Zoological Studies **51**(7): 977-987 (2012)

Zoological Studies

Effects of Sublethal and Lethal Copper Concentrations on the Gill Epithelium Ultrastructure of Nile Tilapia, *Oreochromis niloticus*

Sandra Mariza Monteiro[1,*], Elsa Oliveira[2], António Fontaínhas-Fernandes[1], and Mário Sousa[2,3]

[1]*Department of Biology and Environment and Center for Research and Technology for Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Apartado 1013, Vila Real 5001-801, Portugal*

[2]*Lab Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto 4099-003, Portugal*

[3]*Center for Reproductive Genetics Alberto Barros, Porto 4100-009, Portugal*

(Accepted June 21, 2012)

Sandra Mariza Monteiro, Elsa Oliveira, António Fontaínhas-Fernandes, and Mário Sousa (2012) Effects of sublethal and lethal copper concentrations on the gill epithelium ultrastructure of Nile tilapia, *Oreochromis niloticus*. *Zoological Studies* **51**(7): 977-987. Histopathology is currently being used as a screening method to detect copper toxicity in fish. However, a knowledge gap exists regarding copper's effects on the gill epithelial ultrastructure. To fill in this gap, Nile tilapia *Oreochromis niloticus* were acutely exposed to sublethal and lethal waterborne copper concentrations. Results showed that in the basal region of gill lamellae, a sublethal concentration caused edema, stretching of pillar cells (PLCs), and the disappearance of pericytes (PCTs), whereas in the apical region PLCs remained intact, and PCTs and pavement cells (PVCs) were activated. In the filament epithelium, PVCs showed structural signs of high functional activity, while mitochondrion-rich and mucous cells were degenerated. In the deep filament region, there were edema, loss of neuroepithelial cells, proliferation of undifferentiated cells (UDCs), and transformation of leukocyte-like cells into macrophages. Acclimation was characterized by vasodilatation of the entire lamellar axis and a decrease in the filament epithelial thickness. The protruding lamellae showed PCT proliferation, whereas the superficial filament epithelium became denser due to UDC attachment. All fish exposed to the lethal dose died within 24 h. Gills showed vasodilatation and edema that had lifted the PVCs, and necrosis was evident. In conclusion, our findings contribute to clarification of gill epithelial cell dynamics, and also revealed that apical and basal regions of the lamellae showed different responses, with the apical region exhibiting higher resistance to the toxic actions of copper. Also, superficial and deep regions of the filament epithelium showed dissimilar responses to copper, with the deep epithelium playing important roles in regeneration and protection.
http://zoolstud.sinica.edu.tw/Journals/51.7/977.pdf

Key words: Teleostei, Branchial epithelium, Ultrastructure, Copper, Acute toxicity.

Copper is a trace element essential for the normal growth and metabolism of living organisms. It functions as a cofactor for key enzymes (Puig and Thiele 2002), and in vertebrates, it is indispensable for bone formation, development of connective tissue, and cardiac function (Li et al. 1996). However, this metal becomes toxic to cells when its concentration surpasses certain natural levels (Theophanides and Anastassopoulou 2002). The main sources of copper pollution include mining, industrial discharge, sewage sludge disposal, and fertilizers. Considering this ample range of sources, lixiviation considerably magnifies its levels in aquatic environments, the main final deposit sites for heavy metals of anthropogenic origin (Nor 1987). In freshwater aquatic environments, standard non-toxic values were set to 5-112 μg/L (Chapman 1996); however, higher concentrations are observed in ground and surface waters. Indeed, concentrations of up

*To whom correspondence and reprint requests should be addressed. Tel: 351-259-350108. Fax: 351-259-350266.
E-mail:smonteir@utad.pt

to 560 μg/L were previously reported in polluted waters (Chapman 1996, IPCS 1998, US EPA 2007).

The effects of water copper contamination on fish are of primordial interest. Even sublethal concentrations can induce severe damage, with negative impacts on fish performance and enhanced susceptibility to secondary diseases that potentially cause mortality. Accordingly, copper levels determined during occasional monitoring of aquatic environments might not uncover the severity of contamination, especially in cases of sublethal doses (Canli and Stagg 1996).

Gills represent the largest body surface area of freshwater fish, and in the branchial epithelium, the distance between the water and blood is only a few micrometers (Hughes 1984). This organ is both morphologically and physiologically complex, performing multiple functions, such as gas exchange, ion and water exchange, acid-base balance, nitrogenous waste excretion, toxicant uptake, detoxification, excretion, and several other metabolic transformations (Wood and Soivio 1991, Evans et al. 2005, Tang and Lee 2011). Due to its multiple regulatory functions, delicate structure, and constant exposure to the external environment, gills are the most sensitive target organ of waterborne pollutants (Lauren and McDonald 1987a b, Perry and Laurent 1993, Pelgrom et al. 1997). In copper-polluted water, gills are an important route of metal uptake, and in the initial stages of exposure, retain most of the metal burden, which may change their morphology (Lauren and McDonald 1985, Nowak et al. 1992, Pelgrom et al. 1995a 1997, Campbell et al. 1999). Indeed, histopathological changes are the result of the integration of a large number of interactive physiological processes (van de Oost et al. 2003). The ultrastructure of tissues and organs is altered when the waterborne contaminant is still at low levels. Therefore, histopathological assays may provide a valuable screening method before severe damage occurs (Jiraungkoorskul et al. 2007).

The Nile tilapia, *Oreochromis niloticus*, constitutes a suitable biological model for toxicity studies due to its wide geographic distribution, great economic potential, and ease of brooding in natural and laboratory environments. It can provide a good model to study responses and possible adaptations of fish populations to aquatic pollutants. Indeed, the effects of copper on *O. niloticus* gills were previously studied by our group, who showed the induction of several histopathological and morphometric changes (Monteiro et al 2008, Monteiro et al. 2009a b). However, few reports have been published on the ultrastructure of the gills of *O. niloticus*, with the effects of copper still remaining largely unknown. Copper accumulation in the branchial epithelium (Pelgrom et al. 1995a c), and its effects on ion regulation (Pelgrom et al. 1995b 1997, Dang et al. 2000, Monteiro et al. 2005) and metallothionein concentration (Dang et al. 1999, Olsvik et al. 2001, Wu et al. 2006) were studied in this and other tilapia species, such as *O. mossambicus*, with ultrastructural studies being essentially centered on mitochondrion-rich cells (Li et al. 1998, Dang et al. 2000). Accordingly, in the present study, we evaluated the toxicity of sublethal and lethal copper concentrations on the gill ultrastructure of *O. niloticus*, in order to clarify specific cell-type responses and contribute to establishing a more-efficient and -sensitive risk assessment methodology.

MATERIALS AND METHODS

Animals

Nile tilapia, *Oreochromis niloticus* Linnaeus (1758), were originally obtained from the Institute Nationale de Recherche Agronomique (Rennes, France), and were raised in the Aquaculture Station of the University of Trás-os-Montes e Alto Douro (UTAD) for 3 generations. Fish were maintained at low production densities (< 150 g/L), in glass aquaria with a freshwater flow rate of 5 L/min. Animals were fed daily with commercial fish food (fiber 1.9%, lipids 4.3%, and crude protein 37.2%) to visual satiation, and kept at a constant temperature of 25 ± 1°C under a controlled photoperiod (12-h dark: 12-h light). Supplemental aeration was provided to maintain the dissolved oxygen near saturation. Water quality parameters (pH 6.5-7.5, alkalinity 63 mg/L as HCO_3^-, conductivity 69.5 μS/cm, Na^+ 14 mg/L, K^+ 2.3 mg/L, Ca^{2+} 4.1 mg/L, Mg^{2+} 6.5 mg/L, Cl^- 20.5 mg/L, NO_3^- 27 mg/L, NO_2^- 0.41 mg/L, and SO_4 15.9 mg/L) were maintained at acceptable levels by mechanical and biological filtration. Experiments complied with European Guidelines (86/609/EU) for the correct use of laboratorial animals.

Time course of copper exposure

To analyze the effects of copper on the gill ultrastructure, sublethal and lethal copper concentrations were chosen, based on preliminary results (LC_{50} of 1207 μg/L). Three days before the start of the experiment, sexually mature *O. niloticus* (36.7 ± 7.4 g in mean body weight) were randomly selected from the holding tank and distributed among 9 tanks containing 60 L of fresh water. There were 4 fish per tank in triplicate treatments for each concentration, for a total of n = 36. Fish from 3 aquaria containing water with no copper added served as the control. Fish from the remaining tanks were exposed to concentrations of 600 (range, 628-640) and 1300 (range, 1270-1311) μg/L of copper, supplied as copper sulfate (Merck, Darmstadt, Germany). The experiment was conducted in a semi-static system with 1/3 of the water replaced every 2 d, and carried out at a constant temperature (25 ± 1°C) and with a controlled photoperiod (12-h dark: 12-h light). Supplemental aeration and constant mechanical and biological filtration were provided. The water quality parameters mentioned above were assessed during the experimental period, with no significant changes observed among treatments or acclimation tanks. During the experimental period, fish were fed once daily to visual satiation and were starved for 24 h before sampling.

In the sublethal concentration experiment (600 μg/L), fish were sampled after 3 (acute) and 7 (sub-acute) d of exposure. At each sampling time, 6 fish per treatment (2 from each tank) were anesthetized with 2-phenoxiethanol (Sigma, Barcelona, Spain) (1 ml/L water), weighed, and sampled. In the group exposed to the lethal concentration (1300 μg/L), fish were removed from tanks after the first signs of apparent death (without visible movements or reaction to being touched on the caudal fin). Gill filaments were immediately collected from the anterior and posterior aspects of the medial region of the 2nd gill arch on the right side of each fish.

Transmission electron microscopy (TEM)

Samples (≤ 1 mm^3) were fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.2, for 2 h at 4°C. Following this, they were rinsed in buffer, for 2 h at 4°C, and postfixed with 2% osmium tetroxide in buffer containing 0.08% potassium iron-cyanide, for 2 h at 4°C. Specimens were then washed in buffer, dehydrated through an ascending ethanol series, transferred to propylene oxide, and embedded in Epon. Semi-thin sections (1 mm) were used for a light microscopic analysis after staining with methylene blue. Ultrathin sections were then cut with a diamond knife (Diatome, Hatfield, PA), collected on formvar-coated carbon-reinforced copper grids (TAAB, Berkshire, United Kingdom), double-stained with 5% aqueous uranyl acetate (20 min) and Reynolds lead citrate (10 min), and examined in a JEOL 100CXII TEM (Tokyo, Japan) operated at 60 kV. Chemicals of analytical grade were obtained from Merck.

RESULTS

The gill epithelium of control *Oreochromis niloticus* was formed by a filament multilayered epithelium, periodically sectioned by longitudinal capillary axes that originated in the lamellae, which were covered by a 2-layered epithelium. The superficial layer of the filament epithelium (Fig. 1A) contained mucous cells (MCs), mitochondrion-rich cells (MRCs), their precursors, and intercalating support cells (SCs), which were externally covered by a monolayer of pavement cells (PVCs). The deep layer (Fig. 1B), which was latero-basally lined by myoepithelial cells (MECs) and basal lamina, was formed by a network of undifferentiated cells (UDCs), and also enclosed neuroepithelial cells (NECs), eosinophil-like cells, and MC precursors. Each lamella possessed a central vascular axis, the endothelium of which was composed of pillar cell (PLC) cytoplasmic extensions, externally coated with a basal lamina and a loose interstitial tissue that contained pericytes (PCTs). In the protruding part of the lamellae, the most external coat was composed of lamellar PVCs (Fig. 2).

Although no mortality was registered after acute exposure (3 d) to the sublethal copper concentration (600 μg/L), the initial part of the protruding lamellae showed edema of the interstitial tissue and irregular capillary shapes, whereas vasodilatation was mostly confined to the lamellar basal region and was associated with stretched PLCs (Fig. 3A, B). The external cover of PLCs also exhibited some modifications. In the distal part of the lamellae, PCTs, exhibiting cytoplasm and nuclear characteristics of cell metabolic activation, were observed to be in direct contact with the PLC basement membrane (Fig. 4A, B). However, in the lower part of the protruding lamellae, UDCs were also observed

to be occupying the interstitial lamellar space (Fig. 3A, B). Lamellar PVCs had developed microfolds and exhibited structural characteristics commonly associated with increased functional activity, such as enhanced Golgi complexes, a high quantity of rough endoplasmic reticula, and a consequent increase in dense vesicles. These cells also showed a thickening of the microfilament cortical region and an increased density of the glycocalix (Figs. 3A, 4A). In the lamellar basal region, there were loss of PCTs, a retraction of the ascending-branch of the filament basal lamina, and replacement of the lamellar interstitial tissue with filament MECs and UDCs showing structural characteristics of high metabolic activity (Fig. 3B).

In the superficial layer of the filament epithelium, there was loss of most MRCs and MCs, with occasional degenerating pale-MRCs found near lamellae (Figs. 3, 5A, B). Filamental PVCs were enlarged and displayed apical crypt formation, with conserved microfolding (Fig. 3C). This cell type also exhibited structural signs of high functional metabolic activity, characterized by the presence of a euchromatic nucleus, widening of the cytoplasm compartment, signs of enhanced Golgian and rough endoplasmic reticular activity, and increased formation of dense vesicles (Fig. 6C). The deep layer of the filament epithelium was enlarged, with the network of UDCs apically

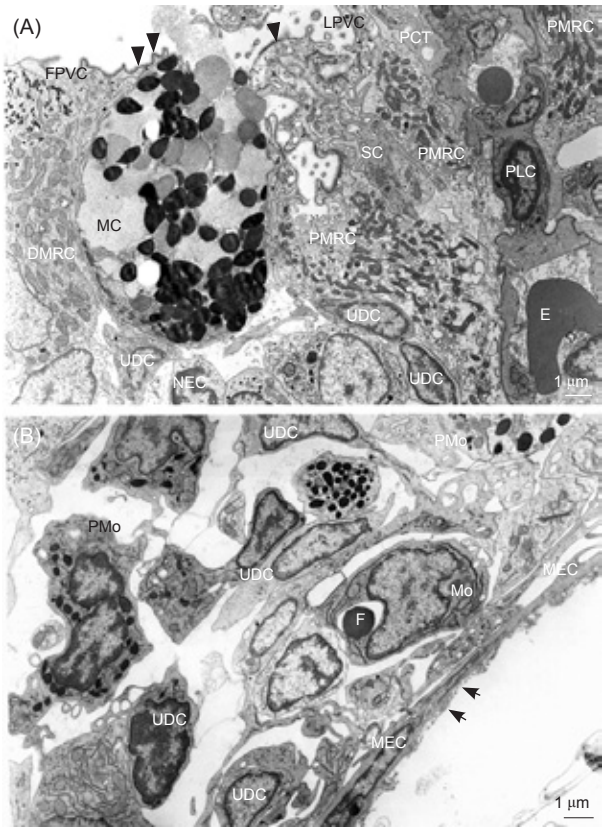

Fig. 1. Ultrastructure of the gill filament epithelium in control *Oreochromis niloticus*. (A) Superficial layer showing a dark mitochondria-rich cell (DMRC) with rod-like vesicles in the submembrane region; a mucous cell (MC) with dense, intermediate-dense, and pale secretory vesicles; pale mitochondria-rich cells (PMRC) with a tubular network, mitochondria, and lipid droplets. The PMRCs are intercalated with support cells (SC) and covered by lamellar (LPVC) and/or filamental pavement cells (FPVC). The presence of a glycocalix (arrowheads) at the FPVCs but not at LPVCs can be noted. PMRCs exhibit cytoplasmic extensions into the lamellar interstice. Note the presence of undifferentiated cells (UDC) and neuroendocrine cells (NEC) in the superficial region of the filament epithelium. Capillary axis at the level of the superficial filament layer. (B) General view of the deep filament layer with UDCs, myoepithelial cells (MEC), precursors of macrophage-like cells (PMo) and mature macrophage-like cell (Mo). Arrows, basal lamina; E, erythrocyte; F, phagosome; PCT, pericyte; PLC, pillar cell.

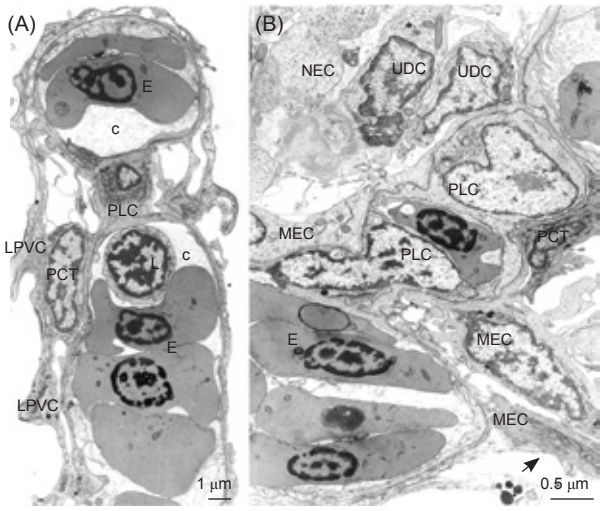

Fig. 2. Ultrastructure of the gill epithelium in control *Oreochromis niloticus*; the capillary axis. (A) Upper limit of the capillary axis. Each pillar cell (PLC) originates 4 cytoplasmic branches that compose the endothelium lining of adjacent capillary rows; it is externally coated by a thin basal lamina and a loose interstitial tissue with pericytes (PCT). Lamellar pavement cells (LPVC), less dense and with reduced numbers of microfolds and organelles than those found in filament pavement cells. (B) Base of the capillary axis, showing myoepithelial cells (MEC) in the deepest region and PCTs from there to the superficial region of the filament epithelium. Arrow, basal lamina; c, capillary; E, erythrocyte; L, lymphocyte; NEC, neuroepithelial cell; UDC, undifferentiated cell.

expanded to establish direct connections with superficial PVCs (Fig. 3A, C). UDCs showed signs of activation, characterized by a lower cytoplasmic electron density and increased numbers of mitochondria, rough endoplasmic reticular cisternae, ribosomes, dictyosomes, and small dense vesicles (Fig. 6A, B). Intercellular spaces, enlarged by the interstitial edema, contained occasional hypotrophic NECs and degenerating MC precursors, which were mainly filled with leukocyte-like cells that convert to active macrophages involved in the phagocytosis of degenerative cells (Fig. 5B, C). In the most basal region of the filament epithelium, ME cells exhibited structural characteristics associated with high functional activity, as evidenced by a more-euchromatic nucleus, widening of the myofilament-rich cytoplasm, and several dictyosomes with abundant cisternae (Figs. 5C, 6D). These changes affected the entire ME cell population, including those that lined the filament epithelium basal region (Fig. 6D), those resting on them (Fig. 5C), and ME cells involved in strengthening the lamellar capillaries (Fig. 3B). The filament basal lamina seemed to have increased in thickness, mainly due

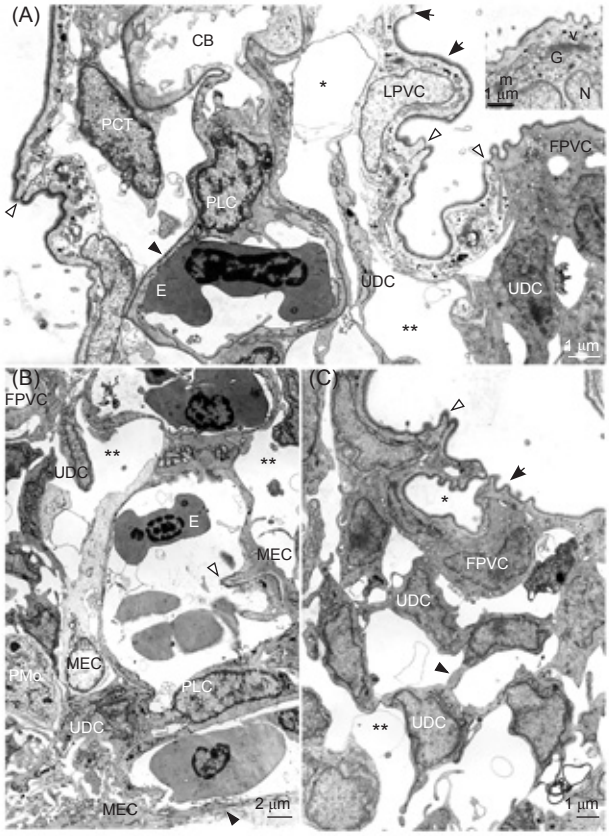

Fig. 3. Ultrastructure of gills from fish exposed to 600 μg/L copper for 3 d. (A) Protruding portion of lamellae. Note the irregular capillary bed (CB), interstitial edema (*), and infiltration by undifferentiated cells (UDC), intact pillar cells (PLC) and their basal lamina (black arrowhead), activated pericytes (PCT), and activated lamellar pavement cells (LPVC) with microfolding (arrows) and tight cell junctions (white arrowheads). In the filament epithelium, note the absence of pale mitochondria-rich cells (**) and the presence of activated filamental pavement cells (FPVC) with attached activated UDCs (the inset shows an activated PVC at higher magnification). (B) Basal half of lamellae with vasodilatation, stretching, and retraction (white arrowhead) of PLCs, the encircling of the capillary base by activated myoepithelial (MEC) and UDCs, and the increased thickness of the filament basal lamina (black arrowhead). (C) Superficial region of the filament epithelium with activated FPVCs, with tight cell junctions (white arrowhead), glycocalix, microfolding (arrows), crypt formation (*), and attached activated UDCs. The deep region of the filament shows a network of interconnected (black arrowhead) activated UDCs separated by large intercellular spaces (**) due to interstitial edema. E, erythrocyte; G, Golgi complex; m, mitochondria; N, nucleus; PMo, precursor of macrophage-like cell; v, vesicles.

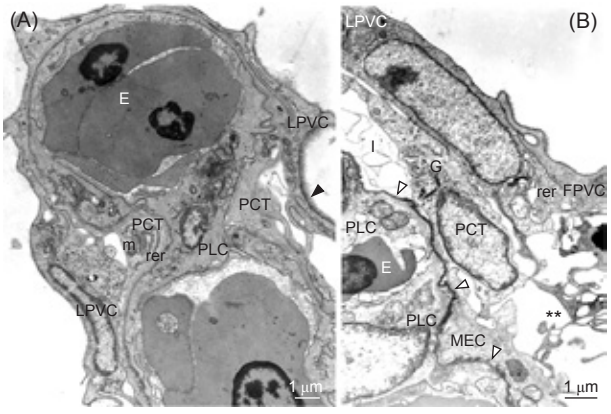

Fig. 4. Ultrastructure of gills from fish exposed to 600 μg/L copper for 3 d. (A) The tip of the protruding lamella, showing intact pillar cells (PLC) and pericytes (PCT) with structural characteristics that suggest high functional activity such as a prominent Golgi complex (G), rough endoplasmic reticulum (rer), and mitochondria (m). Lamellar pavement cells (LPVC) with microfolds and a non-continuous glycocalix (black arrowhead). (B) Lamellar interstice at the limit of the superficial filament epithelium, showing PCTs with structural characteristics of functional activation, the absence of light mitochondria-rich cells (**), and their replacement with myoepithelial cells (MEC), the cytoplasmic extensions of which cover pillar cells (PLC) up to the filament epithelium limit. E, erythrocyte; I, interstice; white arrowheads, basal membrane of pillar cells.

to reticular and collagen fiber deposition (Figs. 5C, 6D).

After 7 d of exposure to 600 μg/L, the entire capillary axis showed vasodilatation, which was especially pronounced in the basal part of the lamellae and had induced extensive stretching of PLCs, with focal signs of necrosis and transformation into squamous cells. In this region, the lamellar interstitial tissue was replaced by filamental MECs and UDCs that showed structural evidence of increased metabolic activity. PCTs and the ascending branch of the filament basal lamina were absent (Fig. 5D). Despite vasodilatation, PLCs in the protruding part of the lamellae showed

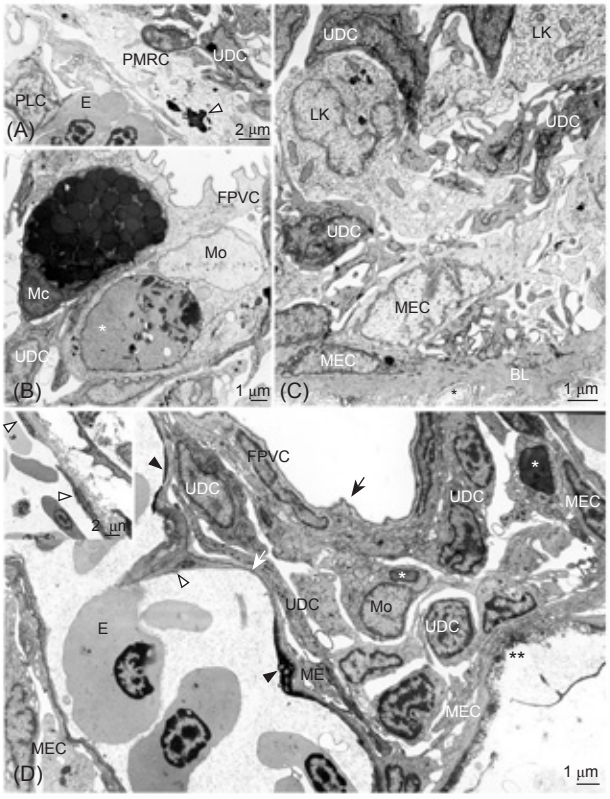

Fig. 5. Ultrastructure of gills from fish exposed to 600 μg/L copper for 3 (A-C) and 7 d (D). (A) Degenerated pale mitochondrion-rich cells (PMRC) next to lamellae, showing autolytic lysosomes (white arrowhead), a decreased number of mitochondria, and loss of the tubular system. (B) Under the filament pavement cell (FPVC) epithelium, note an intact mucous cell (MC), a macrophage (Mo) with a large digestive vacuole (*), and undifferentiated cells (UDC). (C) Basal layer of the filament epithelium. Note the hypertrophic and hyperplastic myoepithelial cells (MEC), the thickening of the basal lamina (*), the network of activated UDCs, and leukocyte-like cells (LK). (D) Filament epithelium and basal part of the lamellae in gills from fish exposed 600 μg/L copper for 7 d. Note the severe vasodilatation, with pillar cells (PLC) showing stretching and retraction (white arrowhead), squamous transformation (inset: white arrowheads), focal necrosis (black arrowheads), intactness of the basal lamina (white arrow), and encircling by activated filamental MECs and UDCs. The filament epithelium is severely decreased in thickness and shows filament pavement cells (FPVC) that are condensed and thin, with intact cell junctions (arrow) and the near absence of microfolds. The surface region is increased in thickness through the attachment to PVCs of activated UDCs. In the deep region, there is a short network of UDCs and macrophages (Mo) with large residual digestive vacuoles (*). The basal lamina is increased in thickness (**). E, erythrocyte.

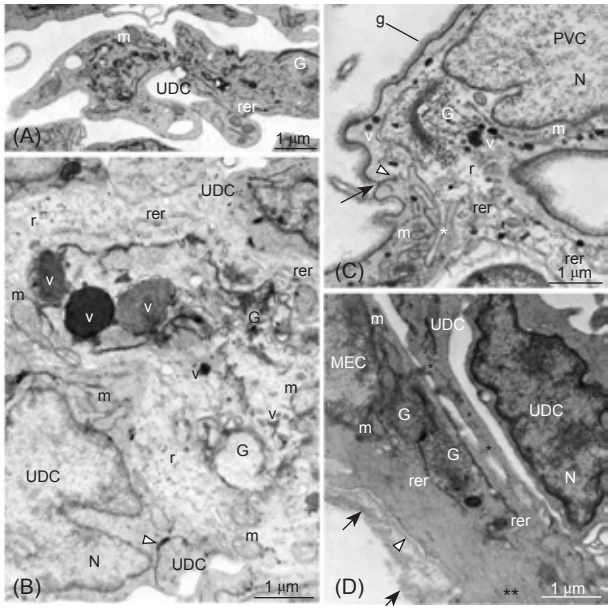

Fig. 6. Ultrastructural characteristics commonly associated with high functional activity in different cell types observed in gills from fish exposed to 600 μg/L copper for 3 d. (A, B) Undifferentiated cells (UDC) from the deep filament epithelium. Metabolic activation is characterized by a less-electron-dense cytoplasm, euchromatic nucleus (N), and enriched mitochondria (m), rough endoplasmic reticula (rer), ribosomes (r), and Golgi complexes (G) very active in dense vesicle (v) formation. Arrowhead, desmosomes. (C) Pavement cells (PVC) showing slight functional activation by the presence of a euchromatic nucleus (N), a wider cytoplasm with high Golgi (G) and rer activities, and a consequent increase in dense vesicles (v). A glycocalix (g), with increased density, and cell unions, with desmosomes (arrowheads) and junctional complexes (arrow), is evident; m, mitochondria; *, cell interdigitations. (D) Myoepithelial cells (MEC) from the deepest region of the filament epithelium, showing structural signs of high functional activation, as evidenced by a wide, euchromatic nucleus (N), elongation of myofilament-rich cytoplasm (**), and the higher Golgi activity (G). Note the basal lamina (arrowhead) thickened by reticular and collagen fibers deposition (arrows) and lateral connections (*) with UDCs.

a rather conserved morphology, although the external covering increased in thickness due to PCT proliferation. The cytoplasm of lamellar PVCs had become more electron-dense, less wide, and had reduced Golgi activity (Fig. 7A).

The filament epithelium was extremely reduced in thickness, showing an almost complete absence of MRCs and MCs, although occasional pale-MRC precursors were observed near lamellae (Figs. 5D, 7A). Filament PVCs were denser and flattened, with decreased microfolding and no signs of crypt formation. The increase in densification of the superficial layer of the filament epithelium was due to the close attachment of UDCs to the base of filamental PVCs (Figs. 5D, 7A). Below this superficial region, the filament epithelium was reduced to a short network of UDCs showing signs of high functional activity, the intercellular spaces of which showed no NECs or precursor cells, but contained leukocyte-like cells and macrophages with large residual digestive vacuoles. The basal lamina of the filament epithelium was apparently thicker, with deposition of collagen and reticular fibers (Figs. 5D, 7B).

Exposure to 1300 μg/L copper was found to be lethal to 100% of the fish in the 1st 24 h. Gill epithelia of fish exposed to a lethal copper concentration exhibited patches of tissues with different degrees of injury, including regions with complete rupture of the filament epithelium and of the lamellar vascular axis (Fig. 8A-D). In most instances, the protruding lamellae showed vasodilatation, with preserved PLCs and their associated basal lamina. However, there were an absence of PCTs, the presence of interstitial edema, and infiltration of the interstitial tissues

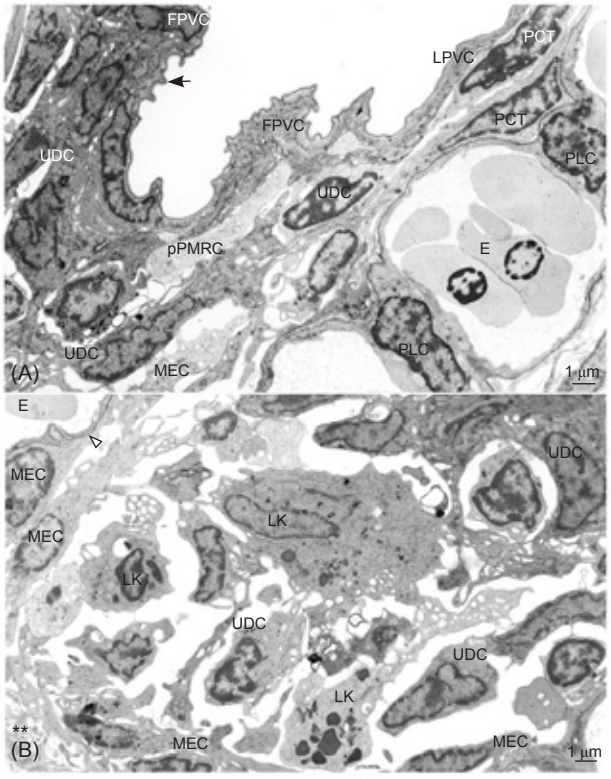

Fig. 7. Ultrastructure of gills from fish exposed to 600 μg/L copper for 7 d. (A) Filament epithelium and apical half of lamellae. Note the vasodilatation, with intact pillar cells (PLC), pericyte (PCT) proliferation, and condensed lamellar pavement cells (LPVC). Filamental pavement cells (FPVC) exhibit a few microfolds (arrow) and appear attached to activated undifferentiated cells (UDC). (B) Basal region of the filament epithelium. Note the short network of UDCs, the activated leukocyte-like cells (LK), the hypertrophic, hyperplastic myoepithelial cells (MEC), and also the thickening of the basal lamina (**). E, erythrocyte; pPMRC, precursor of pale mitochondrion-rich cells; pillar cell basal lamina (white arrowhead).

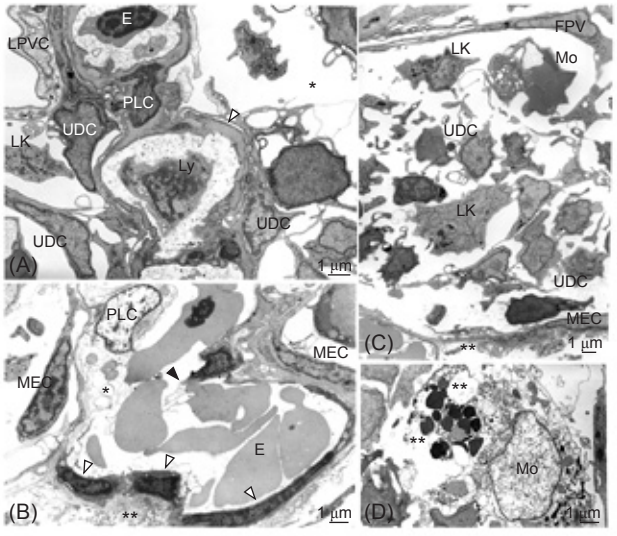

Fig. 8. Ultrastructure of gills from dead fish exposed to 1300 μg/L copper. (A) Apical half of lamellae. Note the vasodilatation, intactness of pillar cells (PLC) and their basal lamina (white arrowhead) and also of lamellar pavement cells (LPVC), the interstitial edema (*), and infiltration of interstitial tissue by activated filamental undifferentiated cells (UDC). (B) Basal half of lamellae with accentuated vasodilatation, PLCs showing stretching and retraction (black arrowhead), necrosis (white arrowheads), and cell lysis (*), encircled by activated filament myoepithelial cells (MEC), and thickening of the basal lamina (**). (C) Filament epithelium, with a lifted pavement cell (FPVC) epithelium that is thin and condensed. Note the intense interstitial edema which has disrupted intercellular connections of activated UDCs. (D) Macrophage (Mo) lysis (**). E, erythrocyte; LK, activated leukocyte-like cells; Ly, lymphocyte; Mo, macrophage filled with large digestive vacuoles.

by filamental UDCs. Lamellar PVCs appeared intact and had developed microfolds (Fig. 8A). In the basal region of lamellae, the vascular axis showed extensive vasodilatation, with stretching and necrosis of PLCs. In this region, the lamellar interstitial tissue was replaced by filament MECs and UDCs which exhibited structural characteristics commonly associated with high functional activity. PCTs and the ascending branch of the filament basal lamina were absent (Fig. 8B).

Due to the marked interstitial edema, the simple squamous epithelium of PVCs was progressively lifted, sometimes up to the tip of the lamellae. Filamental PVCs appeared electron dense and thin, showing loss of microfolds, a reduced glycocalix, and focal ruptures (Fig. 8C). The enlarged filament intercellular spaces contained separated UDCs, leukocyte-like cells, and macrophages with large digestive vacuoles which frequently showed autolysis (Fig. 8C, D). The basal lamina of the filament epithelium also appeared strengthened (Fig. 8B, C).

DISCUSSION

Copper, as with other metal elements, is essential for fish survival but can become toxic when present at high concentrations. Lethality tests provide a useful way to evaluate the toxicity of specific pollutants, and the LC_{50} value was established as an acceptable limiting concentration of a pollutant in water (Nussey et al. 1996). However, these tests cannot accurately predict the concentration at which a pollutant begins to be harmful to an organism, thus underestimating its true effect (Lauren and McDonald 1985 1987a, Taylor et al. 1995). Indeed, in the present study with *Oreochromis niloticus*, we show that a sublethal copper concentration (600 μg/L) exerted sustained negative effects on fish gills, inducing acute toxic damage that seemed to be later compensated for without recovery of the normal cytoarchitecture of the organ.

In lamellae, exposure to sublethal copper levels displayed differential responses, with the basal part being seriously affected, while the protruding region manifested marked resistance to the pollutant. The base of lamellae exhibited vasodilatation with PLC stretching and focal necrosis. Partial detachment of PLCs with subsequent coalescence of capillaries or even the disappearance of most PLCs were changes also observed in other species exposed to copper and other toxicants (Evans et al. 1988, Cardoso et al. 1996, Wilson and Taylor 1993, Pfeiffer et al. 1997). In addition to this, and for the 1st time, the loss of PCTs, was observed.

On the other hand, the protruding intact ultrastructure of the lamellae suggested higher resistance and evidenced a faster compensatory response to the toxic actions, which may have been due to its primordial function in gas exchange, with respiratory cell injury requiring a much-higher toxicant threshold than that observed for cells involved in ion regulation (McDonald and Wood 1993). Furthermore, the finding of PCT and lamellar PVC activation during the acute phase and the development of microfolding of lamellar PVCs and proliferation of PCTs during acclimation suggested a higher capacity of the protruding lamellae for adaptation and regeneration.

In the filament epithelium, the acute toxic effects of copper were described as inducing necrosis and apoptosis of filament PVCs (Pelgrom et al. 1995b, Bury et al. 1998, Li et al. 1998), with these degenerative effects being followed by a reparative action of filamental UDCs, which migrate and differentiate in about 2-5 d (McDonald and Wood 1993, Pelgrom et al. 1995b, Dang et al. 1999). However, other studies referred to the intactness of the squamous surface epithelium (Cardoso et al. 1996). The present experiments with sublethal copper concentrations demonstrated that in the acute phase, necrosis appeared restricted to MRCs and MCs, with the latter showing a higher level of resistance to the toxic actions. On the contrary, filamental PVCs exhibited structural characteristics associated with high functional activity and developed crypts which in addition to the increase in surface area, suggested that PVCs may actively entrap and process copper. This, accompanied by UDCs that closely attached to filamental PVCs during acclimation to a sublethal copper concentration, created a dense multilayered superficial epithelium that made passage of the toxicant to the blood difficult. At this step of adaptation and even if a few precursor cells were found, both MRCs and MCs were absent. Although this was not a quantitative study, in previous work with *O. niloticus* exposed to lower copper concentrations, the stereological quantification of MRCs and MCs showed progressive concentration-dependent declines in these cell types in the gill epithelium, thus supporting the present results (Monteiro et al. 2009b). As all fish remained alive, our results suggested that the ionoregulatory function of MRCs

was replaced by those of filamental PVCs, the only cell type that remained intact and continuously differentiated from filament UDCs. Contrary to our present results, previous studies with copper and other pollutants revealed hyperplasia and hypertrophy of MRCs (Baker 1969, McDonald and Wood 1993, Speare et al. 1997, Karan et al. 1998), associated either with MC hyperplasia and mucus hypersecretion (Mallatt 1985, Karlsson-Norrgren et al. 1986, Speare et al. 1997) or a reduction in MC numbers (Baker 1969, Powell et al. 1995). As polyanionic mucins trap toxic environmental substances, mucus hypersecretion would prevent metal cations from crossing the gill epithelium (Lock and Overbeeke 1981, Reid and McDonald 1991, McDonald and Wood 1993). Thus, the paucity of MCs after 3 d and their total absence after 7 d of copper exposure may be justified by an early mucus hypersecretion with consequent MC degeneration (Miller and MacKay 1982). Declines in MRCs and MCs may be the cause of the apparent decrease in the filament epithelial thickness. Indeed, and supporting this observation, a reduction in the relative epithelial volume was observed after 7 d of *O. niloticus* exposure to waterborne copper (Monteiro et al. 2009b).

The deep region of the filament epithelium also exhibited marked changes after exposure to a sublethal copper concentration. Hypertrophy, hyperplasia, and increased intercellular connections among MECs, which encircled the lamellar capillary up to the region of filament PVCs, were initiated during the acute phase and were maintained under acclimation. These observations clearly show the importance of this cell type, recently described in *O. niloticus* gill epithelium (Monteiro et al. 2010b), in resistance of the organ to toxic insults. The close proximity of pillar cells by these cell types may represent a defense mechanism, by increasing the barrier for copper diffusion, and compensating for the loss of PCTs, of PLC contractility, and of blood flow control. These regenerative effects were in accordance with a high concentration of copper in the blood, as the basal filament epithelium is adjacent to the central venous sinus and capillary base (Pelgrom et al. 1995c). Similarly, gills of *O. mossambicus* exposed to copper showed more metallothionein-positive cells in the basal region than in the median region of the filament epithelium (Dang et al. 1999), reinforcing the idea of a high copper content in the basal compartment.

The proliferation of filamental UDCs is thought to be a compensatory defense response by fish gills, which is unrelated to direct actions of the toxicant but with repair mechanisms that enable recovery and acclimation processes (McDonald and Wood 1993). The repair process is characterized by increased mitotic activity that is assumed to originate in filamental UDCs (Laurent 1984, Pisam and Rambourg 1991, Dutta et al. 1996). These are supposed to differentiate and replace other cell types, thus contributing to a thickening of the filament epithelium, and increasing the distance to external water (Evans et al. 1988). Indeed, a quantitative study of the gill epithelium in copper-exposed *O. niloticus* proved the occurrence of UDC proliferation (Monteiro et al. 2009a). Our present results, although qualitative, suggest that filamental UDCs proliferate and demonstrate that a few had invaded interstitial tissues of the protruding lamellae, where they probably replaced injured PCTs and PLCs. In the basal lamellar region, UDCs totally replaced the missing PCTs and contributed to an increased cell barrier, establishing multiple interconnections with MECs that encircle the basal capillary bed. Upon acclimation, the filamental PVC epithelium was reinforced by 2 or 3 additional cell layers derived from activated UDCs that attached and showed differentiated morphological characteristics typical of filament PVCs.

The presence of macrophages and leukocytes in the filament extracellular space is part of the compensatory repair response to tissue damage that occurs as soon as 3 d after exposure to different types of pollutants (Wendelaar Bonga and Lock 1992, Dutta et al. 1996, Teh et al. 1997, Li et al. 1998), with increased injury being noted when blood leukocyte recruitment fails (Nowak 1992). Our present results, in the acute phase of sublethal copper exposure, clearly demonstrated that macrophages are derived from a resident population of filament leukocyte-like cells, with no recruitment of blood cells noted. This observation supports a previous hypothesis that in contrast to other species, *O. niloticus* possesses a resident granular cell type in the gill epithelium, probably belonging to the immune defense system (Monteiro et al. 2010a). These cells actively phagocyte degenerated cells, and at the time acclimation is established, they remain filled with giant residual digestive vacuoles, whereas leukocyte-like precursor cells had decreased, thus suggesting a very successful adaptive response.

The present study shows for the 1st time that lethal copper concentrations induce deep

degenerative effects on the entire lamellar structure and exert profound toxic actions on all epithelial cell types, which is in accordance with the death of all fish in the 1st 24 h. Indeed, despite the proliferative effort of UDCs and the higher number, size, and phagocytosing efforts of resident leukocyte-like cells, the edema, increased filament intercellular space that disrupted all UDC connections, decreased precursor forms of defense cells, and the frequent autolysis experienced by macrophages, impeded repair activity.

In summary, this study of ultrastructural effects of waterborne copper on gill epithelia revealed that even at sublethal levels, the metal can induce severe damage to fish. The results also allow important deductions about *O. niloticus* gill epithelial cell dynamics. Our findings demonstrate that the apical and basal regions of lamellae show different responses, with PLCs stretching and loss of PCTs in the lamellar base, while in the protruding region, PLCs remained undamaged and PCTs and PVCs were activated, showing higher resistance to the toxic actions of copper. Also, the superficial and deep regions of the filament epithelia showed dissimilar responses to copper, with the deep epithelium having an important role in regeneration and protection. Furthermore, a lethal copper dose induced severe irreversible tissue damage, with PLC retraction and necrosis and the disappearance of PCTs.

Acknowledgments: This work was financially supported in part by FCT (POCI/SAU-MMO/60709/60555/59997/04; UMIB).

REFERENCES

Baker JT. 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleuronectes americanus*). J. Fish. Res. Board Can. **26:** 2785-2793.

Bury NR, J Li, G Flik, RAC Lock, SE Wendelaar-Bonga. 1998. Cortisol protects against copper induced necrosis and promotes apoptosis in fish gill chloride cells *in vitro*. Aquat. Toxicol. (Amsterdam) **40:** 193-202.

Campbell HA, RD Handy, M Nimmo. 1999. Copper uptake kinetics across the gills of rainbow trout (*Oncorhyncus mykiss*) measured using an improved isolated perfused head technique. Aquat. Toxicol. **46:** 177-190.

Canli M, RM Stagg. 1996. The effects of *in vivo* exposure to cadmium, copper and zinc on the activities of the gill ATPases in the Norway lobster, *Nephrops norvegicus*. Arch. Environ. Contam. Toxicol. **31:** 494-501.

Cardoso EL, H Chiarini-Garcia, RM Ferreira, CR Poli. 1996. Morphological changes in the gills of *Lophiosilurus alexandri* exposed to unionized ammonia. J. Fish Biol. **49:** 778-787.

Chapman DV, ed. 1996. Water quality assessments. A guide to the use of biota, sediments and water in environmental monitoring, 2nd ed. Washington, DC: Taylor & Francis.

Dang Z, R Lock, G Flik, SE Wendelaar Bonga. 1999. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. Am. J. Physiol. **277:** R320-R331.

Dang Z, R Lock, G Flik, SE Wendelaar Bonga. 2000. Na^+/k^+-ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to copper. J. Exp. Biol. **203:** 379-387.

Dutta HM, J Munshi, PK Roy, NK Singh, S Adhikari, J Killius. 1996. Ultrastructural changes in the respiratory lamellae of the catfish, *Heteropneustes fossilis* after sublethal exposure to malathion. Environ. Pollut. **92:** 329-341.

Evans DH, PM Piermarini, KP Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. **85:** 97-177.

Evans RE, SB Brown, TJ Hara. 1988. The effects of aluminium and acid on gill morphology in rainbow trout, *Salmo gairdneri*. Environ. Biol. Fish **22:** 299-311.

Hughes CM. 1984. General anatomy of the gills. *In* WS Hoar, DJ Randall, eds. Fish physiology, vol. 10A. New York: Academic Press, pp. 1-72.

IPCS. 1998. Copper: environmental health criteria 200. Geneva: International Programme on Chemical Safety (IPCS), World Health Organization.

Jiraungkoorskul W, S Sahaphong, N Kangwanrangsan. 2007. Toxicity of copper in butterfish (*Poronotus triacanthus*): tissues accumulation and ultrastructural changes. Environ. Toxicol. **22:** 92-100.

Karan V, S Vitorovic, V Tutundzic, V Poleksic. 1998. Functional enzyme activity and gill histology of carp after copper sulfate exposure and recovery. Ecotoxicol. Environ. Saf. **40:** 49-55.

Karlsson-Norggren L, W Dickson, O Ljungberg, P Runn. 1986. Acid water and aluminium exposure: gill lesions and aluminium accumulation in farmed, brown trout, *Salmo trutta* L. J. Fish Dis. **9:** 1-9.

Lauren DJ, DG Mcdonald. 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson: modulation by water hardness and pH. J. Compar. Physiol. **155B:** 635-644.

Lauren DJ, DG McDonald. 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Can. J. Fish. Aquat. Sci. **44:** 99-104.

Lauren DJ, DG McDonald. 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Can. J. Fish. Aquat. Sci. **44:** 105-111.

Laurent P. 1984. Gill internal morphology. *In* WS Hoar, DJ Randall, eds. Fish physiology, vol. 10A. New York: Academic Press, pp. 73-183.

Li J, RAC Lock, SE Quabius, PHM Klaren, HGP Swarts, FMAH Schururmans Stekhoven et al. 1996. Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase. Toxicol. Lett. **87:** 31-38.

Li J, SE Quabius, SE Wendelaar Bonga, G Flick, RAC Lock. 1998. Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquat. Toxicol. **43:** 1-11.

Lock LA, AP Overbeeke. 1981. Effects of mercuric chloride and methylmercuric chloride on mucus secretion in rainbow trout, *Salmo gairdneri* Richardson. Comp. Biochem. Physiol. **69C:** 67-73.

Mallatt J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can. J. Fish. Aquat. Sci. **42:** 630-648.

McDonald DG, CM Wood. 1993. Branchial mechanisms of acclimation to metals in freshwater fish. *In* JC Rankin, FB Jensen, eds. Fish ecophysiology. London: Chapman & Hall, pp. 297-321.

Miller TG, WC Mackay. 1982. Relationship of secreted mucus to copper and acid toxicity in rainbow trout. Bull. Environ. Contam. Toxicol. **28:** 68-74.

Monteiro SM, AA Fontaínhas-Fernandes, M Sousa. 2010a. An immunohistochemical study of gill epithelium cells in the Nile tilapia, *Oreochromis niloticus*. Folia Histochem. Cyto. **48:** 112-121.

Monteiro SM, JM Mancera, AA Fontaínhas-Fernandes, M Sousa. 2005. Copper induced alterations of biochemical parameters in the gill and plasma of *Oreochromis niloticus*. Comp. Biochem. Physiol. **141C:** 375-383

Monteiro SM, E Oliveira, AA Fontaínhas-Fernandes, M Sousa. 2010b. Fine structure of the branchial epithelium in the teleost *Oreochromis niloticus*. J. Morphol. **271:** 621-633.

Monteiro SM, E Rocha, AA Fontaínhas-Fernandes, M Sousa. 2008. Quantitative histopathology of *Oreochromis niloticus* gills after copper exposure. J. Fish Biol. **73:** 1376-1392

Monteiro SM, E Rocha, JM Mancera, AA Fontaínhas-Fernandes, M Sousa. 2009b. A stereological study of copper toxicity in gills of *Oreochromis niloticus*. Ecotox. Environ. Saf. **72:** 213-223.

Monteiro SM, NMS Santos, M Calejo, AA Fontaínhas-Fernandes, M Sousa. 2009a. Copper toxicity in gills of the teleost fish, *Oreochromis niloticus*: effects in apoptosis induction and cell proliferation. Aquat. Toxicol. **94:** 219-228.

Nor YN. 1987. Ecotoxicity of copper to aquatic biota: a review. Environ. Res. **43:** 247-282.

Nowak BF, JG Deavin, BL Sarjito Munday. 1992. Scanning electron microscopy in aquatic toxicology. J. Comput. Assisted Microsc. **4:** 241-246.

Nussey G, JH Van Vuren, HH Preez. 1996. Acute toxicity tests of copper on juvenile Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae), at different temperatures. South Afr. J. Wild Res. **26:** 47-55.

Olsvik PA, P Gundersen, RA Anderson, KE Zachariassen. 2001. Metal accumulation and metallothionein in brown trout, *Salmo trutta*, from two Norwegian rivers differently contaminated with Cd, Cu and Zn. Comp. Biochem. Phys. **128C:** 189-201.

Pelgrom S, L Lamers, J Garritsen, BM Pels, R Lock, P Balm, SE Wendelaar Bonga. 1995a. Interactions between copper and cadmium during single and combined exposure in juvenile tilapia, *Oreochromis mossambicus*: influence of feeding condition on whole body metal accumulation and the effect of the metals on tissue water and ion content. Aquat. Toxicol. **30:** 117-135.

Pelgrom S, L Lamers, R Lock, P Balm, SE Wendelaar Bonga. 1995b. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. Aquat. Toxicol. **32:** 303-320.

Pelgrom S, R Lock, P Balm, SE Wendelaar Bonga. 1995c. Interactions between copper and cadmium modify metal organ distribution in mature tilapia, *Oreochromis mossambicus*. Environ. Pollut. **90:** 415-423.

Pelgrom S, R Lock, P Balm, SE Wendelaar Bonga. 1997. Calcium fluxes in juvenile tilapia, *Oreochromis mossambicus*, exposed to sublethal waterborne Cd, Cu or mixtures of these metals. Environ. Toxicol. Chem. **16:** 770-774.

Perry SF, P Laurent. 1993. Environmental effects on fish gill structure and function. *In* JC Rankin, FB Jensen, eds. Fish ecophysiology. London: Chapman & Hall, pp. 231-264.

Pfeiffer CJ, B Qiu, CH Cho. 1997. Electron microscopic perspectives of gill pathology induced by 1-naphthyl-N-methylcarbamate in the goldfish (*Carassius auratus* Linnaeus). Histol. Histopathol. **12:** 645-653.

Pisam M, A Rambourg. 1991. Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. Int. Rev. Cytol. **130:** 191-232.

Powell MD, GM Wright, DJ Speare. 1995. Morphological changes in rainbow trout (*Oncorhyncus mykiss*) gill epithelia following repeated intermittent exposure to chloramine-T. Can. J. Zool. **73:** 154-165.

Puig S, DJ Thiele. 2002. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem. Biol. **6:** 171-180.

Reid SD, DG McDonald. 1991. Metal binding activity of the gills of rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. **48:** 1061-1068.

Speare DJ, G Arsenault, N Macnair, MD Powell. 1997. Branchial lesions associated with intermittent formalin bath treatment of Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhyncus mykiss* (Walbaum). J. Fish Dis. **20:** 27-33.

Tang CH, TH Lee. 2011. Morphological and ion-transporting plasticity of branchial mitochondrion-rich cells in the euryhaline spotted green pufferfish, *Tetraodon nigroviridis*. Zool. Stud. **50:** 31-42.

Taylor EW, MW Beaumont, PJ Butler, J Mair, MS Mujallid. 1995. Lethal and sub-lethal effects of copper upon fish: a role for ammonia toxicity? *In* EW Taylor, ed. Toxicology of aquatic pollution. Physiological, molecular and cellular approaches. Cambridge: Cambridge Univ. Press, pp. 85-111.

Teh ST, SM Adams, DE Hinton. 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquat. Toxicol. **37:** 51-70.

Theophanides T, J Anastassopoulou. 2002. Copper and carcinogenesis. Crit. Rev. Oncol. Hematol. **42:** 57-64.

US EPA. 2007. Aquatic life ambient freshwater quality criteria - copper. 2007 Revision. EPA-822-R-07-001 (CAS Registry no. 7440-50-8). Washington DC: US Environmental Protection Agency (EPA) and Office of Water.

Van der Oost R, J Beyer, NPE Vermeulen. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. **13:** 57-149.

Wendelar Bonga SE, RAC Lock. 1992. Toxicants and osmoregulation in fish. Netherlands J. Zool. **42:** 478-493.

Wilson RW, EW Taylor. 1993. The physiological responses of freshwater rainbow trout (*O. mykiss*) during acutely lethal exposure. J. Compar. Physiol. **163B:** 38-47.

Wood CM, A Soivio. 1991. Environmental effects on gill function: an introduction. Physiol. Zool. **64:** 1-3.

Wu SM, CC Chen, YC Lee, HT Leu, NSu Lin. 2006. Cortisol and copper induce metallothionein expression in three tissues of tilapia (*Oreochromis mossambicus*) in organ culture. Zool. Stud. **45:** 363-370.