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# Hepatic alterations and induction of micronuclei in rainbow trout (*Oncorhynchus mykiss*) exposed to a textile industry effluent

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**Summary.** Rainbow trout, *Oncorhynchus mykiss*, were exposed to a sublethal dose of a wool shrinkproofing effluent for 15, 30, 45 and 60 days. Liver and blood samples were taken after the exposure time together with samples of control handled fish. A light and electron microscope study was carried out to evaluate the histopathological lesions induced in the liver of treated fish. The genotoxic potential of the effluent was assessed by piscine micronucleus test. Vacuolation of liver bile preductular cells was observed in all exposed fish; abnormal lipid accumulation and basophilic foci were seen in the liver of one 30-days- and one 45-days-exposed fish, respectively. These specific alterations could be related to a pre-carcinogenic process. On the contrary, other lesions also described in all treated fish such as dilatation, vesiculation and degranulation of the rough endoplasmic reticulum, altered mitochondria, increase in myelin bodies and lysosomes and presence of phagosomes in wandering macrophages might be considered as non-specific alterations, similar to those described in fish exposed to different pollutants. Frequencies of micronucleated peripheral erythrocytes showed a significant increase following 30-days exposure.

**Key words:** Rainbow trout, Wool shrinkproofing effluent, Liver histopathology, Micronuclei induction

## Introduction

Textile industry poses rather severe environmental problems mainly due to the large volume of wastewater it generates as well as to the complex composition of the effluents produced, which makes their treatment difficult (Stewart, 1983). At present very few studies are available in the literature with regard to the toxicity of these effluents on aquatic organisms (Walsh et al., 1980; Riva et al., 1993; Riva and Vallés, 1994), and to our knowledge, their genotoxic and/or carcinogenic potential, if any, has not been reported so far.

Wool, one of the most appreciated fibers of the textile industry, has a natural tendency to a shrink and felt. One of the shrinkproofing process developed to diminish the fiber friction responsible for the shrinking phenomenon is a treatment based on covering fibers with a resine of polyamine-epichloridine type (Earle, 1970). The effluent resulting from this process contains a mixture of chlorinated compounds, resins, surfactants, etc, which, after being poured out into natural waters, might affect aquatic organisms.

Fish are widely used as experimental models in aquatic pollution monitoring (Eisler, 1986; Hawkins et al., 1988; Overstreet, 1988). Fish liver studies are particularly relevant because most of the enzyme systems related to activation, deactivation and conjugation of metabolites arising from degradation of noxious substances are found in this organ (Winston et al., 1988; Stegeman et al., 1990; Van Veld et al., 1990; Schoor et al., 1991). Also, the activation of pre-carcinogens in the liver is related to the high sensitivity shown by this organ to experimental exposure to several xenobiotics (Hendricks et al., 1984; Harada et al., 1988). Liver tumour development has been reported in fish from heavily polluted environments (Murchelano and Wolke, 1985; Gadner et al., 1989), hepatocytes and bile pre-ductular cells having been shown to be the most altered cell types (Kölher, 1989; Moore et al., 1989; Bodammer and Murchelano, 1990).

Some industrial effluents such as paper mill ones have also been shown to be genotoxic (Das and Nanda, 1986). Micronulei (MN) assay has been successfully applied in fish to measure the genotoxic activity of several compounds under field and laboratory conditions

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(Hooftman and de Raat, 1982; Dinnen et al., 1987; Al-Sabti and Metcalfe, 1995).

In order to elucidate the hepatic lesions induced in the trout by a sublethal dose of a wool shrinkproofing effluent a light and electorn microscope study was undertaken after treating the fish up to 60 days. The piscine MN test was applied to assess the genotoxic potential, if any, of the effluent tested.

## Materials and methods

### *Fish*

Rainbow trout, *Oncorhynchus mykiss* (sexually immature, 8 to 13 cm long), were obtained from a local hatchery and maintained in well-aerated running dechlorinated tap water for at least 2 weeks before experiments. Water conditions, during acclimation period as well as during treatment, were: total hardness: 520±20 mg/l, pH: 8.0±0.3, temperature: 15.5±1 °C. Ammonia and nitrites never reached levels considered to be toxic for salmonids (De Kinkelin et al., 1985). Fish were fed a commercial fish diet at a rate of 1% live weight/d. Individuals for experimentation were replaced in 201 tanks (10 fish/tank). Fish were sacrificed after 15-, 30-, 45- and 60-days exposure to the effluent, using tricaine methane sulfonate (MS 222, Sandoz); 4-5 control and 9 treated fish were used in each sampling period. Control fish were handled in the same way as the treated group. Blood and liver samples for routine light microscope studies were taken after each exposure time. Liver was sampled at 30 and 60 days for a transmission electron microscope study.

### *Wool shrinkproofing effluent*

Waters resulting from the process of chlorination, acidification, neutralization, rinsing, resin supply and softening (Table 1) were collected during the experimental time and stored in glass containers separately at the factory. Samples were stored at 4 °C in darkness in order to minimize changes in their composition. The effluent to be tested was prepared just before experimentation by mixing waters in the same proportion as it is poured into municipal waters by the factory. Experimental animals were exposed to a sublethal concentration (0.3%) of the final mixture. This concentration corresponds to half the 96 h $LC_{50}$ for tout (Marlasca et al., 1993). Exposure was done under semistatic conditions and solutions were replaced every 2 days in order to remove fish excretions, and to maintain the concentration of the effluent constant.

**Table 1.** Process of the wool shrinkproofing treatment and their main components.

| PROCESS | COMPONENTS |
|---|---|
| Chlorination | Solution of sodium dichloro-isocyanate (70gr/l), 55% of chlorine |
| Acidification | 360 ml/l acetic acid 80% and 15 ml/l sulfuric acid 98% |
| Neutralization | 100gr/l sodium hydroxide and sodium bicarbonate; 25 mg/l anhydrous sodium sulphite anhydrous |
| Rinsing | Mainly water |
| Resin supply | 40 ml/l polyamide-epichlorhidrine resin; 10 gr/l sodium bicarbonate |
| Softening | Non-ionic tensiactive; 5 gr/l sodium bicarbonate |

### *Sample processing*

i) Histological study

Samples of liver were fixed in 10% buffered formalin, dehydrated through ethanol series and embedded in paraffin wax. Sections (2-3 $\mu$m) were cut, stained with hematoxyline-eosine and viewed under a light microscope. Sections, 5 $\mu$m thick, were cut for histo-chemical study. Ziëlh-Nielssen and Perl's Prussian blue staining were used to determine the pigment content of melanomacrophages.

ii) Ultrastructural study

Samples of liver were immediately fixed in 5% glutaraldehyde in 0.1M Na cacodylate buffer (pH 7.3), postfixed in 2% $OsO_4$, dehydrated through ethanol series, stained «en bloc» with uranyl acetate and embedded in araldite. Semi-thins sections (1 $\mu$m) were cut, stained with toluidine blue and viewed under a light microscope. Ultra-thin sections were stained with lead-citrate and observed in a HITACHI H-7000 transmission electron microscope.

iii) Piscine micronucleus test

Peripheral blood was obtained in heparinized micro-hematocrit tubes after severance of the caudal vein. A drop of blood was smeared on the slide, air dried, methanol fixed and stained in May Grünwald-Giemsa solutions. Eight slides per fish were obtained, but only the best four were considered. MN scored after counting 500 mature erythrocytes (ME) per slide were recorded (2000 ME per animal). Erythrocytes were scored «blind» and only those with intact cells and nuclear membranes were included in the counts. Isolated nuclear fragments were counted as MN, following morphological criteria described by Tates et al. (1980). The frequencies of MN in pooled control and experimental erythrocytes at each period studied were compared statistically using the tables of Kastenbaum and Bowman (1970).

## Results

### *Histological and ultrastructural study of the liver*

Macroscopic examination of the liver did not show any change either in colour or in gross morphology of

this organ. Light and transmission electron microscope studies revealed the existence of hepatic alterations in effluent-exposed fish. The most striking feature in the liver of treated fish was the vacuolation of bile pre-ductular cells (Fig. 1). This cell type can be identified on the basis of its location within the hepatic tubule, its cytoplasm, and nuclear morphology according to the descriptions of Hampton et al. (1989). Initial changes

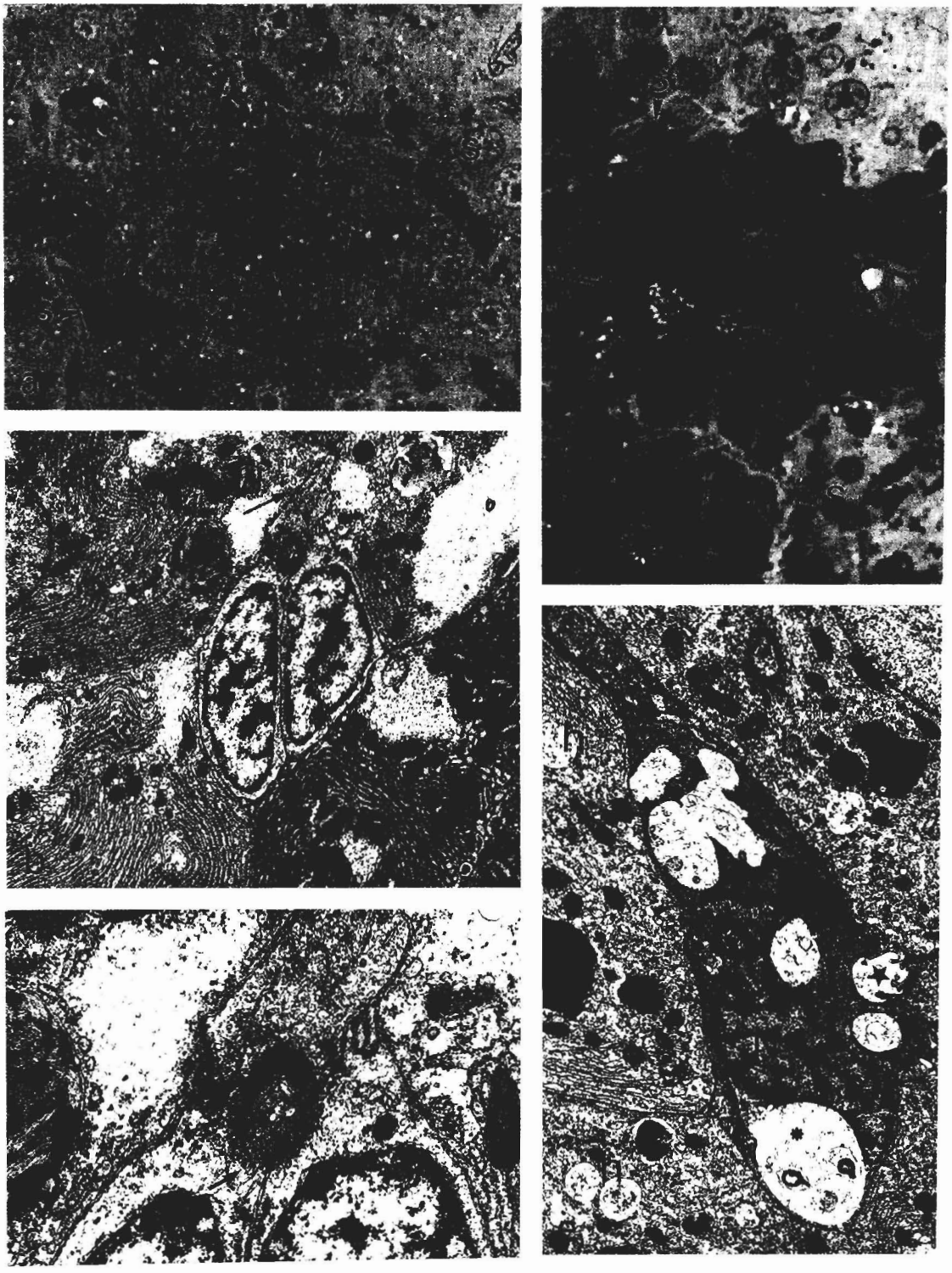

**Fig. 1.** Liver of the rainbow trout, *Oncorhynchus mykiss.* a-c, control fish; d-e, 60 d and 30 days effluent-exposed fish, respectively. **a.** Section of the liver showing hepatocyte cords (arrows), blood sinuses (s) and bile pre-ductular cells (arrowheads). **b.** Electron micrograph of bile ductular cells. Note the junctional complexes between them (arrowhead) and junctions with hepatocytes (arrow). **c.** Electron micrograph illustrating the junctional complexes. Note the presence of microfilaments (arrow) in the cytoplasm next to them. **d.** Section of liver from effluent 60-days-exposed fish. Pre-ductular cells (arrows) are vacuolated. **e.** Electron micrograph of a vacuolated pre-ductular cell. Vacuoles (*) contain amorphous material and whorls of membranes. Note that junctional complex are maintained (arrowheads). N: nucleus; h: hepatocytes; Star: pre-ductule lumen. a,d, x 2,800; b, x 6,000; c, x 18,000; e, x 7,500

appeared in all fish after 30 days treatment and subsisted in all 60-days-exposed fish. Single cells or small groups of cells exhibiting different degrees of vacuolation were seen spread throughout the parenchyma of treated fish liver. Vacuolated pre-ductular cells were enlarged when compared to pre-ductular cells of control fish livers (Fig. 1a,d), their nuclear margin being indented due to the presence of vacuoles (Fig. 1e). These vacuoles showed an amorphous or whorl membranous content. Junctional complexes between pre-ductular cells, or between pre-ductular cells and hepatocytes were maintained despite the great level of vacuolation (Fig. 1e).

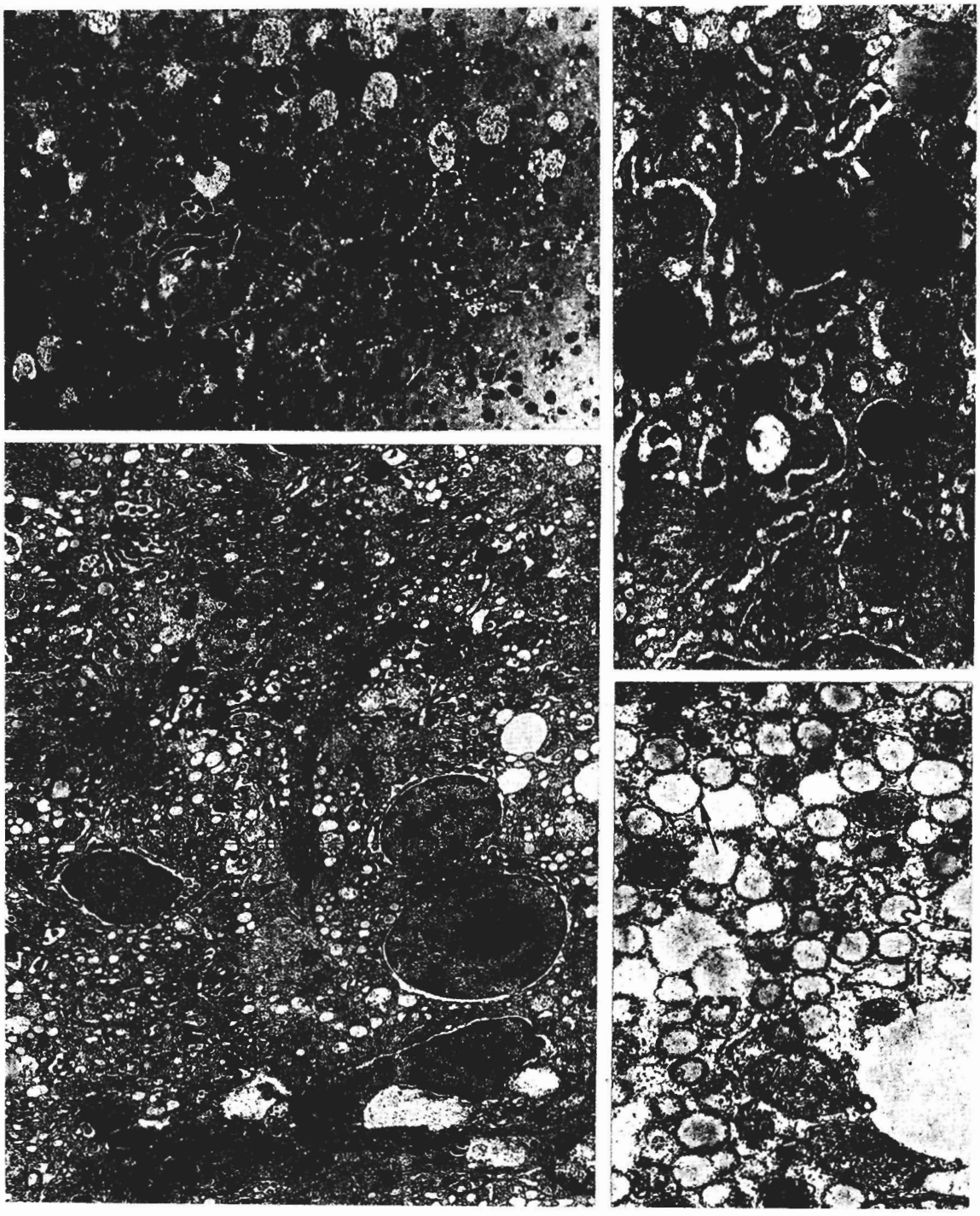

**Fig. 2.** Liver of a 30-days-exposed fish showing lipid accumulation. **a.** Some hepatocytes are completely filled with lipid droplets (arrows) while others show some fat accumulation mainly near sinusoidal space (arrow heads). **b.** Electron micrograph of hepatocytes. Note the increase in collagen fibers (arrowheads) in perisinusoidal space. Pre-ductular cell (pc) is vacuolated. N: nucleus; li: lipid droplet; bc: bile canaliculi; m: mitochondria. **c.** Hepatocytes show anastomosed pattern of the rough endoplasmic reticulum. Note partial degranulation (arrows) of RER. m: mitochondria; l: lipid droplet. **d.** Mitochondria (m) and isolated RER cisternae (arrowhead) are scattered between lipid droplets (arrow). Note the presence of lipid droplets surrounded by a membrane-like structure (arrow). a, x 1,120; b, x 6,000; c, x 15,00; d, x 24,000

One of the 30-days-exposed fish showed abnormal levels of lipids. Many hepatocytes were packed with large quantities of fat (Fig. 2a). Lipid accumulation was so high that nuclei were displaced to a basal or lateral locations, some of the droplets being surrounded by membrane-like structures (Fig. 2d). Although the great majority of hepatocytes displayed the two cord-like arrangement, altered zones could be observed in which the typical hepatic arrangement was lost and hepatic nucleus anaplasia was evident. In these zones sinusoids could only be observed in places where large quantities of collagen fibers completely filled the space of Disse (Fig. 2b). Anatomosed cisternae and circular profiles of RER were spread throughout all the hepatic cytoplasm (Fig. 2c).

One of the 45-days-exposed fish showed basophilic cell foci containing cells within the hepatic sinusoids possibly of inflammatory origin (Fig. 3). The basophilic cells were almost normal in appearance, being arranged in cords of 2-cell thickness. No mitotic figures were observed.

Although both control and treated fish showed hepatocytes with various degrees of cytoplasmic electrodensity (Fig. 4), dark cells were more abundant in liver sections of effluent-exposed fish. These cells were characterized by a minor size, an irregular outline and shrinkage of the cytoplasm. Dark cells displayed different degrees of shrinkage and cytoplasmic condensation. While cells with a relative «light» cytoplasm (Fig. 4a) showed cellular organelles mostly unaltered, darker cells exhibited dilatation of RER cisternae, swollen mitochondria and very irregular nucleus shape (Fig. 4b). Cytopathological changes such as nuclear picnosis and karyolisis, high-amplitude mitochondrial swelling and extensive dilatation of RER cisternae were frequently observed in very dark hepatocytes (Fig. 4c).

Hepatocytes of all treated animals exhibited a large number of lysosomes near the bile canaliculi. Wandering macrophages, mainly located in the space of Disse or between hepatocytes, showed phagosomes containing a great variety of materials and secondary lysosomes (Fig. 5b). Macrophages of control fish mainly exhibited primary lysosomes (Fig. 5a). Ziëhl-Nielsen and Perl's stainings revealed no changes either in the number of melanomacrophages or in their pigment content following treatment. Hepatic cells of all 30-days-exposed animals showed a cribiform appearance due to the dilatation and vesiculation of RER cisternae as well as mitochondrial alterations (Fig. 6a). RER cisternae and vesicles were partially demonstrated (Fig. 6b). A great number of myelin bodies and cytophagosomes containing myelin bodies could be noted (Fig. 6c). In all 60-days-exposed fish, only a light dilatation of RER cisternae was observed.

**Table 2.** Micronuclei frequency (‰) in the peripheral mature erythrocytes of the rainbow trout, *Oncorhynchus mykiss*, after sublethal effluent exposure.

| EXPOSURE TIME | CONTROL | TREATMENT |
|---|---|---|
| | F(n MN) | F(n MN) |
| 15 days | 0.125 (l) | 0.563 (9) |
| 30 days | 0* | 0.500* (8) |
| 45 days | 0 | 0.439 (7) |
| 60 days | 0.125 (l) | 0.250 (4) |

F: frequency; n MN: micronucleated erythrocytes; *: significant difference ($p<0.05$, Kastembaun and Bowman, 1970).

### *Piscine micronucleus test*

In all the periods evaluated the incidence of MN was higher in effluent-exposed fish than in control ones, but the difference was only significant in the 30-days exposure period (Table 2). In effluent-exposed fish a significant decrease of MN numbers with increasing exposure time was observed ($r=-0.95$, $p=0.004$) (Fig. 7). Each affected cell contained only one MN generally located close to the main nucleus.

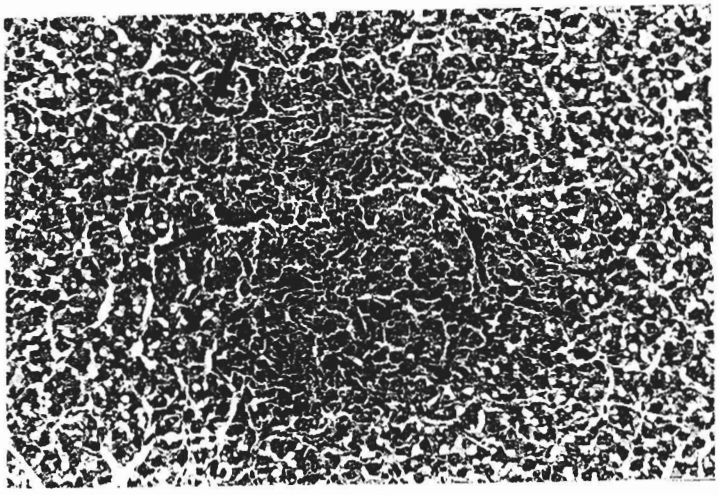

**Fig. 3.** Section of a liver from a 45-days-exposed trout showing the presence of a basophilic cell focus. Small cells with very basophilic nuclei are shown in sinusoids (arrows). x 520

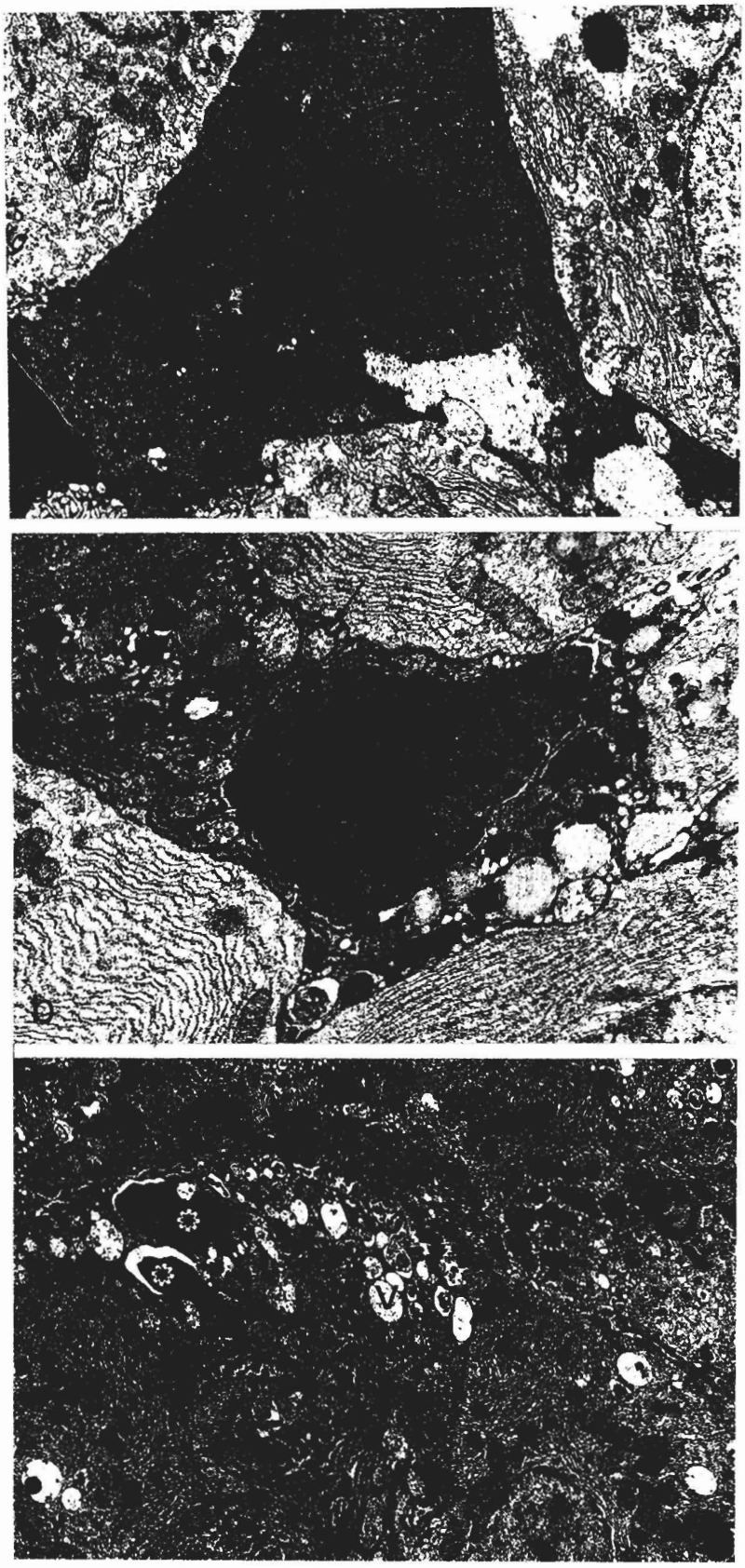

**Fig. 4.** Electron micrographs showing «dark hepatocytes» exhibiting different degrees of organelle alterations. Control (a) and 60-days-effluent-exposed (b,c) fish. **a.** RER cisternae show a slightly dilated appearance (arrowheads) while mitochondria exhibit normal morphology (arrows). **b.** Some lipid droplets (li) are found intermingled with swelling mitochondria (arrows) and dilated RER (arrow head). **c.** Fragments of picnotic nucleus (*), large and swelling mitochondria (arrows) and vacuoles (v) are observed in very «dark cells». a, x 7,500; b, x 12,000; c, x 5,520

## Discussion

### *Specific changes related to pre-carcinogenic alterations*

Vacuolation of bile pre-ductular cells has been related to hepatocellular and cholangiocellular neoplasia in teleost inhabiting contaminated environments (Murchelano and Wolke, 1985; Moore et al., 1989; Bodammer and Murchelano, 1990) and also, after experimental exposition to pesticides (Rojik et al.,

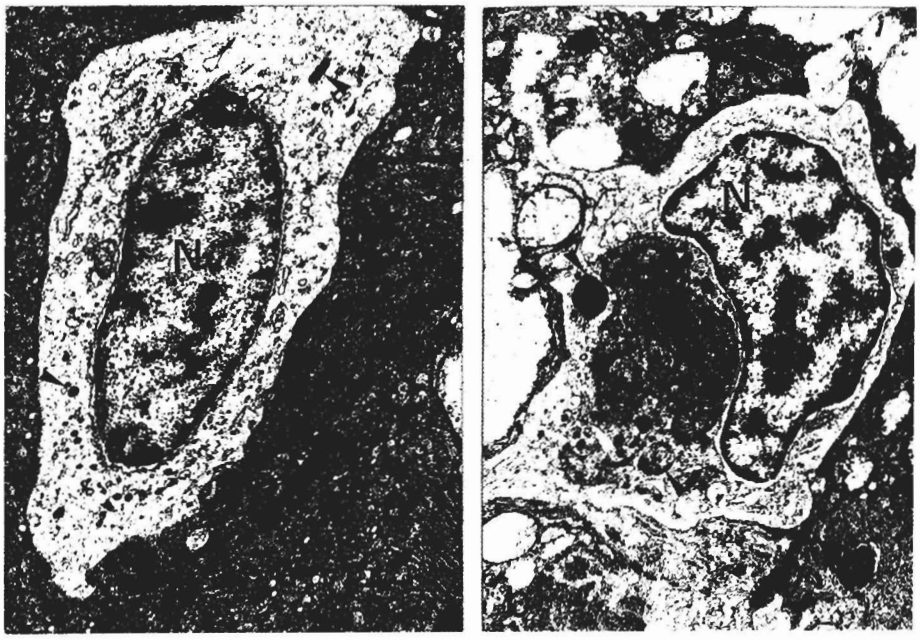


**Fig. 5.** Wandering macrophages in the liver of control (a) and 30-days effluent exposed (b) fish. **a.** Note the presence of primary lysosomes (arrowheads). N: nucleus; arrow: collagen fibers. **b.** Note the presence of phagosomes containing various materials (star) and secondary lysosomes (arrowheads). a, x 8,400; b, x 9,000

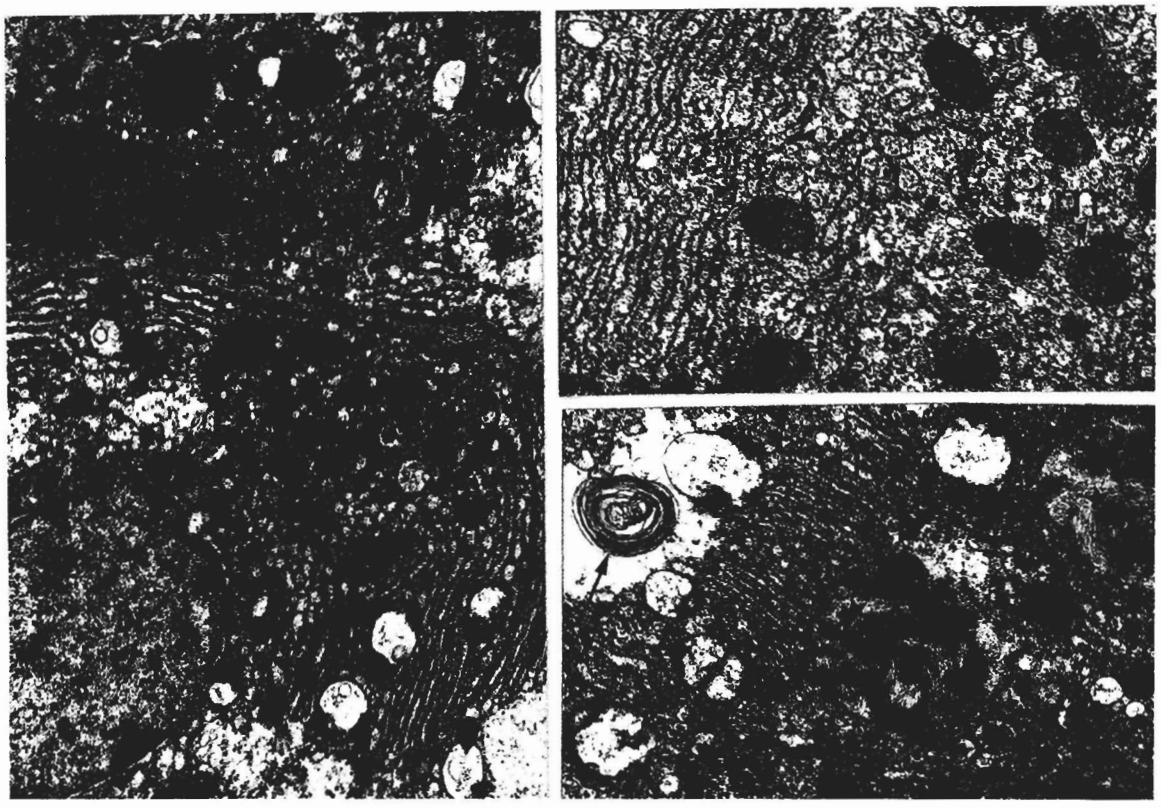

**Fig. 6.** Electron micrographs of 30-days-exposed fish liver. **a.** Dilatation and vesiculation of the RER (arrowheads) and alteration of numerous mitochondria (arrows). N: nucleus; L: lysosomes; bc: bile canaliculi. **b.** Degranulation of the RER vesiculae and cisternae (arrow heads). m: mitochondria. **c.** Myelin body (arrow) and cytophagosomes containing myelin bodies (stars). m: mitochondria. a, x 7,500; b, x 20,400; c, x 10,800

1983). From our work it is apparent that exposure of the trout (*O. mykiss*) to the wool shrinkproofing effluent tested induces vacuolation of the live bile pre-ductular cells. It has been shown by biochemical studies carried out in winter flounder (*Pseudopleuronetctes americanus*) that vacuolation of the liver cells might be associated with their proliferative capacity, either by activation of the oncogenic *Ki*-ras sequences (McMahon et al., 1990) or by the presence of high levels of bromodeoxyuridine and ornithine decarboxylase they contain (Koza et al., 1993), which suggests that vacuolated cells are involved in tumour development. In the present work, vacuolation was only observed affecting isolated or small groups of pre-ductular cells. No other lesions such as hepatocyte vacuolation, bile duct proliferation, fibrosis, and macrophage aggregation described in advanced stages of hepatocellular neoplasia (Moore et al., 1989) were encountered. Our results would indicate that the alteration described might correspond to the first step of a carcinogenic process.

The basophilic cell foci observed by us in one of the 45-days-treated fish correspond to the less malignant basophilic foci type described by Hendricks et al. (1984), given the lack of mitosis and the normal hepatocyte size. As no signs of hepatic necrosis or small hepatocytes were found, we assume that these foci are not related to cellular regeneration (Hinton et al., 1988). The occurrence of basophilic cell foci has been reported in fish from polluted areas (Murchelano and Wolke, 1985; Cormier et al., 1989; Bauman et al., 1990) and in those exposed to carcinogens (Harada et al., 1988; Nuñez et al., 1989). This lesion is considered as a pre-neoplastic lesion (Hendricks et al., 1984; Murchelano and Wolke, 1985; Bauman et al., 1990) and its progression to hepatocellular nodules, a benign intermediate phase in hepatocellular carcinoma, has been described (Harada et al., 1988).

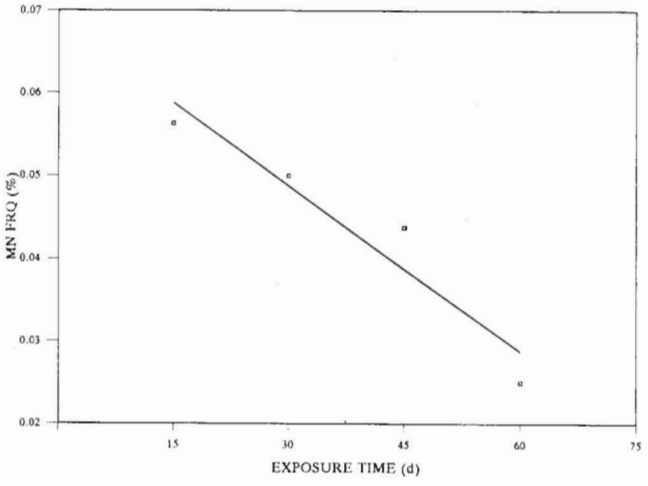


**Fig. 7.** Regression line of the incidence of micronuclei in peripheral erythrocytes of rainbow trout, *O. mykiss*, in wool shrinkproofing effluent-exposed fish (MNFRQ(%)=0.00069-0.0000070 D; R=-0.96; P=0.044).

*Non-specific alterations*

Light-dark cell phenomenon was observed in our study in both control and exposed-fish livers. Dark cells are considered by several authors as the «shrunken» version of light cells. Fixation artifacts, pathological changes induced by noxious agents, differences in functional and hydration status and cell apoptosis have been proposed to explain this phenomenon (Ghadially, 1982; Bodammer and Murchelano, 1990). The fact that in our study, dark cells were more abundant in exposed fish and that darker hepatocytes exhibited unequivocal cytological changes indicative of cell death (nuclear picnosis and karyolysis, high-amplitude mitochondrial swelling and extensive dilatation of RER cisternae) points out the cytotoxic activity of the effluent tested. Dark cell phenomenon was also described in the dogfish gill chloride cells following treatment with heavy metals (Crespo and Sala, 1986). The apoptotic origin of dark cells could be rejected as no apoptotic bodies (Meseguer et al., 1996) were described in our study.

Dilatation and vesiculation of RER and its posterior degranulation could be due either to ingress of water or to storage of secretory products (Ghadially, 1982). The presence of amorphous material within the RER cisternae described by electron microscopy in effluent-treated fish would indicate an accumulation of RER-secreted products related to the suppression of ATP production by altered mitochondria. In addition, the occurrence of myelin bodies as well as cytolysosomes containing myelin figures in the hepatocyte cytoplasm could be associated with mitochondrial involution (Ghadially, 1982). Similar changes have been reported either in starved fish (Segner and Braunbeck, 1988) or in fish exposed to different kinds of toxic substances (Rojik et al., 1983; Köhler, 1989; Arnold et al., 1996).

The presence of macrophages containing phagosomes and secondary lysosomes in effluent-exposed fish might be related ot the activation of the phagocytic mechanisms to eliminate damaged organelles and cells.

Although several authors (Agius, 1985; Marlasca et al., 1992) have reported the role played by melano-macrophages in detoxification of xenobiotics, in the present work we did not find any differences between control and effluent-exposed fish with regard to the number and pigment content of liver melanomacrophages.

One of the 30-days-exposed fish showed abnormal lipid accumulation of the liver. Lipid accumulation might be considered as a rather unspecific alteration since it has been related to different etiologies such as peroxidized fatty acids in the diet (Ferguson, 1989), nutritional deficiencies (Mosconi-Back, 1991), toxic agents (González et al., 1994; Hendricks et al., 1984) and environmental pollution (Cormier et al., 1989; Köhler, 1989). Nevertheless, fat accumulation can also be related to the development of liver neoplasia by allowing the bioaccumulation of precarcinogenic compounds or by acting as tumour promoter (Cormier et

al., 1989).

*Micronuclei*

The rate of MN spontaneous induction in trout (0.125%) was very low, similar to the value previously reported for this species (Marlasca et al., 1992) and to the values described in mudminnows (*Umbra limi)* and bullheads (*Ictalurus nebulosus*) (Metcalfe, 1988). The significant increase of micronucleated erythrocytes after 30 days effluent exposure suggests the genotoxic potential of the effluent at the concentration tested. However, a more detailed work (more animals or cells/animal studied, different concentrations, etc) must be done to clearly demonstrate the genotoxic activity of this effluent.

Although erythropoiesis time in fish is unknown, Dinnen et al. (1987) observed an increase in MN numbers of trout 23 days after a radiation treatment, suggesting an erythrocyte maturation period of 17-23 days for this species. In goldfish, a maturation period of 16-20 d was proposed by Murad and Houston (1992). Thus, the time elapsed since the last mitotic cell division to erythrocyte maturation could explain the delay in detecting a significant increased of micronucleated erythrocytes (Dinnen et al., 1987). This period could be even longer if the effluent exerts an inhibitory effect on the mitosis process (Das and Nanda, 1986; Scarpato et al., 1990). The decrease of MN numbers with exposure time has also been reported in aquatic organisms inhabiting heavily polluted environments, MN frequencies being always above the basal levels (Das and Nanda, 1986; Scarpato et al., 1990).

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