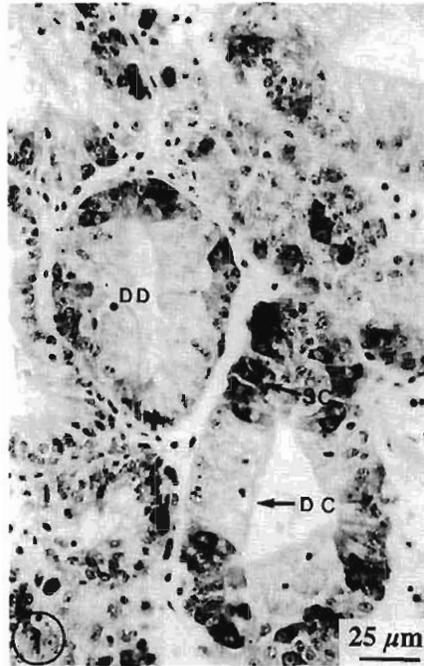
The observed cumulative mortalities are presented in figure 1. In each one of the treated groups, mortality was high when compared to the control group. Up to treatment day 13, it was roughly dose-dependent, but occurred regardless of TBT concentration from day 13 to day 18. It should be mentioned that some oysters began to spawn at day 6, which might indicate a reproduction-linked individual variability in response to TBT.

Days of experiment	Contamination phase			Depuration phase		
	3	10	13	6	14	20
TBTF ng/l 2	No effect	VCT moderate increase in hemocyte number in blood sinuses.	DD:CD some tubules are flattened, weak PAS positivity. VCT hemocytic infiltration still moderate.	Digestive gland returns to normal.	-	-
13.1	No effect	DD:CD fragmentation of apical cytoplasm in some digestive tubules, PAS positivity reduced. VCT infiltration of hemolymph spaces by numerous hemocytes and "brown cells".	DD:CD general decrease in height of epithelia digestive tubules, cuboidal cells presenting large unstained empty vacuoles and dilated lumen, some necrotic tubules. VCT strong decay in PAS positivity (<i>fig. 2.10</i>) hemocytosis in blood spaces increased. G. vacuolated cells, mucous cells staining faint, erosion of frontal cells in some filaments acidophilic cells unaffected. K. swelling of epithelial cells, erosion of apical cytoplasm - no signs of necrosis.	Repair pattern basically the same as in oysters exposed to higher concentration (64.8 µg/l).		
64.8	DD:CD pale staining of digestive cells with EHE some with apical cytoplasm fragmentation, acidophilic cell debris in lumen (<i>fig. 2.2</i>). PAS positivity reduced in digestive and branchial cells (<i>fig. 2.13</i>).	DD:CD apical fragmentation increased, cuboidal aspect of some digestive cells, lumen empty and dilated, basal part retained, crypt cells unaffected (<i>fig. 2.5</i>), PAS positivity strongly reduced (<i>fig. 2.4</i>). VCT hemolymph spaces moderately infiltrated with hemocytes and brown cells, Leydig cells: PAS positivity strongly decreased. G. high vacuolization, spongy cytoplasm, weak PAS positivity.	DD:CD necrosis of digestive epithelial cells (<i>fig. 2.6</i>), basal lamina ruptured in some tubules. VCT hemolymph spaces heavily infiltrated with hemocytes (<i>fig. 2.8</i>), increased number of brown cells. G. general decrease staining of mucous and acidophilic cells break off of frontal and lateral cilia, apical erosion of cytoplasm of frontal cells (<i>fig. 2.12</i>). K. hypertrophy of epithelia of nephridial tubes, cells with small acidophilic granulations and apical swelling, some tubules necrotic, hemocytes in the lumen (<i>fig. 2.16</i>).	DD:CD some digestive tubules lined by strong basiphilic cells - others still necrotic. VCT heavy hemocytic infiltration tendency of pigmented granules of brown cells to fuse. G. epithelial cells remain vacuolated - normal staining of mucous and acidophilic cells. K. epithelia remain swollen - heavy accumulation of +/- pycnotic hemocytes in the lumen.	Tissue appearance returns normal (<i>fig. 2.17</i>) except vacuolization of gill epithelia (<i>fig. 2.18</i>) and hemocytosis (<i>fig. 2.19</i>).	No evolution compared to day 14.

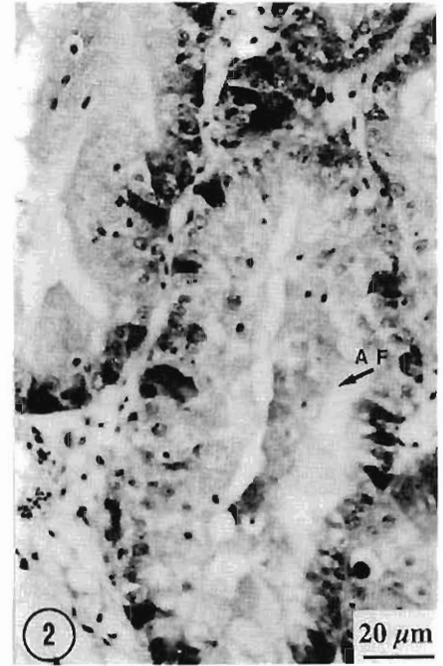
Table 1. - Histopathological observations in digestive diverticula (DD) and collecting ducts (CD), vesicular connective tissues (VCT), gills (G) and kidney (K) during the contamination and depuration phase.

Figure 2. – TBT-induced cell injury in target tissues.

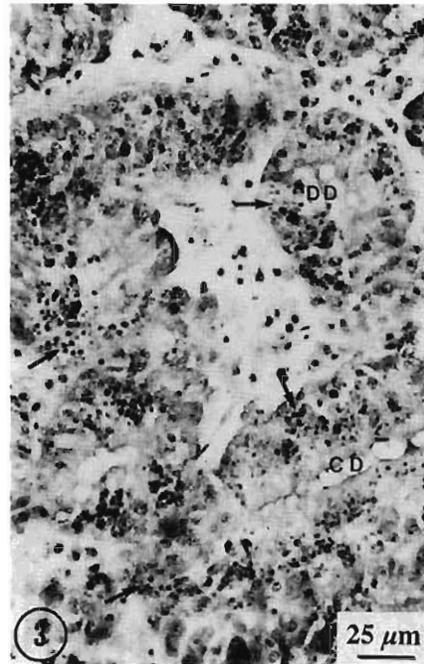
2.1. Ehrlich's hematoxylin eosin (EHE).
Control digestive gland (contamination phase, day 13).
DD = digestive diverticula.
DC = digestive cell.
SC = secretory cell.



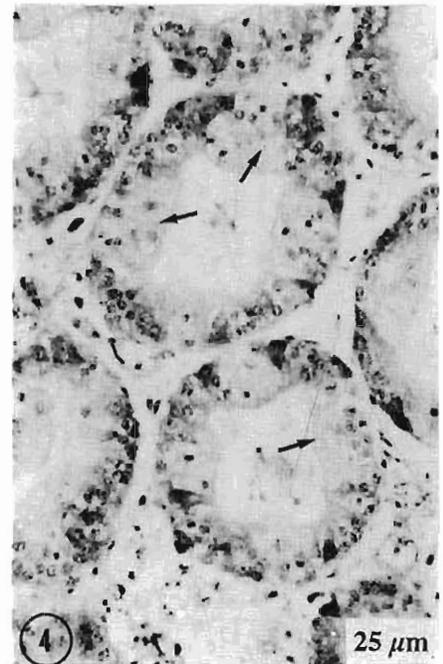
2.2. EHE.
64.8 ng/l TBT-treated digestive gland (contamination phase, day 3).
Apical cytoplasm fragmentation (AF).



2.3. Periodic acid-Schiff (PAS).
Control digestive gland (contamination phase, day 3).
DD = digestive diverticula.
CD = collecting ducts.
Arrows indicate PAS-positive granules.



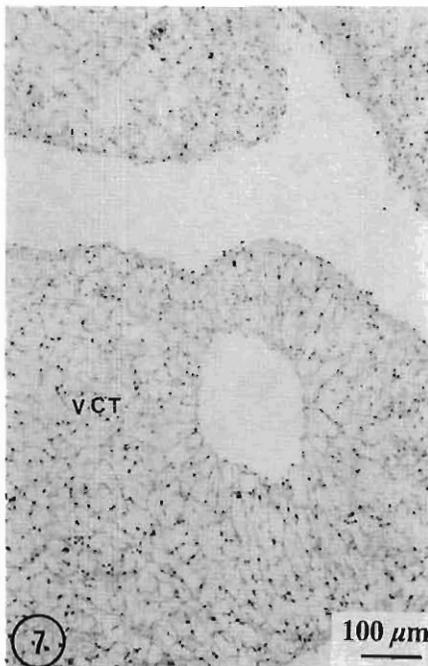
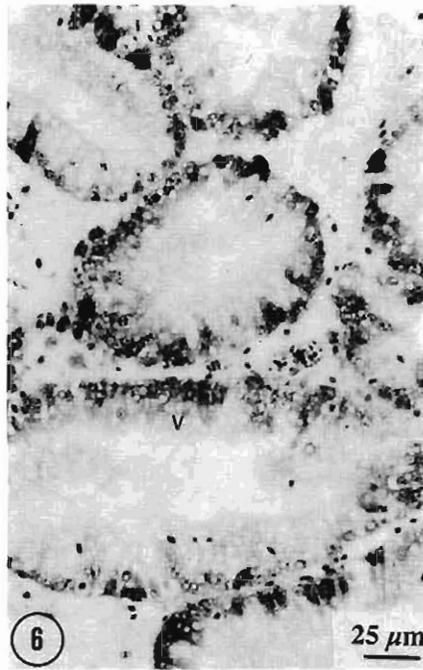
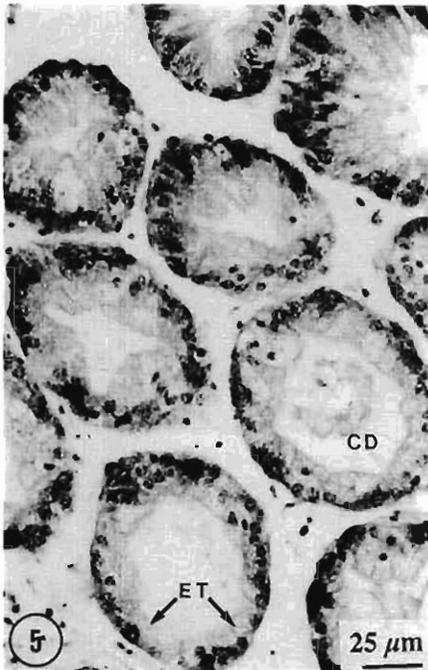
2.4. PAS.
64.8 ng/l TBT-treated digestive gland (contamination phase, day 10).
No PAS positivity in digestive cells (arrows).



It is well known that the spawning period corresponds to a phase of increased mollusc sensitivity to changes in the environment (salinity, oxygen, toxicity...). In our experiment, mortalities appeared in the control group from the first day of spawning and remained thereafter at a very low level. Conversely, in the case of TBT-exposed specimens, mortalities were observed as early as the first days and increased significantly following spawning. This enables us to regard the observed mortalities as attributable to TBT action and not to any experimental artifacts.

Bioaccumulation

During the accumulation phase, a plateau of TBT contamination was not reached by any of the analysed groups. However, during this period, TBT content in tissue increased from 6.5 ng/g dry weight to 12.6 and 49.8 ng/g respectively for exposures of 2 and 13.1 ng/l. At the end of the depuration phase, levels were 7.3 and 27.7 ng/g, showing a slow elimination rate. Comparatively, contamination of the control groups remained constant during both phases: mean value 4.6 ± 0.8 ng/g. Dibutyltin was the major degradation



2.5. EHE.

64.8 ng/l TBT-treated digestive gland (contamination phase, day 10) showing epithelial thinning (ET) and cell debris (CD) in the lumen of digestive diverticula. The general aspect is prenerotic.

2.6. EHE.

64.8 ng/l TNT-treated digestive gland, (contamination phase, day 13). High vacuolisation (V) and necrosis.

2.7. EHE.

Control vesicular connective tissue (VCT) (contamination phase, day 13).

2.8. EHE.

64.8 ng/l TBT-treated vesicular connective tissue showing massive infiltration (arrows) with hemocytes (H).

product measured in tissues; its concentration increased sharply during the exposure period and then decreased slowly. Thus for the group exposed to 13.1 ng/l of TBT, DBT contamination which had risen from 2.8 ng/g dry weight to 35.6 ng/g during the exposure phase, remained very high even after 21 days of depuration (26.7 ng/g dry weight).

Histological observations

Detailed results are presented in *table 1* which shows the histopathological changes recorded for each

group, at specific time intervals for each target organ. Pathological effects were roughly similar in the different groups treated, but the time sequence and the severity of injury differed. No histopathological effect was observed in the control animals during the experiment: *see figures 2.1, 2.3, 2.7, 2.9, 2.11, 2.14 and 2.15.*

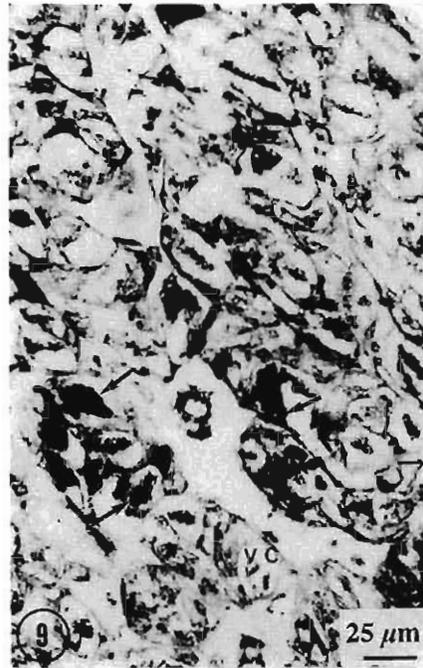
Shell malformations

Shell chamber formation was not observed during the accumulation phase, and none of the control

2.9. PAS.

Control VCT (contamination phase, day 10).

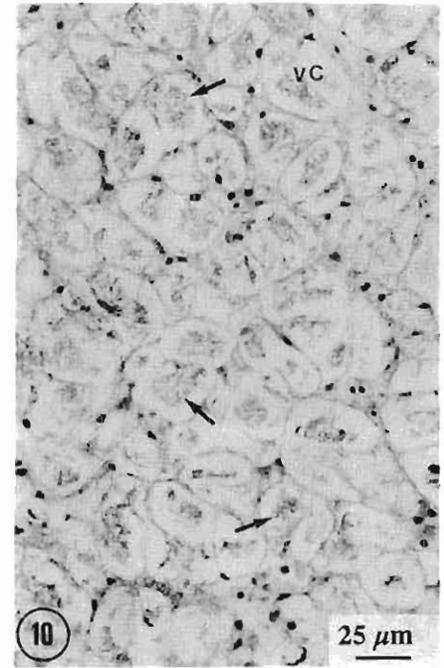
VC=vesicular cells, arrows show PAS-positive glycogen storage areas.



2.10. PAS.

13.1 ng/l TBT-treated VCT (contamination phase, day 10).

Glycogen content is strongly reduced (arrows).



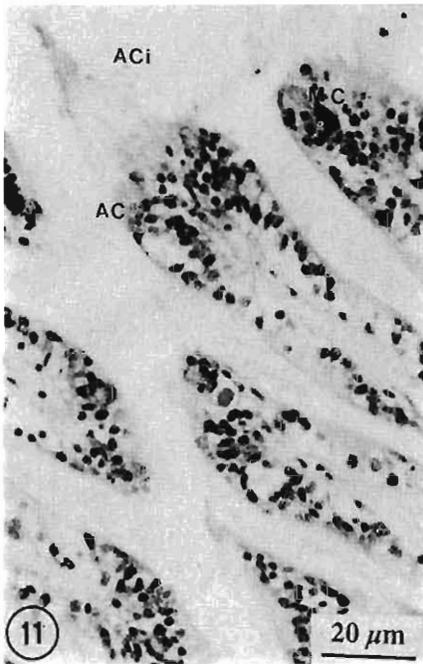
2.11. EHE.

Control branchial filaments (contamination phase, day 13).

AC=acidophilic cell.

MC=mucous cell.

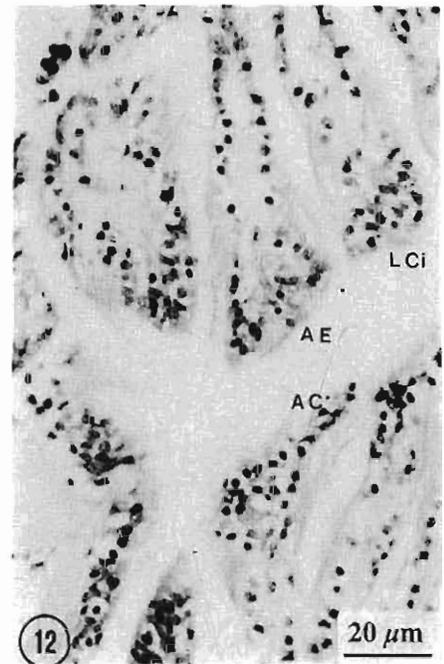
ACi= Apical cilia.



2.12. EHE.

64.8 ng/l TBT-treated gills (contamination phase, day 13).

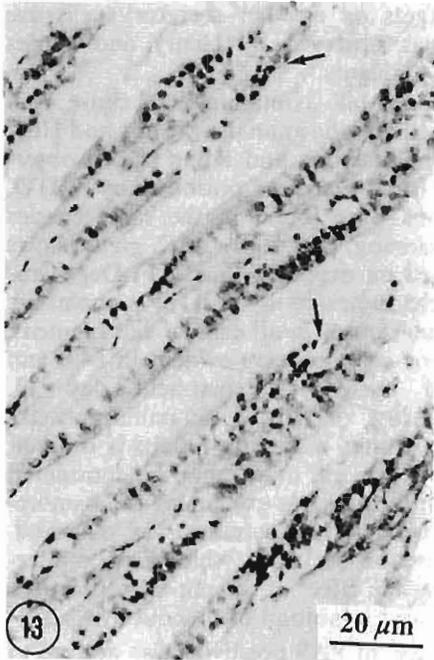
Filaments show apical erosion (AE) and loss of cilia (LCi). Faint staining of acidophilic cells (AC). No evidence of any mucous cells.



animals had chambers during the two experimental phases. However, 7 of the 15 animals sacrificed during the depuration phase presented this abnormality in the 64.8 ng/l treated group, compared to one in the 13.1 ng/l group and none in the 2 ng TBTF/l group. Nevertheless, in a separate assay, where TBT concentrations varied between 2 and less than 2 ng/l, anomalies in calcification were observed in a significant number of individuals (13%).

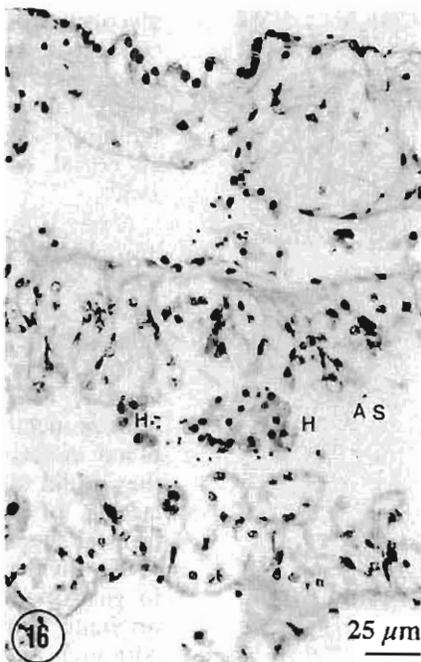
DISCUSSION

The present report provides evidence for TBT-induced cell injury in defined target tissues of the Pacific oyster *C. gigas*, such as digestive diverticula, gills, vesicular connective tissue, hemal tissue and kidney. Although the observed histopathological changes cannot be linked with the relatively high mortalities observed during exposure and probably due to spawning, their severity appears to be both dose and time-



2.13. PAS.
64.8 ng/l TBT-treated gill filaments (contamination phase, day 3). Absence of PAS-positive mucous cells (arrows).

2.14. PAS.
Control gill filaments (contamination phase, day 3) showing numerous PAS-positive areas, mostly representing mucous cells (arrows).



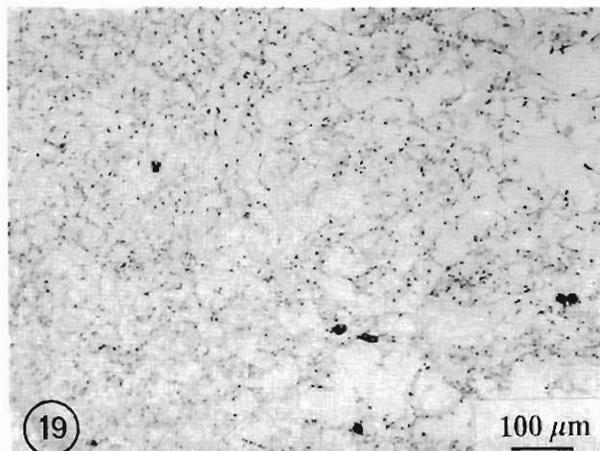
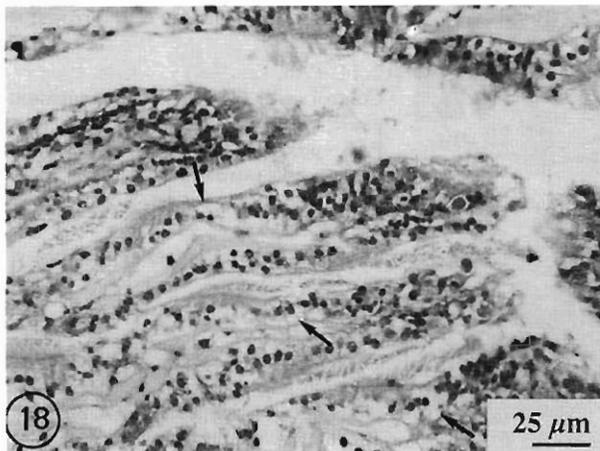
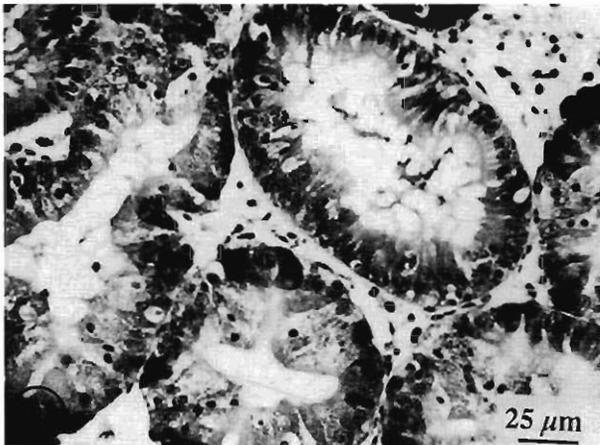
2.15. EHE.
Control kidney (contamination phase, day 13).

2.16. EHE.
64.8 ng/l TNT-treated kidney (contamination phase, day 13). Epithelial cells show apical swelling (AS) and necrosis. The lumen contains hemocytes (H).

dependent. Furthermore, the data acquired suggest that poisoning results from different mechanisms.

Although there are few studies devoted to histopathological effects of pollutants in marine molluscan species, digestive diverticula modifications such as intensive fragmentation, vacuolization, epithelial thinning have been noted (Tripp *et al.*, 1984; Couch, 1984; Rasmussen, 1982; Rasmussen *et al.*, 1985). Such modifications could be considered as a general molluscan response to stress (Moore *et al.*, 1979; Lowe *et al.*, 1981) and have been interpreted as a physiological

survival mechanism of bivalves subjected to stress (Moore *et al.*, 1979; Henry, 1987). This hypothesis is consistent with the observed rapid regeneration capacity of the organ. However limited necrosis, indicative of a degenerative syndrome was also conspicuous in digestive cells, and longer exposure might have led to extensive and irreversible breakdown, by affecting the potential regenerative cells of the crypts. Digestive cells appear to be the first ones affected on a structural scale. However, an ultrastructural study will be required to determine whether they are truly



2. 17. EHE.
64.8 ng/l TBT-treated digestive gland (deuration phase, day 14) - aspect normal.

2. 18. EHE.
64.8 ng/l TBT-treated gills (deuration phase, day 14) - vacuolisation still present (arrows) and cilia lost in apical part of some filaments.

2. 19. EHE.
64.8 ng/l TBT-treated VCT (deuration phase, day 14) - hemocytic infiltration still present.

the primary targets or whether secretory cells are more sensitive, as observed by Henry and Carles (1985) for other toxicants.

Most environmental contaminants cause gill damage in exposed aquatic animals (Meyers and Hendricks, 1982). Chliamovitch and Kuhn (1977) observed gill lesions in *S. gairdneri* poisoned by TBTO, but no alterations were found in the same species after TBTCI poisoning. Our study in *C. gigas* shows gills to be affected on exposure to TBTF. Degenerative changes were indicated by loss of cytoplasmic density and vacuolization in all cells of the filament. Advanced necrotic changes were evident in 64.8 and 13.1 ng/l-exposed oysters at treatment day 13. Whether or not loss of cilia accompanies general autolysis, or constitutes a specific lesion, is difficult to ascertain. However, gill injury may be considered to severely impair feeding, gas exchanges and osmoregulation. No mucous hypersecretion was observed. Instead, hyposecretion occurred, which can be interpreted either as a side effect of the pre-necrotic phase or as a TBT-induced inhibition of mucous synthesis.

A strong decrease of PAS positivity was noticed in branchial and digestive cells, as well as in the main glycogen storage cells, *i.e.* the vesicular cells of the connective tissue. Reduced glycogen storage has been noted in carp and in *S. gairdneri* (Thompson *et al.*, 1985). Glycogen breakdown could arise from the inhibitory effect of triorganotin compounds on mitochondrial energy conservation (Thompson *et al.*, 1985).

Other effects in *C. gigas* include hemocytic and "brown cells" infiltration and kidney hypertrophy, which we feel could be discussed together. The hemocytes of molluscs have various physiological functions including metal storage (Ruddell and Rains, 1975; George *et al.*, 1978; Cheng, 1981; Simkiss and Mason, 1983). It may be possible that hemocytes remove metals from the hemolymph, thereby keeping blood concentration below toxic level. In addition, they could take the compounds expelled in the basal lamina of digestive and branchial epithelia and transfer them to the kidney (Ballan-Dufrançais *et al.*, 1985). Bivalve "brown cells" or serous cells are similar to gastropod pore cells (Martoja *et al.*, 1985) and originate from pericardial glands (Cheng, 1981). Although they do not concentrate silver as sulfide in *C. gigas*, they do concentrate this compound in *Pecten maximus* (Martoja *et al.*, 1985). The physiology of the excretory system in bivalves is largely unknown, but numerous studies have shown evidence for concentration of metals in the kidney (Simkiss and Mason, 1983). Herwig and Holwerda (1986) have demonstrated the accumulation of DBTCI in kidney cells of the freshwater mussel *Anodonta anatina*. Lack of extensive kidney damage, presence of hemocytes in the lumen of renal tubules, a presumed "throwing up" (Owen, 1972), accumulation of hemocytes and brown cells in blood spaces and under basal lamina could all indicate an extensive detoxifying process. It

is worth mentioning that tissue recovery was relatively slow at medium and high dose, with gill vacuolation and hemocytosis persisting as long as 20 days after poisoning.

No damage was detected under the light microscope in the mantle epithelia. However, normal metabolism was affected as assessed by chamber formation, due to abnormal conchiolin secretion. This could confirm Krampitz experiments (Krampitz *et al.*, 1983) establishing that abnormal protein secretion is caused by the perturbation of molecular genetic mechanisms.

CONCLUSION

Observations of shell malformations lead us to consider that the threshold limit for induction of chambers in *C. gigas* shells should be roughly 2 ng/l. This value is consistent with the field observations on Arcachon Bay oysters, showing anomalies in areas where TBT contamination was in the range of less than 2 to 5 ng/l (Alzieu *et al.*, 1989).

Our results confirm the extreme harmfulness of TBT for adult Pacific oyster *C. gigas*. Histological modifications on the cells of digestive diverticula, the primary target organ, are observed when oysters are exposed to concentrations as low as 2 ng/l. This contamination is now considered as a usual level in well-flushed mariculture areas of the French Atlantic coast. Nevertheless, in case of exposure up to 2 weeks, tissues will regain their normal aspect within 6 days. In contrast, cell damage is more severe after 2 weeks of exposure at 13.1 ng/l, a contamination level occasionally found in certain mariculture area. In this case, gill epithelia do not recover even after 20 days following the end of exposure. Assuming that severe digestive and gill cell injuries could have a negative effect on oyster metabolism, and considering the probable threshold for shell malformations, our data strongly suggest that safe TBT levels in the waters of oyster culture areas should be lower than 2 ng/l.

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