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# A stereological study of copper toxicity in gills of *Oreochromis niloticus* ☆, ☆☆

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**Abstract**

Stereological methods were used to estimate the volumetric density ($V_V$) of the filamentar epithelium (FE, 39%), lamellae (L, 28%), central venous sinus (CVS, 14%), central axis (16%), mucous cells (MC, 2%) and chloride cells (CC, 1%) in the gill filament of control Nile Tilapia. The relative volumes of FE and L, and the relative volumes of CVS and central axis, varied inversely under exposure to copper, with high copper toxic levels declanching a chronic defence mechanism that was, nevertheless, overcome, and low copper toxic levels causing adaptation within a moderate acute phase type of response. Copper also induced a decrease of the $V_V$ (MC, gill filament) due to reduction of surface MC, despite the marked increase of stem MC at chronic exposure to high copper toxic levels. Diminution of the numerical density of filamentar CC was responsible for the decreased $V_V$ (CC, gill filament), although lamellar CC significantly increased at chronic exposure to low copper toxic levels. The present results demonstrate that cell relative volumes, mean volumes and numerical densities are dependent on the variations of the FE and L, which without a quantitative approach may be misinterpreted, thus stressing the importance of using stereological tools for analyzing histopathological patterns.


*Keywords:* Copper toxicity; Gills; Histopathology; Stereology; Teleostei; Tilapia

## 1. Introduction

Gills are a critical organ to fish as they represent the primary site for gas exchange, ion regulation, and excretion of metabolic waste products. With a wide surface area open to the external milieu, gills are also the first target to waterborne pollutants (Mallatt, 1985; Perry and Laurent, 1993). When present at high concentrations, copper causes histopathological changes in gills of Teleost fish, such as epithelial necrosis, vasodilatation, epithelial lifting, hypertrophy of the respiratory epithelium and hyperplasia of the filamentar and lamellar epithelium (Bury et al., 1998; Olsson et al., 1998; Arellano et al., 1999; Cerqueira and Fernandes, 2002).

To quantitatively characterize the histopathological changes caused by waterborne toxicants, several studies have used morphometric methods to analyze the thickness of the filamentar epithelium (FE) (Cerqueira and Fernandes, 2002) as well as the size and number of chloride and mucous cells (MC) (Lock and Overbeeke, 1981; Wendelaar bonga et al.,1990; Pelgrom et al., 1995; Cerqueira and Fernandes, 2002; Lease et al., 2003; Alvarado et al., 2006), whereas others have used stereological methods to evaluate

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the thickness of the blood-oxygen diffusion barrier (Hughes and Perry, 1976; Pinkney et al., 1989; Pane et al., 2004). Stereology is a precise tool for acquiring quantitative three-dimensional information based on two-dimensional data obtained from microscopic structures in tissue sections (Gundersen et al., 1988a, b). Thus, knowing the relative volumes of the various gill filament constituents, the nature of the changes following an experimental exposure can be more precisely described.

The Nile tilapia *Oreochromis niloticus* is a teleost fish with wide distribution around the world, and economic importance for fisheries and aquaculture. Due to its easy handling, culture and maintenance in the laboratory, and because it promptly responds to environmental alterations, this species is also a well-established model for toxicological research (Almeida et al., 2002; Figueiredo-Fernandes et al., 2006). In the present study, we applied current stereological methods to the analysis of the histopathological changes caused by different concentrations of waterborne copper in gills of *O. niloticus*. The relative volumes of the various gill filament constituents, their variation under acute and chronic copper exposure, and the usefulness of the stereological parameter volume-weighted mean volume for detecting size-related changes in chloride cells are presented.

## 2. Materials and methods

### 2.1. Fish

Nile tilapia, *O. niloticus* (Linnaeus, 1758) were raised in the Aquaculture Station of the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real, Portugal), kept in 100 L recirculating tanks (5 L/min) filled with dechlorinated tap water whose quality parameters (84/449/EEC Directives, Annex 5, method c1) were maintained by mechanical and biological filtration: pH 6.5–7.5, 60 mg $HCO_3^-$/L (alkalinity), 63 μS/cm (conductivity), 14 mg $Na^+$/L, 2.3 mg $K^+$/L, 4.1 mg $Ca^{2+}$/L, 6.5 mg $Mg^{2+}$/L, 19.5 mg $Cl^-$/L, 27 mg $NO_3^-$/L (nitrate), 0.5 mg $NO_2^-$/L (nitrite), 74.5 mg $CaCO_3$/L (hardness), 6.2 mg dissolved $O_2$/L, 21 mg $CO_2$/L, 0.1 mg $H_2S$/L (hydrogen sulfide), 0.7 mg $NH_4^+$/L (ammonia) and 12 mg suspended solids/L. Supplemental aeration was provided to maintain the dissolved oxygen near saturation, the temperature was kept at $25 \pm 1$ °C and the photoperiod was controlled (12D:12L). Fish were fed daily once to visual satiation, with commercial fish food dry pellets (Aquasoja-Sorgal, Ovar, Portugal): 1.9% fiber, 4.3% lipids, 37.2% crude proteins, 2.2% $Ca^{2+}$, 1.4% P and vitamins A, C, $D_3$ and E.

### 2.2. Experimental design

Experiments complied with European (86/609/EU) Guidelines for the correct use of laboratorial animals. Sixty sexually mature male and female fish of $36.3 \pm 7.7$ g mean body weight were randomly distributed among 12 tanks (5 fish/100 L). Four tanks served as controls and had water without added copper (3.9 μg Cu/L tap water). Copper (copper sulfate, $CuSO_4$, Merck, Darmstadt, Germany) was added to nominal concentrations of 40 μg Cu/L (mean 39.8, range 36–45) in 4 tanks and 400 μg Cu/L (mean 395.8, range 375–420) in 4 other tanks after preliminary results shown to be sublethal during a 21-day period of exposure. Experiments were carried out in semi-static systems, with a rate of 1/3 water renovation every 2 days. Water samples were taken at collecting days and actual copper concentrations were measured by graphite-furnance atomic absorption spectrophotometry (Unicam 939 AA Spectometer, equipped with a Unicam GF 90 furnance and FS 90 furnance autosamples, UK). Water quality parameters were also reassessed at collecting days during the experimental period, with no significant changes being observed. No mortality was observed during experiments. Fish were fasted for 24 h before collection at days 3, 7, 14 and 21. Five fish per treatment were anesthetized with 1 mL of 2-phenoxiethanol/L water (Sigma, Barcelone, Spain), euthanized by decapitation and the second gill arch of the right side of each fish collected (Bury et al., 1998; Handy et al., 2002; Pane et al., 2004; van Heerden et al., 2004).

### 2.3. Immunohistochemistry

Tissues were fixed for 24 h in Bouin fluid (Panreack, Barcelone, Spain), dehydrated and embedded in paraffin wax. Five paraffin blocks per treatment were serially sectioned (5 μm thick) parallel to the long axis of the gill filament. Two consecutive sections were chosen and mounted on poly-L-lysine-coated glass slides (Sigma). The first section was used for MC localization using the periodic acid-Schiff (PAS) reaction (Merck) and the second section was used for chloride cell (CC) identification using a mouse monoclonal antibody raised against a chicken synthetic peptide corresponding to part of the highly conserved region of the $Na^+/K^+$-ATPase α-subunit (Ura et al., 1996). After deparaffinization and rehydration, slides were rinsed in tap water and dipped for 15 min in 0.3% $H_2O_2$ for endogenous peroxidase blocking (Merck). After rinsing 5 min each in tap water, distilled water and Tris buffer (Merck), sections were incubated for 18 h at 22 °C with 1:500 primary antibody (Department of Biological Sciences, University of Iowa, USA). After $3 \times 5$ min washes in Tris buffer, sections were incubated for 1 h with 1:40 goat-anti-mouse IgG1 secondary antibody and then for 45 min in 1:100 PAP complex (Sigma). After rinsing in Tris buffer, sections were immersed for 10 min in 0.05% of 3,3′-diaminobenzidine in Tris buffer containing 0.03% $H_2O_2$ (Sigma). Subsequently, sections were rinsed for 10 min in tap water, dehydrated, cleared and mounted. Antisera and PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenen, 0.5% Triton X-100 and 0.02% sodium azide (Sigma). For incubations, Coplin jars were used for antisera, whereas, PAP was carried out in a closed dark moist chamber. For negative controls, the primary antibody was omitted.

### 2.4. Stereological analysis

Volume density ($V_V$) is defined as the percentage of the total volume of a well-defined reference space occupied by any given component within it. Accordingly, a stereological approach was designed to estimate the $V_V$ of the different structural elements within the gill filament (the reference space): central axis (cartilaginous and subepithelial connective tissue), central venous sinus (CVS), lamellae (L) and FE and, within the latter, CC and superficial and deep MC. The $V_V$ were estimated by point counting (Freere and Weibel, 1967) using the formula

$$V_v(\text{structure}, \text{reference}) = [P(s) \times 100]/P(r)$$

where $P(s)$ is the number of test points within each structural component and $P(r)$ is the total number of test points lying over the reference space. Counting was done in a microscope (Olympus, BX-50) equipped with a motorized stage (Prior) for stepwise displacements in $x$–$y$ directions (1 μm accuracy) and a CCD camera (Sony) connected to a 17″ PC monitor (Sony). The whole system was controlled by CAST-Grid software version 1.5 (Olympus Denmark A/S, Albertslund, Denmark). The first field of vision was randomly selected. Following fields were systematically sampled by stepwise movements of the stage in the $x$- and $y$-directions ($\text{step}_{x,y} = 165$ μm). A software-generated counting frame, with two sets of points (ratio 1:12), was superimposed on the monitor live image (Fig. 1). To estimate the $V_V$ of central axis, CVS, FE, and L, the 16-encircled points of the lattice were used, whereas, for the $V_V$ of CC and MC, the 192 points of the lattice were used. Counting was made at a final magnification of ×400, with analysis of an average of 230 fields per fish.