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# Effects of the Fungicide Mancozeb on Liver Structure of Nile Tilapia, *Oreochromis niloticus*: Assessment and Quantification of Induced Cytological Changes Using Qualitative Histopathology and the Stereological Point-Sampled Intercept Method

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Aquatic ecosystems receive large inputs of household, industrial and agricultural pollutants leading to water quality decline and adversely affecting animal health. Sources of agricultural contaminants are numerous and include insecticides, herbicides, and fungicides — all of them ready to be taken by fish from water, food, sediments, and suspended particulate material (Hardersen and Wratten 1998). Mancozeb is a fungicide that after being exposed to water or oxygen is rapidly and spontaneously degraded to ethylene thiourea (its main metabolite) which is teratogenic, goitrogenic, and carcinogenic (US Environmental Protection Agency 1988).

Fish are useful experimental models that have been widely used for evaluating the health of aquatic ecosystems and for studies on toxicological pathology (Law 2003). Tilapia is a good model for toxicological experiments because these fish have high growth rates; adapt easily to commercial diets; are resistant to diseases and injury from handling practices; reproduce well in captivity; and are tolerant of various environmental conditions.

The fish liver is progressively being exploited as a model in research, such as experimental carcinogenesis (Ding et al. 1989) or histological assessment of changes caused either by natural ambient stressors (Balasubramanian et al. 1999) or by pollutants (Desai et al. 1984; Zapata-Perez et al. 2000). The liver has a key role in metabolizing xenobiotics. Nevertheless, these compounds may induce both functional and structural changes. The prompt identification and quantification of abnormal changes is worthy in such studies. In particular, data about the effects of the pesticide mancozeb on fish hepatic histological and biochemical parameters are scarce (Bisson and Hontela 2002; Jarrard et al. 2004).

Based on the above information, our first objective was to determine the effects of waterbone exposure to sublethal amounts of mancozeb for a short time period (four days) on histopathological changes in the livers of Nile tilapia, *Oreochromis niloticus*. Our second objective then was to evaluate the usefulness of the stereological parameter "volume-weighted mean volume" (Gundersen and Jensen

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1985) for detecting size-related cytological changes in fish liver. Stereology focused on hepatocytes, intra-hepatic pigmented macrophages (PMs), and eosinophilic granular (mast) cells (EGCs).

## MATERIALS AND METHODS

The young adult Nile tilapia used originated from stocks reared and maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Fish were maintained in 100 L flow-through tanks supplied with oxygenated water at a rate of 5 L/min, at 22 (±1) ºC, and controlled photoperiod (light: dark cycle of 12L:12D). Water quality parameters were maintained at adequate levels by filtration. Fish were fed daily to visual satiation with a diet previously tested in tilapia (Fontaínhas-Fernandes et al. 1999). Two replicates of 4 animals of both sexes were randomly assigned to each treatment (n=8 per group) and then continuously exposed to waterborne mancozeb for 4 days, kept at the nominal concentrations of 0.5, 1.0, 2.5, and 5.0 $mg.L^{-1}$. Considering the average fish weight (30 g) and the tanks used, the biological load for this experiment was $1.2g.L^{-1}$. Mancozeb (80% pure) was purchased from Riedel de Haën (Germany) and, prior to use, was dissolved in absolute ethanol. The control group was exposed only to the vehicle (ethanol nominal concentration in water was 0.001%). The selected test concentrations of mancozeb ranged from 2.5 to 25% of the LC50 ($20\ mg.L^{-1}$); as estimated at 4 days, using the Probit analysis program based on Finney (1971). Reproductive behaviour or gender effects on results were not noticed.

At the end of the experiment, tilapia were anaesthetized by immersion in a $0.2ml.L^{-1}$ aqueous solution of 2-phenoxyethanol (Merck, Germany) and thereafter measured and weighed. Then, fish were rapidly killed by decapitation. The liver was quickly dissected, sliced into 3 mm thick slabs, and immersed in Bouin's fixative for 24 h, dehydrated, and embedded in paraffin; a minimum of 5 pieces resulted. Histological sections (5 µm-thick) were cut and stained with haematoxylin-eosin (H&E); parallel serial sections were stained with the periodic acid Schiff (PAS) reaction for the demonstration of glycogen in hepatocytes. A qualitative histopathological evaluation was made before applying the stereological approach.

The "volume-weighted mean volume" ($\bar{v}_v$) is a shape-independent stereological parameter, that can be defined as the mean volume of any defined type of bounded structure, each unit being sampled proportional to their volume. Thus, the parameter contains information on mean particle size as well as on variation in size, and it has been widely used either in normal conditions (Henrique et al. 1997) or in pathological ones, including patient prognosis (e.g., Matsui et al. 2005). Cell disturbances (either non-neoplastic or neoplastic) frequently lead to increased nuclei size and/or cell size and related variability; thus, the $\bar{v}_v$ is consequently increased. We estimated the $\bar{v}_v$ of the: 1) hepatocytes (cell body and nucleus); 2) PMs (containing diverse pigment types, as denoted by the brown to yellowish tone); and 3) EGCs. The $\bar{v}_v$ was obtained with the unbiased point-sampled intercepts (PSI) method, according to Gundersen and Jensen (1985). The procedure was performed semi-automatically, by using the Olympus DK A/S computer assisted stereological toolbox; C.A.S.T.-Grid system (ver. 1.6). Under oil immersion (100x objective lens), analysed fields were chosen by the very

efficient systematic uniform random sampling strategy (as detailed in Marcos et al. 2004), using a microscope equipped with a fully motorized high-precision stage (Prior), controlled by the C.A.S.T.-Grid.

Quantitative data are presented as group means, accompanied by the respective coefficients of variation (CV = standard deviation / mean). This ratio provides immediate information about interindividual variability, and allows comparing the relative amounts of variation across the diverse target parameters. Dose effect was examined with analysis of variance (ANOVA), using the software Statistica (ver. 6.0), once confirming normality and variance homogeneity. After a significant ANOVA, the Neuman-Keuls post-hoc test revealed the relevant differences among the groups. The significance level ($\alpha$) was set at 0.05.

## RESULTS AND DISCUSSION

Nile tilapia have a single-lobed liver (often called the hepatopancreas) that harbours pancreatic exocrine acini, which extends from the hilum and that is essentially located adjacent to portal veins and derived venules. In addition to the hepatocytes, there are other important fish liver cell families (Rocha and Monteiro 1999), including the well-known PMs and the EGCs; the latter also involved in defense mechanisms in tilapia (Matsuyama and Iida 1999).

Liver histology from control and exposed animals is briefly illustrated in Fig. 1. In the control group, the liver exhibited a normal architecture and there were no pathological abnormalities, with hepatocytes presenting a homogenous (basically lipid and glycogen rich) cytoplasm, and a large central or subcentral spherical nucleus (Fig. 1A). In animals exposed to concentrations equal or higher than 1.0 $mg.L^{-1}$ of mancozeb it was evidently observed a gradual depletion of hepatocytic glycogen, which attained a minimum amount at 5.0 $mg.L^{-1}$ dosage (Fig. 1B-D).

Concomitantly, there was an increasing hepatocellular basophilia. Such combined loss of hepatic glycogen and increased basophilia is a common, although non specific, liver response to many toxicants (Wolf and Wolfe 2005). Most commonly, the greater cell basophilia results (at least partially) from an increased relative amount of rough endoplasmic reticulum in the cytoplasm, as previous correlative studies have demonstrated in fish hepatocytes (Hinton 1993; Rocha and Monteiro 1999; Wolf and Wolfe 2005). No other qualitative alteration could be depicted in relation to the control group, such as signs of cell death (necrosis) or inflammatory reaction. Moreover, the careful qualitative evaluation did not reveal evidence of nuclear or cellular hypertrophy either in hepatocytes, PMs or EGCs.

As to the stereological approach, the estimated $\overline{v}_v$ values are presented in Table 1. Only the $\overline{v}_v$ of the hepatocyte nucleus showed significant differences, with increased values when exposed to 2.5 and 5.0 $mg.L^{-1}$; although at the latter concentration the value of the $\overline{v}_v$ was lower than at the former concentration.

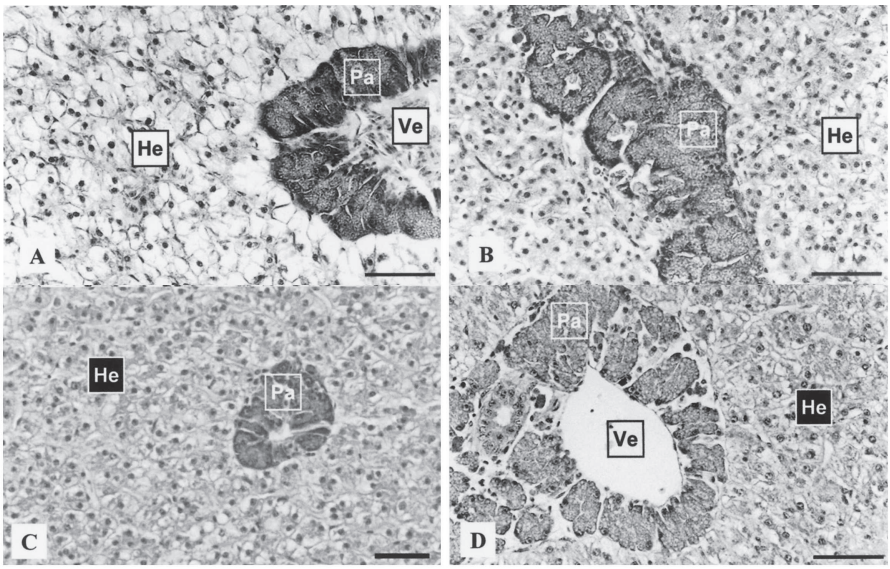


**Figure 1.** Histological images of Nile tilapia (*Oreochromis niloticus*) liver. Control fish (**A**) and fish exposed to mancozeb (**B** - 1.0 mg.L$^{-1}$; **C** - 2.5 mg.L$^{-1}$; **D** - 5.0 mg.L$^{-1}$), showing a gradual increase in basophilia of the hepatic parenchyma (as illustrated by the increasingly darker hepatocytes - He); the darkest (slightly granulated) areas correspond to exocrine cells of the (intrahepatic) pancreas (Pa), typically surrounding afferent veins (Ve). H&E, bars = 50 µm.

Considering established relationships in hepatology (Hildebrand 1980; Deschenes 1981), such an increase in the nuclear $\bar{v}_v$ compared to controls and to lower concentrations is eventually the end result of a higher hepatocellular metabolic activity; a hypothesis that nicely correlates with the increased basophilia. In fact, previous reports showed that the nuclear volume was directly related to DNA content and to the level of cellular activity in non-mitotic cells (Hildebrand 1980). Even in cells in which mitosis is a normal occurrence, such as hepatocytes, changes in nuclear size without modifications in ploidy have been related to altered protein synthesis and nucleolar activity (Christie and LePage 1961; Deleener et al. 1987). Our current data thus calls for a future correlative structural and biochemical approach, for tackling the above cited hypothesis.

Although there were no differences in the hepatocyte body, there was a marked decrease in interfish variation with increasing mancozeb doses. The CVs decreased from 15% in the control group to 1% in both the 2.5 and 5.0 mg.L$^{-1}$ groups (Table 1). This occurrence suggests that, when adapting to the chemical stressor, hepatocytes decreased their natural size variability — usually high among specimens as seen in our own control animals and also as reported for other fishes (Rocha et al. 2001).

**Table 1.** Values of the $\bar{v}_v$ ($\mu m^3$) for the hepatocyte nucleus ($Hep_{nuc}$), hepatocyte cell body ($Hep_{cell}$), pigmented macrophages (PMs), and eosinophilic granular cells (EGCs), both in fish exposed to mancozeb at different dosages and in the control. Data are presented as: Mean (CV)*.

| | **Control** | | **0.5 mg.L$^{-1}$** | | **1.0 mg.L$^{-1}$** | | **2.5 mg.L$^{-1}$** | | **5.0 mg.L$^{-1}$** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **$Hep_{nuc}$** | 47[a] | (0.01) | 45[a] | (0.05) | 39[a] | (0.06) | 70[b] | (0.06) | 58[c] | (0.05) |
| **$Hep_{cell}$** | 3869 | (0.15) | 4005 | (0.10) | 3330 | (0.17) | 4231 | (0.01) | 4000 | (0.01) |
| **PMs** | 156 | (0.12) | 197 | (0.30) | 270 | (0.28) | 128 | (0.03) | 187 | (0.15) |
| **EGCs** | 402 | (0.01) | 299 | (0.32) | 335 | (0.67) | 314 | (0.02) | 375 | (0.14) |

This study proved that Nile tilapia survives and apparently easily handle short--term exposure to the tested doses of mancozeb. These results are quite different from long-term exposure effects on liver by compounds such as glyphosate. After a 3-month exposure, the mancozeb caused evident swelling of hepatocytes and their hydropic and hyaline degeneration, and also severe leucocytic infiltration (Jiraungkoorskul et al. 2003).

We demonstrated that short-exposure to sublethal concentrations of mancozeb evoked in Nile tilapia hepatocellular changes suggestive of an adaptive response, namely as revealed by the evident glycogen depletion and augmented basophilia, by the increased nuclear $\bar{v}_v$, and, finally, by the decreased interanimal variability of the hepatocyte cellular $\bar{v}_v$. No histopatological changes were evoked, correlating well with the unchanged $\bar{v}_v$ of either the PMs or the EGCs. We established that the unbiased stereological PSI method might well be easily usable in fish liver toxicology, providing fast results and detecting relevant structural changes that would otherwise pass totally unnoticed. This study supports the concept that increases in the hepatocellular nuclear $\bar{v}_v$ can be a warning sign for pollutant stress in fish. Consequently, the usefulness of this parameter in ecotoxicology deserves further evaluation.

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