

Sublethal effects of synthetic dyes on rainbow trout *Oncorhynchus mykiss*: a light and electron microscope study

M. J. Marlasca¹, B. Vallés², M. C. Riva², S. Crespo^{1, *}

¹ Laboratori de Biologia, Facultat de Veterinària, U.A.B., E-08193 Bellaterra, Barcelona, Spain

² Institut d'Investigació Tèxtil i Cooperació Industrial de Terrassa, Barcelona, Spain

ABSTRACT: Subchronic exposures to sublethal concentrations of the synthetic, metal-complex dye C.I. Acid Violet 66 and its azo compound C.I. Acid Red 217 did not cause severe histological damage to rainbow trout *Oncorhynchus mykiss*. Fish exposed to C.I. Acid Violet 66 for 30 d exhibited the most pronounced morphological alterations, which included spongiosis of the gill tissue and degeneration of chloride cells. Both dyes triggered adaptative responses which involved granulocytes, macrophages and granular cells in the gill, and melanomacrophages in the kidney, liver and spleen. Hepatocytes displayed altered endoplasmic reticulum after treatment. Frequencies of micronucleated peripheral erythrocytes did not increase following dye treatment, suggesting that these chemicals, at least at the doses tested, are not genotoxic following a 30 d exposure.

INTRODUCTION

The environmental problems posed by dyes are rather moderate in severity compared with those caused by other chemicals such as pesticides, detergents, industrial oils, heavy metals, etc. (Anliker 1977). Dyes have been shown to be mildly to moderately toxic to aquatic organisms: 98 % of more than 3000 commercial dyes tested on fish by the ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry) exhibited 48 h LC₅₀ values greater than 1 mg l⁻¹ (Clarke & Anliker 1980). However, it must be taken into account that more than 100 t of dyes (50 % corresponding to the textile sector) are released daily into the environment, primarily dissolved or suspended in water, and evaluations of their sublethal effects on aquatic organisms are not available in the literature.

Toxicity tests carried out in our laboratory demonstrated that the 48 h LC₅₀'s of the metal-complex dye C.I. Acid Violet 66 (see Fig. 1b) and the azo compound utilized in its synthesis (C.I. Acid Red 217; see Fig. 1a) for rainbow trout *Oncorhynchus mykiss* were 8.20 and 71.04 mg l⁻¹ respectively (Riva et al. 1990), revealing that toxicity was mainly due to the presence of chromium (Cr III) in the dye molecule. It was also

shown that, following treatment with sublethal concentrations, levels of these chemicals increased in the bile and accumulation in the tissues occurred (Riva 1989). In order to elucidate the morphological effects, if any, of a sublethal dose of the chromium-complex dye and its azo compound on trout, a light and electron microscope study was undertaken after treating the fish up to 30 d with both chemicals. Since a few dyestuffs, particularly benzidine derivatives, have been shown to be mutagenic in mammals (Anliker & Steinle 1987, Arcos et al. 1988) and recent experimental data (Bianchini & Levis 1988) indicate that chronic exposures even to low doses of insoluble Cr (III) produce genotoxic consequences, the piscine micronucleus test (Hooftman & de Raat 1982) was used to assess the genotoxicity, if any, of both dyes.

MATERIAL AND METHODS

Experimental treatment. Rainbow trout *Oncorhynchus mykiss* (sexually immature, ca 10 to 15 cm long) were obtained from a local hatchery and maintained in well-aerated running freshwater (total hardness 316 mg l⁻¹; pH 8 ± 0.1; temperature 14 ± 1°C) for at least 2 wk before experiments. Fish were fed a commercial fish diet at a rate of 1 % live weight d⁻¹ during the acclimation period as well as during treatment.

* Addressee for correspondence

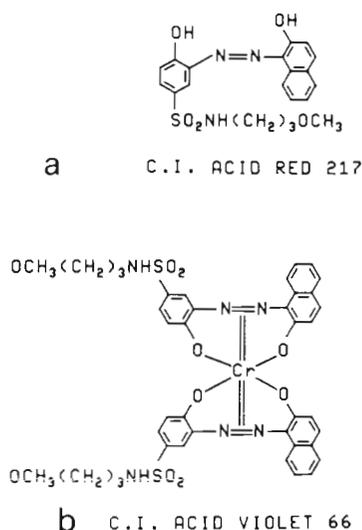


Fig. 1. Chemical structure of the dyes used in this study

Individuals for experiments were placed in 20 l tanks (10 fish tank⁻¹). Metal-complex dye solutions were prepared by adding 4.1 mg l⁻¹ of the commercial dye (Fig. 1a). This concentration corresponds to half the 48 h LC₅₀ for trout (Riva et al. 1990). In order to remove fish excretions and to maintain a relatively constant concentration of the toxicant in experimental tanks, solutions were replaced every 2 d. Fish were sacrificed after a 14 and 30 d exposure to the toxicant. In order to compare the histopathological effects of the metal-complex dye (Fig. 1b) and its azo compound (Fig. 1a), a group of 10 fish was exposed to 2.9 mg l⁻¹ of C.I. Acid Red 217 for 14 and 30 d (2.9 mg corresponds to the amount of this compound present in 4.1 mg of the final

metal-complex dye). Both chemicals were supplied by Sandoz S.A. (Basel). Ten fish were used as controls and were handled in the same way as treated ones.

Histological and electron microscope study. Treated specimens were killed with tricaine methanesulfonate (MS 222, Sandoz) and samples of the liver, kidney, spleen and gills were fixed in 5% glutaraldehyde in 0.1 M Na cacodilate buffer (pH 7.3), post-fixed in 2% OsO₄ dehydrated through ethanol series, stained 'en block' with uranyl acetate and embedded in araldite. Semi-thin sections (1 μm) were cut, stained with toluidine blue and viewed under a light microscope. Ultrathin sections were stained on the microscopical grids with lead citrate and observed in a Hitachi H-7000 transmission electron microscope. Several samples were fixed in 10% buffered formalin, embedded in paraffin wax, cut at 6 μm and stained using the Perl's Prussian Blue method (Luna 1968). This staining procedure allows differentiation of the 3 pigments present within the melanomacrophages (Wolke et al. 1985): the ferric ion of hemosiderin stains bright blue, the lipofuscin pigment remains unstained and melanin, also unstained, appears as dark brown granules.

Piscine micronucleus test. After severing the caudal vein, a drop of blood was smeared on the slide, air dried, methanol fixed, and then stained in May-Grünwald-Giemsa solutions. Eight slides were obtained from each fish. The number of micronuclei scored after counting 500 mature erythrocytes per slide was recorded. Erythrocytes were scored blind and only cells with intact cell and nuclear membranes were included in the counts. We only counted as micronuclei isolated nuclear fragments, following the morphological criteria described by Tates et al. (1980) (see Fig. 2). The fre-

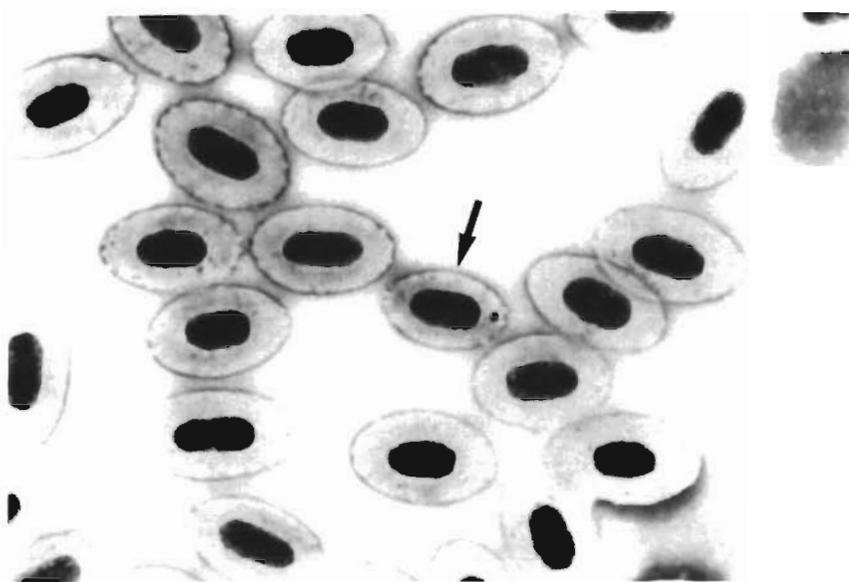


Fig. 2. *Oncorhynchus mykiss*. Micronucleated erythrocyte (arrow) of rainbow trout. Micronuclei are visible as isolated nuclear fragments. May-Grünwald Giemsa, × 1320

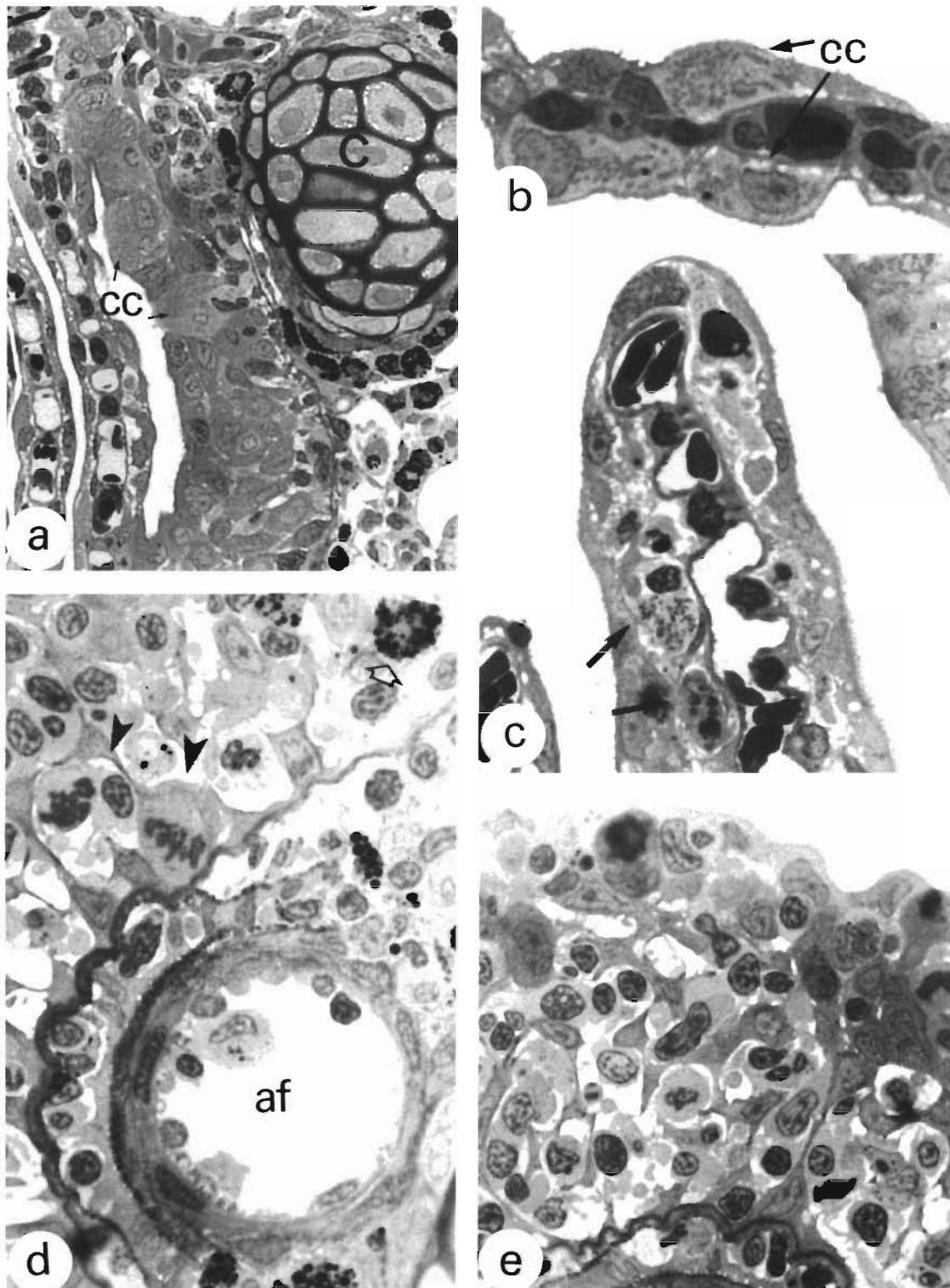


Fig. 3. *Oncorhynchus mykiss*. Gill morphology of (a, b) untreated and (c to e) dye-treated rainbow trout. Note that following treatment with metal-complex dye for 30 d, lymphoid spaces (c) are dilated and invaded by granuloctes (arrows). Spongiosis of the filament epithelium (d, e) is apparent after treatment, and mitotic figures (d; arrowheads) are more frequent than in control fish. Granular cells, which in untreated fish are only located in the connective tissue (a) surrounding the cartilage, are observed invading the epithelial layer in treated fish (d; open arrow). C: cartilage; cc: chloride cells; af: afferent artery. Toluidine blue; (a) $\times 610$, (b) $\times 1950$, (c) $\times 1320$, (d) $\times 1750$, (e) $\times 1300$

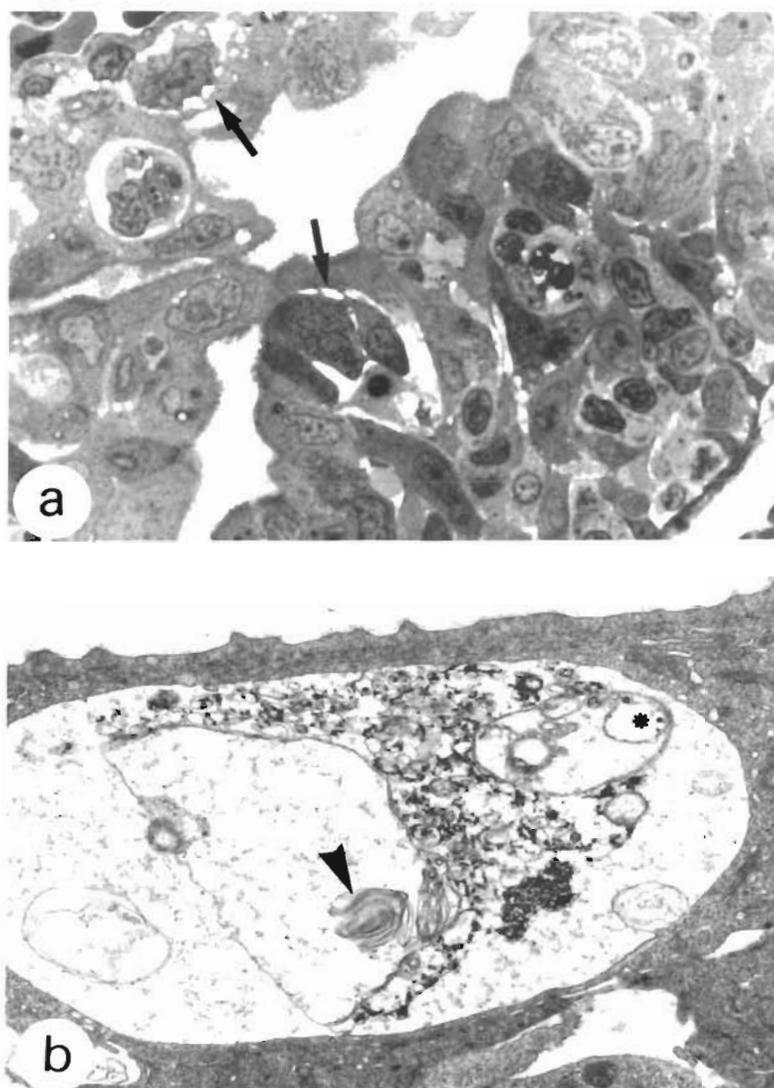


Fig. 4. *Oncorhynchus mykiss*. Degeneration of chloride cells in the gill filament epithelium of rainbow trout exposed to sublethal doses of metal-complex dye. (a) Light micrograph (Toluidine blue; $\times 1200$) shows shrinkage of chloride cell cytoplasm (arrows). (b) Electron micrograph of a degenerate chloride cell ($\times 9000$) shows vesicular (*) and whorled membranous bodies (arrowhead), probably derived from involuting mitochondria

quencies of micronuclei in pooled control and experimental erythrocytes were compared statistically using the tables of Kastenbaum & Bowman (1970).

RESULTS

None of the fish displayed any apparent toxicity symptoms (overturning, erratic movement, increased coughing, flared operculum, etc.) during experimentation. The lamellar epithelium of trout gill is composed of 2 layers of epithelial cells separated by lymphoid spaces; chloride cells are located in the interlamellar spaces of the filament and in the lamellae (Fig. 3a, b). In control fish, granular cells were mainly found in the connective layer surrounding the cartilage (Fig. 3a). Following treatment with either the metal-complex dye or its azo compound, gill epithelium was spongiotic and

displayed enlarged lymphoid spaces (Fig. 3c to e). Spongiosis was more severe in fish treated with the metal-complex dye for 30 d, and mitotic figures were more abundant (Fig. 3d). In all treated fish, numerous granulocytes and macrophages were seen infiltrating the lymphoid spaces (Fig. 3c) and granular cells appeared in the epithelial layer as well as in the connective tissue (Fig. 3d, e). Rodlet cells were found in the filament and in the lamellae of both control and treated fish. No differences in the number of mucous cells were detected following treatment. Degeneration of epithelial cells (mainly chloride cells) was particularly apparent after metal-complex dye treatment (Fig. 4).

Histological examination of the liver, kidney and spleen did not reveal any pathological features. However, following a 30 d treatment with the metal-complex dye, it was apparent that melanomacrophages increased in number in the kidney intertubular tissue,

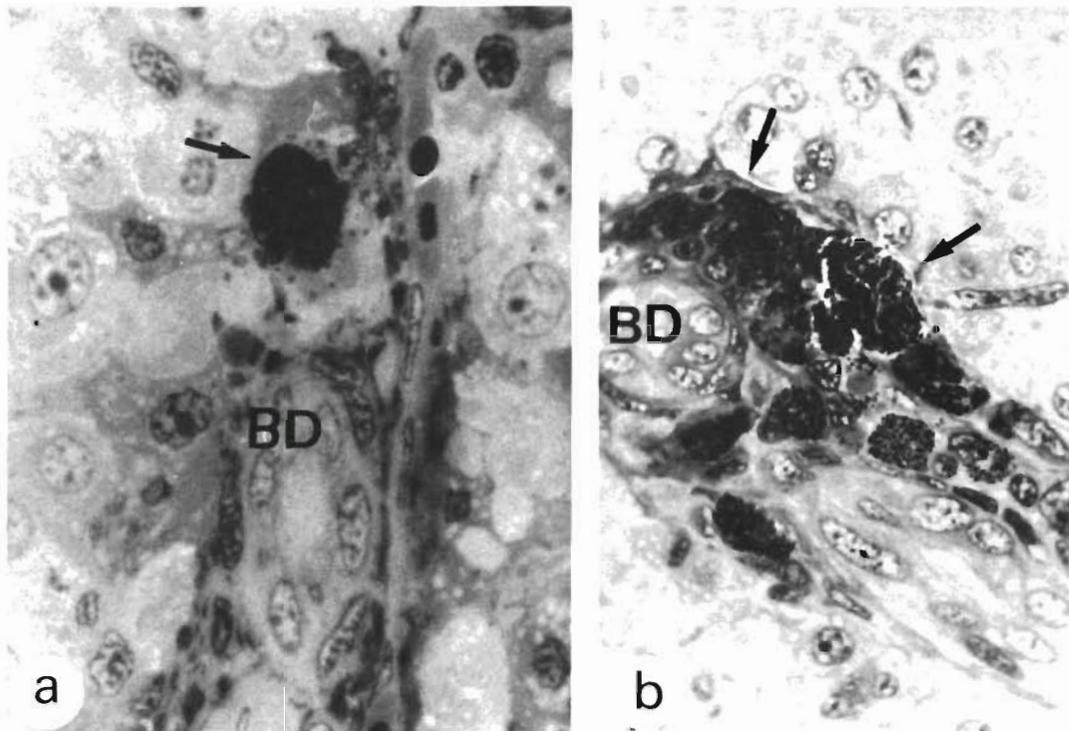


Fig. 5. *Oncorhynchus mykiss*. Light micrograph observation of the liver of (a) untreated (Toluidine blue, $\times 1400$) and (b) metal-complex dye treated (Toluidine blue, $\times 1200$) rainbow trout. No apparent alterations of the hepatocyte structure are observed. Note that melanomacrophages (arrows) surrounding the bile duct (BD) increase in number following treatment.

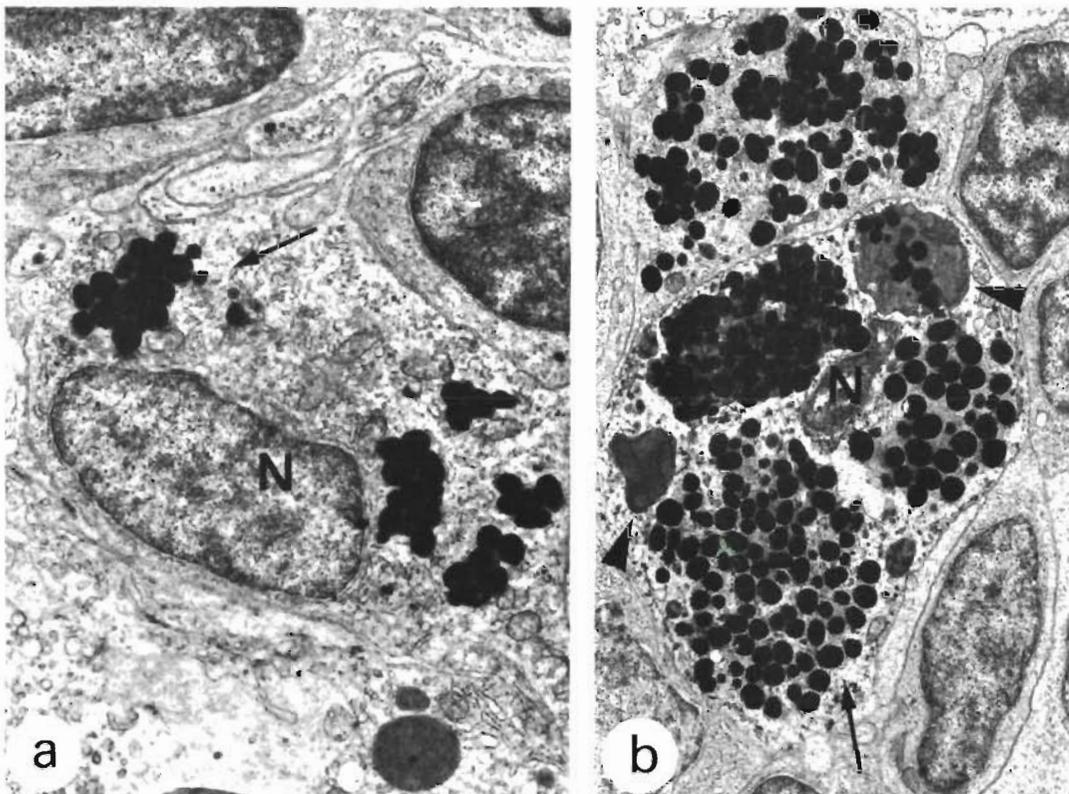


Fig. 6. *Oncorhynchus mykiss*. Melanocytes in the intertubular tissue of the kidney in (a) control ($\times 5300$) and (b) metal-complex dye exposed trout ($\times 6500$). Note that renal melanocytes of treated fish are packed with melanosomes at all stages of development (b), whereas early stages of development are less frequently observed in control fish (a). N: nucleus, arrowheads: early stages of melanosome development; arrows: melanin granules.

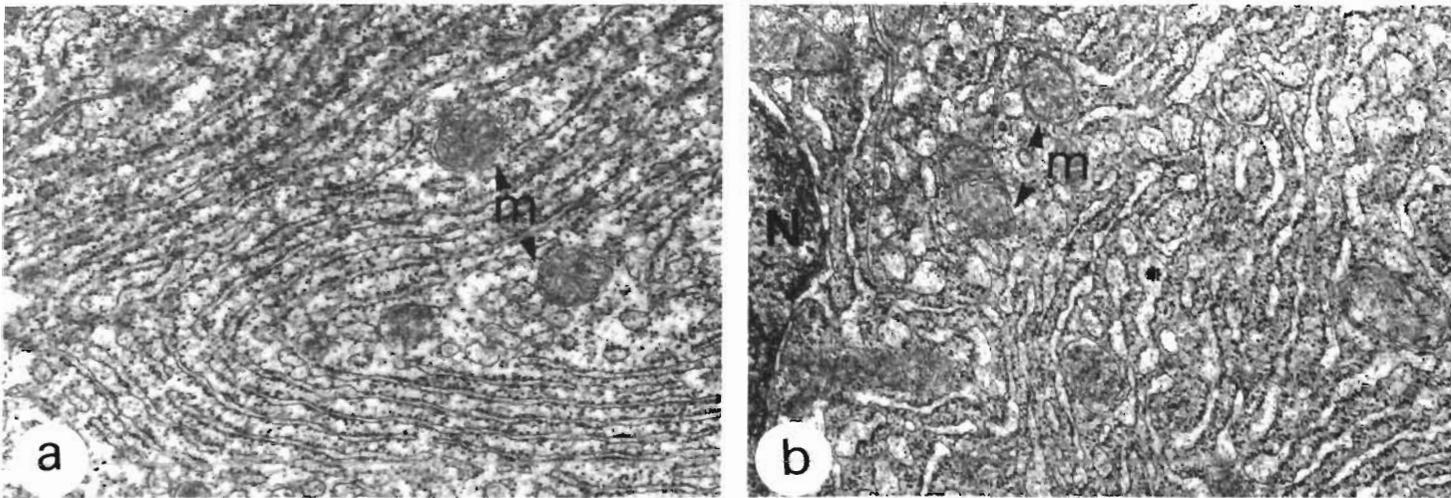


Fig. 7. *Oncorhynchus mykiss*. Electron micrograph of the rough endoplasmic reticulum of hepatocytes in (a) control ($\times 21\,500$) and (b) treated rainbow trout ($\times 21\,000$). Note the dilation and degranulation (\star) of the reticulum in treated fish. N: nucleus; m: mitochondria

spleen and, particularly, in the liver, surrounding the biliary ducts (Fig. 5). Perl's Prussian Blue staining revealed that there was an increase in the amount of melanin pigment, whereas hemosiderin remained unaltered. From ultrastructural observations it was apparent that in treated fish, renal melanocytes were packed with melanosomes which displayed all stages of development, whereas in control fish, melanosomes in early stages of development were rare (Fig. 6). After 30 d exposure of fish to the metal-complex dye, hepatocytes exhibited dilation and degranulation of the endoplasmic reticulum (Fig. 7), although the reticular nature of the organelle was not lost. No changes were found in mitochondria and other hepatocyte organelles. Table 1 shows the frequencies of micronuclei found in mature peripheral erythrocytes of *Oncorhynchus mykiss* exposed to both dyes for 30 d. Although the number of micronucleated cells was

higher in the group exposed to C.I. Acid Red 217, no statistically significant differences (Kastenbaum & Bowman 1970) were found relative to control values.

DISCUSSION

Our observations provide evidence of histological and ultrastructural alterations, although not severe, in fish exposed to dye solutions. Gill spongiosis, granulocyte infiltration in the enlarged lymphoid spaces and increases in the number of granular cell and mitotic figures were observed following all treatments. However, these changes were more pronounced after treatment of fish with the metal-complex dye for 30 d, and degeneration of epithelial cells (mainly chloride cells) was particularly apparent in this group. Chloride cells have been shown to be the most sensitive gill cell type to a great variety of agents such as heavy metals (Crespo & Sala 1986), acid water (Tietge et al. 1988), imbalanced diet (Bell et al. 1985) and stress (Peters & Hong 1985). Our observations accord with the fact that the 48 h LC_{50} of the metal-complex dye for trout is one-ninth that of its azo compound (Riva et al. 1990) and suggest that dye toxic effects might be mainly due to the presence of the metal (Cr III). Nevertheless, it must be taken into account that gill morphological alterations described in the present work are non-specific and have been reported previously in fish exposed to other toxicants (Mallatt 1985, Evans 1987). The enlargement of the lymphoid spaces and the infiltration of granulocytes and macrophages might correspond to an inflammatory response to the toxicants. Although the lamellar structure was not severely altered after

Table 1. *Oncorhynchus mykiss*. Frequencies of micronuclei in peripheral erythrocytes of trout exposed to dyes. No statistically significant differences were recorded when comparing treated and control values (Kastenbaum & Bowmann 1970)

	Control ^a	Treatment	
		C.I. Acid Violet 66 ^b	C.I. Acid Red 217 ^a
Frequency (%)	0.083	0.036	0.250

^a 36 000 erythrocytes scored in control and C.I. Acid Red 217 treated fish (9 fish, pooled)
^b 28 000 erythrocytes scored in metal-complex dye treated fish (7 fish, pooled)

treatment, the length of the water-blood pathway might be increased in dye-exposed animals, which might account for an unbalanced gas transfer. Branchial granular cells, histologically similar to the eosinophilic cells found in the submucosa of the trout intestinal tract (Bergeron & Woodward 1983), have been shown to increase in number following all dye treatments, which might be consistent with their hypothesised inflammatory role (Ferguson 1989). Cells undergoing mitosis were more abundant following dye treatment. The same observation was reported by Crespo et al. (1986) for trout intestinal epithelium after oral administration of lead and cadmium and was related to an increased renewal rate of absorptive cells.

The histological and ultrastructural examination of the kidney, liver and spleen revealed that melanomacrophages increased in number, melanin was more abundant within cells, and melanosomes, in early stages of development, were more apparent in treated specimens, which might indicate increased melanin synthesis in these fish. Pigment-containing macrophages are a prominent feature of piscine haemopoietic tissues, and the role of melanin might be related to detoxification mechanisms, since this pigment is able to absorb free radicals and cations (Agius 1985). Pigmented macrophage accumulations were proposed as possible indices of fish health (Wolke et al. 1984) and several authors described increased pigmented macrophage numbers in fish caught from polluted waters (Wolke et al. 1985) and experimentally treated with polluted waters (Poels et al. 1980). However, reduced numbers of melanomacrophage centers were reported in flounder exposed to hydrocarbon-contaminated sediments (Payne & Fancey 1989), and it was suggested that at low levels of pollution the cellular defense system would be capable of removing debris by means of increased phagocytic activity, whereas at higher levels of pollution phagocytosis might be impaired, leading to a decrease in melanomacrophage centres.

Ultrastructural observations of the liver hepatocyte showed dilation and degranulation of rough endoplasmic reticulum following metal-complex dye exposure, although the reticular nature of the organelle was not lost. Ultrastructural changes in the endoplasmic reticulum of fish exposed to other toxicants have also been described by Braunbeck et al. (1989). It is assumed that dilation of endoplasmic reticulum can be due to an ingress of water and/or a storage of secretory products (Ghadially 1982). However, the fact that mitochondria were not swollen and the lumen of the reticulum displayed the same electron density following treatment does not support either of these hypotheses. Rather, we suggest that these changes might correspond to the initial events leading to formation of new smooth endoplasmic reticulum, since a great variety of drugs (includ-

ing azo compounds) have been reported to induce hypertrophy of smooth endoplasmic reticulum in mammalian hepatocytes (Ghadially 1982).

Since the application of the micronucleus test to assess genotoxic damage to fish using the peripheral erythrocytes of the eastern mudminnow *Umbra pygmaea* (Hooftman & de Raat 1982), there have been numerous reports on the induction of micronuclei following fish exposure to genotoxic agents (Al-Sabti 1986, Das & Nanda 1986, Metcalfe 1988). However, although the micronucleus test has been used to assess environmental pollution (Hose et al. 1987, Scarpato et al. 1990), recent studies by Carrasco et al. (1990) point out that this assay lacks sensitivity to the presence and effects of chemical contaminants, mainly due to the fact that nuclear lesion frequencies are very low and variable. Our study shows that neither C.I. Acid Violet 66 nor C.I. Acid Red 217 induced micronuclei formation in mature peripheral erythrocytes of trout at the doses tested. It could be argued that the period of exposure was not long enough. However, Das & Nanda (1986) have described statistically significant increases in the number of micronucleated cells after exposing catfish *Heteropneustes fossilis* to paper mill effluents for the same period of time (30 d), and Metcalfe (1988) has reported the induction of micronuclei in mudminnows *Umbra limi* and bullheads *Ictalurus nebulosus* 96 h after intraperitoneal injection with ethyl methane-sulphonate and benzo-(a)-pyrene. Moreover, Al-Sabti (1986) has described increased micronucleus frequencies in carp 48 h after treatment with several chemicals. As previously pointed out by Metcalfe (1988), the low incidence of micronuclei in peripheral fish erythrocytes (ranging from 0.036 to 0.25‰ in our study) creates a problem for statistical analysis. However, in the present work a large sample of erythrocytes was included in the counts: up to 28 000 cells (7 fish, pooled) and 36 000 cells (9 fish, pooled) were scored after treatment with C.I. Acid Violet 66 and C.I. Acid Red 217 respectively; even so, no differences were found when comparing these values to those for control micronuclei (36 000 cells scored; 9 fish, pooled). These results might suggest that neither dye, at least at the doses tested, is genotoxic following a 30 d exposure.

Acknowledgements. This work received financial support from the DGICYT (Dirección General de Investigación Científica y Técnica del Ministerio de Educación y Ciencia), PB 87-0180.

LITERATURE CITED

- Agius, C. (1985). The melano-macrophage centres of fish: a review. In: Manning, M. J., Tatner, M. F. (eds.) Fish Immunology. Academic Press, London, p. 85-105
- Al-Sabti, K. (1986). Clastogenic effects of five carcinogenic-mutagenic chemicals on the cells of the common carp, *Cyprinus carpio* L. Comp. Biochem. Physiol. 85C: 5-9

- Anliker, R. (1977). Colour chemistry and the environment. *Ecotoxicol. Environ. Saf.* 1: 211-237
- Anliker, R., Steinle, D. (1987). Prevention of risks in the use of handling colorants. In: Proceedings of the 14th IFATCC Congress. Tampere, Finland, 3-5 June 1987. Tampere University of Technology, Tampere, p. 325-346
- Arcos, J. C., Woo, Y., Lai, D. Y. (1988). Database on binary combination effects of chemical carcinogens. *Envir. Carcinogenesis Rev. (J. Envir. Sci. Health)* 6: 1-150
- Bell, M. V., Henderson, R. J., Pirie, J. S., Sargent, J. R. (1985). Effects of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot *Scophthalmus maximus*. *J. Fish. Biol.* 26: 181-191
- Bergeron, T., Woodward, B. (1983). Ultrastructure of the small intestine of the rainbow trout (*Salmo gairdneri*) before and after stratum granulorum formation. *Can. J. Zool.* 61: 133-138
- Bianchini, V., Levis, A. G. (1988). Review of genetic effects and mechanisms of action of chromium compounds. *Sci. total Environ.* 71: 351-355
- Braunbeck, T., Storch, V., Nagel, R. (1989). Sex-specific reaction of liver ultrastructure in zebra fish *Brachydanio rerio* after prolonged sublethal exposure to 4-nitrophenol. *Aquat. Toxicol.* 14: 185-203
- Carrasco, K. R., Tilbury, K. L., Myers, M. S. (1990). Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. *Can. J. Fish. Aquat. Sci.* 47: 2123-2136
- Clarke, E. A., Anliker, R. (1980). Organic dyes and pigments. In: Hutzinger, O. (ed.) The handbook of environmental chemistry, Vol. 3, Part A. Springer-Verlag, Berlin, p. 181-215
- Crespo, S., Nonnotte, C., Colin, A., Leray, L., Nonnotte, L., Aubrée, A. (1986). Morphological and functional alterations induced in trout intestine by dietary cadmium and lead. *J. Fish Biol.* 28: 69-80
- Crespo, S., Sala, R. (1986). Ultrastructural alterations of the dogfish (*Scyliorhinus canicula*) gill filament related to experimental aquatic zinc pollution. *Dis. aquat. Org.* 1: 99-104
- Das, R. K., Nanda, N. K. (1986). Induction of micronuclei in peripheral erythrocytes of fish *Heteropneutes fossilis* by mitomycin C and paper mill effluent. *Mutation Res.* 175: 67-71
- Evans, D. M. (1987). The fish gill: site of action and model for toxic effects of environmental pollutants. *Environ. Hlth Perspectives* 71: 47-58
- Ferguson, H. W. (1989). Systemic pathology of fish. A text and atlas of comparative tissue responses in disease of teleosts. Iowa State University Press, Ames
- Ghadially, F. N. (1982). Ultrastructural pathology of the cell and matrix. Butterworths, London
- Hooftman, N., de Raat, W. K. (1982). Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow *Umbra pygmea* by ethyl methane-sulphonate. *Mutation Res.* 104: 147-152
- Hose, J. E., Cross, J. N., Smith, S. G., Diehl, D. (1987). Elevated circulating erythrocyte micronuclei in fishes from contaminated sites off southern California. *Mar. environ. Res.* 22: 167-176
- Kastenbaum, A., Bowman, K. (1970). Tables for determining the statistical significance of mutation frequencies. *Mutation Res.* 9: 527-549
- Luna, L. G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company, New York
- Mallat, J. (1985). Fish gill structural changes induced by toxicants and other irritants: a statistical review. *Can. J. Fish. Aquat. Sci.* 42: 630-648
- Metcalfe, C. D. (1988). Induction of micronuclei and nuclear abnormalities in the erythrocytes of mudminnows (*Umbra limi*) and Brown bullheads (*Ictalurus nebulosus*). *Bull. environ. Contam. Toxicol.* 40: 489-495
- Payne, J. F., Fancey, L. F. (1989). Effect of polycyclic aromatic hydrocarbons on immune responses in fish: change in Melanomacrophage Centres in Flounder (*Pseudopleuronectes americanus*) exposed to hydrocarbon-contaminated sediments. *Marine environ. Res.* 28: 431-435
- Peters, G., Hong, L. Q. (1985). Gill structure and blood electrolyte levels of European eels under stress. In: Ellis, A. E. (ed.) Fish and shellfish pathology. Academic Press, London, p. 183-189
- Poels, C. L. M., Van Der Gaag, M. A., Van De Kerkhoff, J. F. J. (1980). An investigation into the long-term effects of Rhine water on rainbow trout. *Wat. Res.* 14: 1029-1035
- Riva, M. C. (1989). Toxicidad, acumulación y efectos fisiológicos del colorante premetabilizado C.I. acid violet 66 y su base azoica C.I. acid red 217 en la trucha arcoiris *Salmo gairdneri* R. Ph.D. thesis, Universitat Autònoma de Barcelona
- Riva, M. C., Flos, R., Crespi, M. (1990). Evaluación Toxicológica de un colorante de complejo metálico y su base azoica en la trucha arcoiris *Oncorhynchus mykiss* W. y elección de la dosis. *Revta. Toxicol.* 7: 319-328
- Scarpato, R., Migliore, L., Alfinito-Cognetti, G., Barale, R. (1990). Induction of micronuclei in gill tissue of *Mytilus galloprovincialis* exposed to polluted marine waters. *Mar. Pollut. Bull.* 21: 74-80
- Tates, A. D., Neuteboom, I., Hofker, M., den Engelse, L. (1980). A micronucleus technique for detecting clastogenic effects of mutagens, carcinogens (DEN, DMN) in hepatocytes of rat liver in vivo. *Mutation Res.* 7: 11-20
- Tietge, J. E., Johnson, R. D., Bergman, H. L. (1988). Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. *Can. J. Fish. Aquat. Sci.* 45: 1643-1648
- Wolke, R. E., George, C. J., Blazer, V. S. (1984). Pigmented macrophage accumulations (MMC; PMB): possible monitors of fish health. NOAA tech. Rep. NMFS 25: 93-97
- Wolke, R. E., Murchelano, R. A., Dickstein, C. D., George, C. J. (1985). Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. *Bull. environ. Contam. Toxicol.* 35: 222-227

Responsible Subject Editor: G. Peters, Hamburg, Germany

Manuscript first received: June 11, 1991

Revised version accepted: October 21, 1991