BIOACCUMULATION AND HISTOPATHOLOGY

OF COPPER IN

Oreochromis mossambicus

Submitted in fulfillment of the requirements for the degree of

MASTER OF SCIENCE

of Rhodes University

By

IRENE NAIGAGA

March 2002

ACKNOWLEDGEMENTS

I wish to acknowledge my supervisor, Dr. Horst Kaiser for his constant guidance and support during experimental work and write-up phase.

My appreciation to The Third World Organisation for Women in Science and The Liberty Life Education Foundation for the financial support that enabled this study.

I am grateful to the technical staff of The Eastern Cape Medical Laboratories of the SAIMR, Port Elizabeth, in particular Jenny Grewer and The Department of Histology, Makerere University, Uganda for the assistance with processing histology samples. I extend the same appreciation to Robert Van Hille in the metal lab, Rhodes University for his help with Cu analysis; R.H.M Cross and S.C. Pinchunk of the Electron Microscope Unit, Rhodes University for the assistance with photographing and scanning of micrographs. I also acknowledge James Sales for his assistance with laboratory analysis during the experimental phase.

To all graduate students in the Ichthyology Department thank you for the support and company. Special thanks to my colleagues and friends, Sloans Chimatiro, Margaret Jjuuko, Emmanuel Kaunda, Imran Klotz-Shiran, Monica Mwale, Irene Muriuki, Justus Rutaisire and Sylvia Tshivunge for being a constant source of encouragement, inspiration and advice.

I wish to thank my colleagues at The Faculty of Veterinary Medicine, Makerere University Uganda, the dean, Prof. Katunguka-Rwakishaya, the associate dean, Prof. Johnson Acon and the head WARM department, Dr. Christine Dranzoa, for being supportive. Special thanks to Prof. Okot Bwangamoi, Department of Veterinary Pathology for refreshing my histopathology, your comments were highly valuable.

Lastly to my sisters and brothers, my cousins Jennifer Nantale and Charlotte Gulyetonda your love and support is indispensable.

TABLE OF CONTENTS

SHORT TITLES OF TABLES

SHORT TITLES OF FIGURES

SHORT TITLES OF PLATES

ABSTRACT

Cu is one of the most toxic elements that affect fish populations when the fish are exposed to concentrations exceeding their tolerance. To investigate the effects of elementary Cu on aspects of bioconcentration, histology and behaviour, *O. mossambicus* were exposed to 0 and 0.75 ± 0.20 mg/l of Cu for 96 hours (short-term study), and 0, 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l of Cu for 64 days (long-term study) under controlled conditions in the laboratory. For the long-term study fish were sampled for gills, liver, and kidney Cu accumulation analysis after 1, 32 and 64 days of exposure and after 1, 2, 4, 16, 32, and 64 days for gills, liver and spleen histology analysis.

Cu accumulation was concentration-duration dependent with the highest accumulation capacity in the liver. A multifactor linear model was developed for the relationship between exposure dose, exposure duration and Cu accumulation in the organs with the liver model: $Log\ L = 3.35 + 0.85W + 0.31T$ ($r^2 = 0.892$) giving a better fit than the gills: $G = -35.09 + 10.58W + 17.58T$ ($r^2 = 0.632$). Where L = Cu accumulation values in the liver, G = Cu accumulation values in the gills (both in µg/g dry mass); W = exposure dose in water (mg/l); and T = exposure time (days). Using this model Cu accumulation in organs can be estimated when exposure concentration and duration is known. This model should be tested under different conditions to determine the potential of the model in monitoring Cu toxicity in the environment.

Lesions were observed in the liver, gills and spleen in all Cu treatments at all exposure concentration and exposure durations. However, the incidence and the degree of alteration was related to the concentration of Cu and duration of exposure. The sequential appearance of lesions in the order of, hepatic vacuolar degeneration, fatty degeneration and necrosis indicated a gradual increase in liver damage with larger duration of exposure time and increasing Cu concentration. The initial lesions in the gills were manifested as hypertrophy and hyperplasia of the gill epithelium causing increase in the thickness of the secondary lamellae, mucous cell hypertrophy and proliferation, mucous hypersecretion, proliferation of eosinophilic granule cells

and hyperplasia of interlamellar cells. With increase in exposure time, necrosis of the eosinophilic granule cells, lamellar oedema, epithelial desquamation and increase in severity of lamellar hyperplasia were observed. These lesions indicated an initial defence mechanism of the fish against Cu toxicity followed by advanced histological changes that were related to Cu concentration and duration of exposure. Changes in the spleen were haemosiderosis, increase in the white pulp and macrophage centres, reduction in the red pulp, and necrosis suggesting that fish exposed to environmentally relevant levels of Cu may be histopathologically altered leading to anaemia and immunosuppression.

Regression analysis was used to quantify the relationship between the total activity of the fish, and duration of exposure. There was a gradual decline in fish activity related to Cu concentration and duration of exposure before introducing food into the tanks. There was a constant activity after introducing food in the tanks at the control and 0.11 ± 0.02 mg/l Cu exposure levels irrespective of exposure time. Analysis of covariance (ANCOVA) was used to test for the difference in slopes between treatments. There was no significant difference ($p > 0.05$) between slopes of the control and 0.11 ± 0.02 mg/l Cu, and between 0.29 ± 0.02 and 47 ± 0.04 mg/l Cu before and after introducing food in the tanks. The slopes of both the control and 0.11 ± 0.02 mg/l Cu were significantly different from those of 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu ($p < 0.05$). There were significant differences in the mean opercular movements per minute between treatments ($p < 0.05$). There was hyperventilation at 0.11 ± 0.02 mg/l Cu i.e. 87 ± 18 opercular movements per minute (mean ± standard deviation) and hypoventilation at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu i.e. 37 ± 34 and 13 ± 6 opercular movements per minute compared to the control. Hypo- and hyperventilation were related to the lesser and greater gill damage, respectively.

In conclusion Cu accumulation and effects on histology of the liver, gills and were related to the concentration of Cu in the water and duration of exposure showing a gradual increase in incidence and intensity with larger duration of exposure time and increasing Cu concentration. The fish were initially able to homeostatically regulate and detoxify Cu. However, as the exposure continued, the homeostatic mechanism appears to have failed to cope with the increasing metal burden causing advanced histologicalchanges.

CHAPTER 1

General introduction and literature review

1.1 General introduction

Copper (Cu) has been documented as one of the most toxic elements occurring in polluted natural waters (Jezierska and Slomińska 1997). This metal is plentiful in the environment and it is also one of the most commonly used metals (Carbonell and Tarazona 1994, Goyer 1996). Cu is a soft heavy metal, atomic number 29, with an atomic weight of 63.55, a melting point of 1083.4 ^{o}C, a boiling point of 12567 ^{o}C, and a density in elemental form at 20^{o}C of 8.92 g/cc (United States Environmental Protection Agency [USEPA] 1980, Eisler 1998). Mason (1981) defined a heavy metal as any metal with an atomic number greater than 20 excluding, alkali metals, alkaline earths, lanthanides and actinides. Cu occurs naturally in many minerals such as cuprite (Cu_2O), malachite ($CuCO_3Cu\ (OH)_2$), azurite ($2CuCO_3Cu(OH)_2$), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), and bornite (Cu_5FeS_4), the three important sources being, chalcocite, chalcopyrite, and malachite (Agency for Toxic Substances and Disease Registry [ATSDR] 1990, Eisler 1998). It also occurs uncombined as a metal existing in four oxidation states, elemental (Cu^0), cuprous (Cu^{1+}), cupric (Cu^{2+}), and trivalent (Cu^{3+}). The cupric copper is generally encountered in water (ATSDR 1990, Eisler 1998).

There are numerous definitions of water pollution. For example, in a report by the National Research Council Committee on Pollution quoted by Warren (1971), water pollution was defined as “an undesirable change in the physiological, chemical, or biological characteristics of water that may or will harmfully affect human life or that of other desirable species, industrial processes, living conditions, and cultural assets, that may or will waste or deteriorate raw material resources”. Heath (1995) quoted a definition of water pollution given by Lloyd (1992), as “the addition by humans of something to the water that alters its chemical composition, temperature, or microbial composition to such an extent that harm occurs to resident organisms or to humans”. However, this study will follow the definition given by Mason (1981) who defined

pollution as “the introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources and ecological systems, damage to structure or amenity, or interference with legitimate uses of the environment”. Moriarty (1988) defined a pollutant as a substance that occurs in the environment at least in part as a result of man’s activities, and which has a deleterious effect on living organisms. Chemical pollution including heavy metal pollution has implications for human health, both directly from toxic chemicals in drinking water, and indirectly from the accumulation of toxic compounds by organisms that are eaten by people (Heath 1995).

To assess pollutants or a group of pollutants ecotoxicologists and environmentalists have focused on the pollutant’s fate, persistence and toxic properties to the environment and man (Kroes 1988). Considerable attention has been devoted to identifying various chemicals among which are commonly used pesticides and heavy metals, and assessing their effects on aquatic animals (Kroes 1988). Whereas fish physiologists traditionally had little interest in aquatic toxicology (Heath 1995), their concern has been the understanding of the organ systems of selected fish species and their physiological adaptations to environmental changes such as temperature and dissolved oxygen. Heath (1995) further points to the multivolume work entitled *Fish Physiology* (Hoar and Randall 1969 – 1992) where the effects of pollution on fish are rarely mentioned. He further speculated that neglect may be due to at least three factors: (1) most authors are not professionally involved with the work on pollution, (2) the database has until recently been limited and (3) it is customary in physiology books to spend little, if any, time on the effects of toxicants. Fish physiology is now becoming an integral part of aquatic toxicology. The pollutants in the environment at sub-lethal concentrations are an important variable to which a fish respond physiologically.

Although the amount of environmental information collected to date is impressive, there are still gaps in our knowledge of pollutants, affected organisms and the related environmental conditions. Such gaps need to be filled in order to obtain a complete and integrated picture of the effects caused by heavy metal pollution, to predict further dynamics of these effects, and to come up with a strategy to contain these effects. The qualitative and quantitative description of harmful toxic effects is

essential for an evaluation of the potential hazard posed by a particular chemical. It is also valuable to understand the mechanisms responsible for the manifestations of toxicity, i.e. how a toxicant enters the organism, how it interacts with target molecules, how it exerts its effects, and how the organism deals with the exposure (Gregus and Klaassen 1996). Such information provides the basis for interpreting the effect of a toxic substance, estimating the probability that a chemical will cause harmful effects, establishing procedures to prevent or antagonize the toxic effects, designing drugs and industrial chemicals that are less hazardous, and developing pesticides that are selectively toxic for their target organisms. Elucidation of the mechanisms of chemical toxicity has led to a better understanding of fundamental physiological processes (Gregus and Klaassen 1996).

The test species and its sex and age can influence the toxicity of heavy metals (Gardner and LaRoche 1973, Atchison *et al*. 1987, Eaton and Klaassen 1996, Goyer 1996, Crompton 1997). For example, Cu accumulation and lethal concentrations vary between species and age within species at the top of the trophic chain such as birds and mammals being relatively resistant as compared to fish (Banerjee and Homechaudhuri 1990, Lin and Dunson 1993, Eisler 1998). A few studies that dealt with field pollution or experimental exposure considered *O. mossambicus* as a test animal (Du Preez *et al*. 1996, Robinson and Avenant-Oldewage 1997, James *et al*. 1998, Usha Rani and Ramamurthi 1989, Kotze *et al*. 1999). These authors reported that Cu accumulated in *O. mossambicus* mainly in the liver and gills. Minnows, *Phoxinus phoxinus*; goldfish, *Carassius auratus*; sticklebacks, *Gasterosteus aculeatus* and *Pygosteus pungitius* and trout, *Oncorhynchus mykiss* have been used in experimental pollution studies (Kleerekoper *et al*. 1972, Bilinski and Jonas 1973, Kleerekoper *et al*. 1973, Sellers 1975, Saucier *et al*. 1990, Bieniarz *et al*. 1997). Popular American species are the bluegill, *Lepomis macrochirus*; the fathead minnow, *Pimephales promelas*; the largemouth bass, *Micropterus salmoides*; the guppy, *Poecilia reticulata*; the channel catfish, *Ictalurus punctatus*; Chinook salmon, *Oncorhynchus tshawytscha*; and coho salmon, *Oncorhynchus kisutch* (Mount 1968, O'Hara 1971, Griffin *et al*. 1997). *O. mossambicus* is abundant in African tropical and subtropical fresh water bodies and this fish is a potential source of income and nutrition, supplementing protein in the diet of many people (Merron *et al*. 1994). This study demonstrates the effect of copper on this species.

Copper was used in this study because it represents an essential homeostatically regulated metal in all living organisms (Goyer 1996, Heath 1995). Cu as a microelement is essential for the normal growth and metabolism of plants, animals and most microorganisms (Schroeder *et al*. 1996, Carbonell and Tarazona 1994). This metal is incorporated into numerous enzymes including tyrosine (melanin production), dopamine beta-hydroxylase (catecholamine biosynthesis), copper-zinc superoxide dismutase (free radical detoxification), cytochrome oxidase and ceruloplasmin (iron conversion), and ascorbic acid oxidase (ascorbic acid oxidation) (Goyer 1996, ATSDR 1990, Eisler 1998). Cu compounds, such as copper sulphate, are widely used as biocides to control macrophytes, freshwater snails that may harbour schistosomiasis or other disease pathogens, ectoparasites of fish and mammals, marine fouling organisms, and mildew and other diseases of terrestrial crop plants (Eisler 1998). Cu compounds are also used in agricultural fertilizers, in veterinary and medical products, in the food industry, and in preservatives of wood and other materials (Eisler 1998). As a malleable metal, Cu is used by humans for domestic and industrial purposes (ATSDR 1990).

Exposure of fish to heavy metals in concentrations exceeding their tolerance may adversely affect fish populations and Cu belongs to the most toxic elements in polluted natural waters (Jezierska and Slomińska 1997). This metal causes a wide range of physiological effects in fishes after acute or chronic exposure (McKim and Benoit 1971, Daoust *et al*. 1984). These effects include behavioural changes such as inappetance, lethargy, incoordination, increased ventilation frequency, gasping (Kleerekoper *et al*. 1973, Cardeilhac and Whitaker 1988, Sellers *et al*. 1975, Nelson *et al*. 1999). Copper-induced lesions occurred in kidneys, lateral line, liver, gills and olfactory organs of several fish species (Gardner and LaRoche 1973, Saucier *et al*. 1990, Nelson *et al*. 1999) and are associated with changes in blood chemistry (decrease in haemoglobin and haematocrit values), enzyme activities and corticosteroid metabolism (ATSDR 1990, Hodson *et al*. 1979).

In humans, copper poisoning has been reported to cause respiratory effects such as mucosal irritation of the nose, gastrointestinal irritation manifested as vomiting, nausea, diarrhoea, anorexia, and haematological effects like decreased haemoglobin and erythrocyte levels (Goyer 1996, ATSDR 1990). Deaths were attributed to

extensive hepatic centrilobular necrosis and necrosis and sloughing off of tubular cells in the kidney following ingestion of large amounts of copper (Goyer 1996, ATSDR 1990).

1.2 Literature review

1.2.1 Input of copper into aquatic ecosystems

Human activities contribute increasingly to environmental levels of Cu. Input of Cu into aquatic ecosystems increased sharply during the past century and includes sources such as agricultural and forestral activities; waste discharges and sludge from publicly owned treatment works into saline waters; leaching from antifouling marine paints and wood preservatives; anthropogenic sources like mining and milling operations, and industrial discharges into freshwater (ATSDR 1990, Eisler 1998, Skjelkvåle *et al*. 2001). Cu and its compounds are naturally present in the earth's crust. Natural discharges to water include air-borne contribution from long-range transport, windblown dust, volcanic eruptions, decaying vegetation, forest fires, bedrock geology and soil weathering (Skjelkvåle *et al*. 2001, ATSDR 1990). Of special concern is Cu that gets into the drinking water from the water distribution system. When the system has not been flushed after a period of disuse, the concentration of Cu in tap water may exceed 1.3 mg/l, the Environmental Protection Agency drinking water limit (ATSDR 1990). The human population may be exposed to high concentrations of Cu in drinking water that has picked up Cu from the distribution system, using Cu fungicides and algicides, eating Cu contaminated foods, and skin contact with soil, water, and other copper-containing substances (ATSDR 1990). Workers in agriculture, copper production and metal plating industries are exposed to copper (ATSDR 1990).

1.2.2 Factors affecting the bioavailability and toxicity of copper

A multitude of environmental factors has been reported to affect the bioavailability of Cu in the aquatic environment consequently affecting the bioaccumulation and toxicity of this metal (table 1.1).

Table 1.1 The effect of environmental conditions on the bioavailability and toxicity of copper in the aquatic environment

Environmental factor	Effect on copper		Toxicity	Reference
	Increased bioavailability	Reduced bioavailability		
Organic and inorganic compounds		Forms complexes	Complexed forms are considered less toxic than the free metal ions	USEPA 1980, ATSDR 1990, Heath 1995, Robinson and Avenant-Oldewage, 1997, Olsson 1998, Avenant-Oldewage and Marx 2000
Water hardness		Forms insoluble carbonates or absorbs on calcium carbonate	The harder the water, the lower the toxicity of Cu to aquatic organisms	Kallanagoudar and Patil 1997 Olsson 1998
High pH (pH > 6.0)		Cu precipitates	Cu becomes less toxic	Playle and Dixon 1993, Nussey *et al.* 1999
Low pH (pH < 6.0)	Cu becomes mobile and soluble		Cu becomes more toxic	Pynnönen 1995, Nussey *et al*. 1999, Tao *et al*. 2001
Increase in temperatures from ambient	Cu becomes mobile and soluble		Cu becomes more toxic	Crompton 1997, Prasada and Khan 2000
Oxygen concentration			Cu causes an initial increase in O_2 consumption followed by a decrease	O'Hara 1971, Felts and Heath 1984

Environmental parameters interact to influence the toxicity of metals and should always be monitored and reported whenever toxicity studies are carried out. For example for a given pH, less copper was required to reduce growth of rainbow trout (*Oncorhynchus mykiss*) at low levels of hardness exposed to different copper levels,

pH, and hardness combinations for 10 days (Waiwood and Beamish 1978). At a given hardness, copper-induced depressions in growth were more pronounced and recovery slower at a low pH level.

Depending on the pH and concentration of competing ligands, there is variation in association of copper with inorganic and organic ligands (ATSDR 1990). For example, in river water from the northwest United States with a relatively high pH (pH 7.0 – 8.0) and hardness between 24 and 219 mg/l $CaCO_3$, CO_3^{2-} and OH^- (inorganic ligands) were the most common Cu-associating factors at high copper concentrations. In water from lakes and rivers in southern Maine with a relatively low pH (4.6 – 6.3) and hardness (1 – 30 mg/l $CaCO_3$) the same role was played by organic matter ligands (ATSDR 1990).

1.2.3 Predictors of copper concentration

Concentration of copper in water is a better predictor of fish tissue contamination than the concentration in sediment or benthic invertebrates (Miller *et al*. 1992, Nussey *et al*. 1999).

For example, Cu bioconcentration factor values for water were higher than those for sediment in moggel, *Labeo umbratus* from Witbank Dam suggesting that waterborne copper is the better predictor of copper concentration (Nussey *et al*. 1999). Bioconcentration factor is a unitless value obtained by dividing the concentration in one or more of the tissues by the average concentration in the water (Veith *et al*. 1979). Cu bioconcentration factor values for water, ranged between 84.4 in the muscle to 44071.0 in the liver, and were consistently higher than those in sediment which ranged between 0.02 in the muscle and 26.2 in the liver. This trend was also found by Miller *et al*. (1992) who worked on white sucker, *Catostomus commersoni*. The concentration of Cu and Zn in the water was a better predictor of white sucker tissue contamination than the concentration of these metals in either sediment or benthic invertebrates. Cu and Zn concentrations in tissues were significantly correlated with copper concentrations in water but not with those in sediments or benthic invertebrates (Miller *et al*. 1992). Conversely, zinc concentration in invertebrates was positively correlated with sediment but not with water zinc

concentration. The lower bioconcentration factor for copper in the sediment could be a result of this metal forming complexes with sediments.

1.2.4 Effect of Cu on fish

Studies on copper toxicity dealt with either copper salts (copper sulphate and copper chloride) or effluent as the source of copper. No study was found that considered the effects of elementary copper on fish. Studies that dealt with bioaccumulation found that the liver, gill and kidney were better indicators of copper exposure than muscle. A summary of studies, stating the test species, copper source, types of test and effects of Cu on fish is given in table 1.2.

Table 1.2 A summary of studies undertaken to determine the toxicity of copper in fish

Species	Cu source	Test(s)	Effects	Reference
Fathead minnows *Pimephales promelas*	Copper sulphate	Median tolerance limit (TL_m)	The concentration of Cu that does not affect growth and reproduction lies between 3 and 7% of the 96 hour TL_m	Mount 1968
Brook trout *Salvelinus fontinalis*	Copper sulphate	Haematology	Transient increase in red blood cell count, haematocrit, haemoglobin, plasma glutamic oxalacetic transaminase and total proteins	McKim *et al*. 1970
Brook trout *Salvelinus fontinalis*	Copper sulphate	Growth Reproduction	Decreased survival and growth in adult fish, reduced number of viable eggs produced and hatchability	McKim and Benoit 1971
Bluegill *Lepomis macrochirus*	Copper sulphate	Respiration	Initial stimulation in O_2 consumption followed by inhibition	O'Hara 1971
Rainbow trout *Oncorhynchus mykiss*	Copper chloride	Enzymatic tests	Inhibition of oxidation of lactate by gills Mortalities	Bilinski and Jonas 1973
Brook trout *Salvelinus fontinalis*	Copper sulphate	Behaviour	Initial stimulation of locomotor activity followed by a return to normal	Drummond *et al*. 1973

Table 1.2 Continued

Species	Chemical	Test(s)	Effects	Reference
Mummichog *Fundulus heteroclitus* Atlantic silverside *Menidia menidia*	Not given	Histopathology	Degenerative changes in the mechanoreceptor cells of the lateral line canals	Gardner and LaRoche 1973
Sockeye salmon *Oncorhynchus nerka*	Copper sulphate	Haematology	Rapid corticosteroid stress response to lethal and sublethal Cu exposure	Donaldson and Dye 1975
Rainbow trout *Oncorhynchus mykiss*	Copper sulphate	Respiration	Increased ventilatory activity with increase in Cu concentration	Sellers *et al.* 1975
Catfish *Mystus bleekeri*	Copper sulphate	LC_{50}	LC50's were 4.17, 1.85, 0.95 and 0.85 mg/l Cu for 24, 48, 72 and 96 hours, respectively	Gupta and Rajbanshi 1981
Rosy Barb *Puntius conchonius*	Copper sulphate	Histopathology	Enlarged lamellae epithelial cells and lamellae fusion Vacuolation and necrosis in the liver	Kumar and Pant 1981
Flounder *Platichthys flesus*	Copper nitrate	Bioaccumulation	Cu accumulated in the order of liver > kidney > gills > muscle > plasma	Stagg and Shuttleworth 1982
Goldfish *Carassius auratus*	Copper sulphate	Histopathology	Partial and complete vacuolation of hepatocytes, Vacuolation and necrosis of gill tissue, mucous cell hypertrophy and collapse of blood capillaries	Sultan and Khan 1983

Table 1.2 Continued

Species	Chemical	Test(s)	Effects	Reference
Rainbow trout *Oncorhynchus mykiss*	Copper sulphate	Histopathology	Hypertrophy of lamellar epithelial cells and lamellar fusion	Daoust *et al.* 1984
Brown bullhead *Ictalurus nebulosus*	Copper chloride	Histopathology Histoenzymatic	Derangement and focal necrosis of the gill epithelium Mild onset and slow evolution of liver distress	Benedetti *et al.* 1989
Sharptooth catfish *Clarias gariepinus*	Industrial effluent	Bioaccumulation	Cu accumulated in the order of brain > gills > heart muscle > kidney > spleen > liver	Bezuidenhout *et al.* 1990
Rainbow trout *Oncorhynchus mykiss*	Copper sulphate	Histopathology	Degeneration and epithelial necrosis of the olfactory organ	Saucier *et al.* 1990
Tigerfish *Hydrocynus vittatus*	Industrial effluent	Bioaccumulation	Cu accumulated in the order of liver > stomach > intestine > muscle > gonads > gill > body fat	Du Preez and Steyn 1992
Sharptooth catfish *Clarias gariepinus*	Sewage effluent	Bioaccumulation	Cu accumulated in the order of liver > kidney > muscle	van den Heever and Frey 1994
Common carp *Cyprinus carpio*	Copper sulphate	Growth (early life stages)	Cu concentration-dependent increase in percentage of deformed larvae and decrease in percent of normal larvae	Mis *et al.* 1995 Jezierska and Slomińska 1997

Table 1.2 Continued

Species	Chemical	Test(s)	Effects	Reference
Common carp *Cyprinus carpio* Goldfish *Carassius auratus*	Copper sulphate	Reproduction	Decreased hatchability and increased fractions of larval deformities	Bieniarz *et al*. 1997
Channel catfish *Ictalurus punctatus*	Copper sulphate	Bioaccumulation	Cu accumulated in the order of liver > muscle	Griffin *et al*. 1997
Common carp *Cyprinus carpio*	Copper sulphate	Growth (early life stages)	Reduced swelling of the eggs and time of spermatozoa motility, and extension of developmental time	Jezierska and Slomińska 1997
Tilapia *Oreochromis mossambicus*	Industrial effluent	Bioaccumulation	Cu accumulated in the order of liver > gill > skin > muscle	Robinson and Avenant-Oldewage 1997 Kotze *et al*. 1999
Sharptooth catfish *Clarias gariepinus* Moggel *Labeo umbratus*	Industrial effluent	Bioaccumulation	Cu accumulated in the order of liver > gill > skin > muscle	Nussey *et al*. 1999, Avenant-Oldewage and Marx 2000
Sharptooth catfish *Clarias gariepinus*	Tannery effluent	Bioaccumulation	Cu accumulated in the order of liver > gills > intestinal tissue > muscle tissue	Gbem *et al*. 2001

1.2.5 Interaction of Cu with other metals

Studies regarding copper toxicity have dealt with the effect of effluent and copper salts on fish (Griffin *et al.* 1997). Using effluent as a source of copper in laboratory studies is ambiguous since other metals in the effluent could be exerting a combined action thus making it difficult to give a definite diagnosis of the effective metal. For example, copper sulphate and zinc sulphate dissolved in soft water have been shown to have a synergistic action, i.e. their combined action is more than additive (Jones 1964, Birge and Black 1979). Jones (1964) quoted studies on fathead minnows (*Pimephales promelas*) that survived for about 8 hours in an 8 mg/l zinc solution or a 0.2 mg/l copper solution. A mixed solution containing only 1 mg/l zinc and 0.025 mg/l copper was relatively more toxic. Lead has been reported to reduce the toxicity of copper to freshwater invertebrates, thus having an antagonistic effect (Jones 1964). Metals can interact with one another and this can be further influenced by the presence of other ions in the water such as calcium, magnesium, sodium, manganese, iron, lead, sulphur, selenium and nickel (Olsson 1998). These are known to affect the toxicity of cadmium, copper, zinc and mercury in different ways. The effects of Cu-Zn mixtures on percent survival and hatchability produced closer to additive effects at low concentrations (the mixture and individual metals caused higher survival and hatchability) and synergistic effects at higher concentrations (higher survival in the mixture than in individual metals) in catfish, *Ictalurus punctatus* and goldfish *Carassius auratus* (Birge and Black 1997). Mixtures of copper and mercury have more diverse effects, changing from antagonistic at low concentrations to additive at intermediate concentrations and finally to synergistic at high concentrations (Birge and Black 1979).

Using Cu salts like copper sulphate to investigate the effects of Cu on fish could lead to ambiguous results. For example, copper sulphate may hydrolyse. Yet, the acidity of the solution resulting from this hydrolysis may only be sufficiently high to be lethal at high concentrations. Salts of chromium, aluminium and iron hydrolyse to a much greater extent (Jones 1964). Chromium sulphate and aluminium nitrate are markedly toxic within the tolerated pH range of pH 5.0 – pH 11.0 of most fish species. Ferric chloride solutions can be as toxic to threespine stickleback (*Gasterosteus aculeatus*) as hydrochloric acid solutions of the same pH (Jones 1964).

Many heavy metal pollution studies have been field-based, thus making experimental induction of heavy metal deformities a research priority. To substantiate the field results and to obtain reliable dose-response relationships, experimental studies need to be carried out to objectively assess heavy metal toxicity (Kendall *et al*. 1996). In the field pinpointing the exact culprit is often difficult when a problem arises because the contaminants may be reduced before biologists are notified. The effects of individual contaminants may be worsened as a result of their interactions with each other or with normally inert factors in the environment. Experiments allow maximum control over external variables thereby maximising exposure and bioavailability of the chemical, and a concentration-response function can be established (Kendall *et al*. 1996). However, many laboratory tests on Cu toxicity produced LC_{50} values (Crompton 1997). These values lack information because they reflect only the total metal concentration thus ignoring the importance of the chemical effect on fish health, metabolism and organ histology, which in this case is not defined.

Harmful chemicals such as heavy metals exert primary effects at the enzyme level, or they may alter the permeability of the membranes. These changes affect cell integrity, ultramicroscopic structure, and metabolism such as energy expenditure or secretion of hormones (Heath 1995). As these changes become more severe, cells may die causing histological lesions that are visible using light-microscopic techniques. Organs are composed of many types of cells, and effects on one or more of these will be reflected in changes in organ function. Thus, a pathological effect may lead to one or more physiological responses (Heath 1995). For example, chronic exposure to a pollutant may cause changes in function of the nervous system and this can alter behaviour (Heath 1995).

1.2.6 Aim and objectives

This study dealt with the effect of elementary Cu on the histology of gills, liver and spleen of *O. mossambicus* under experimental conditions. The overall aim of this study was to investigate the effects of copper on aspects of bioaccumulation, histology and behaviour in *O. mossambicus* under experimental conditions in the laboratory.

Specific objectives:

- To establish a relationship between exposure and copper accumulation in the selected organs.
- To establish sequential histological transformation in relation to sub-lethal copper exposure in the gills, liver and spleen.
- To quantify the effect of copper on the behaviour (general activity and respiration) as a function of concentration and duration of exposure.

The study was carried out in two parts. For the short-term study, 60 *Oreochromis mossambicus* yearlings were used with 10 fish per treatment exposed to 0 mg/l (control) and 0.75 ± 0.20 mg/l copper for 96 hours. The long-term study involved exposing fish to three copper levels, including a control with no copper, and three treatments with 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l copper. Each treatment was replicated twice. For each treatment, 14 fish above 40 g body mass were used (sex ratio of 1:1).

CHAPTER 2

General methods

2.1 Materials and Methods

This study was conducted in two parts, a short-term study (4-day Cu exposure) and a long-term study (64-day Cu exposure). Fish used for the experiments were obtained from the freshwater fish farm of the Department of Ichthyology and Fisheries Science, Rhodes University, South Africa.

2.2 Short-term acute study

2.2.1 System design of the short-term exposure

Six 50-litre glass tanks, each with a recirculating under-gravel filter system to allow mixing of the water were used in this experiment. Details of the systems are presented in a schematic diagram (figure 2.1). The filter substrate (E) was a mixture of small stone gravel and broken shells that covered the porous under-gravel filter (F) at the bottom of the tank (A). Air delivered through an air tube C 1 allowed for continuous mixing of the water (see direction of water flow). Water flow was determined at the beginning of the experiment and ranged from 2.5 to 2.9 litres per minute depending on the amount of air pumped.

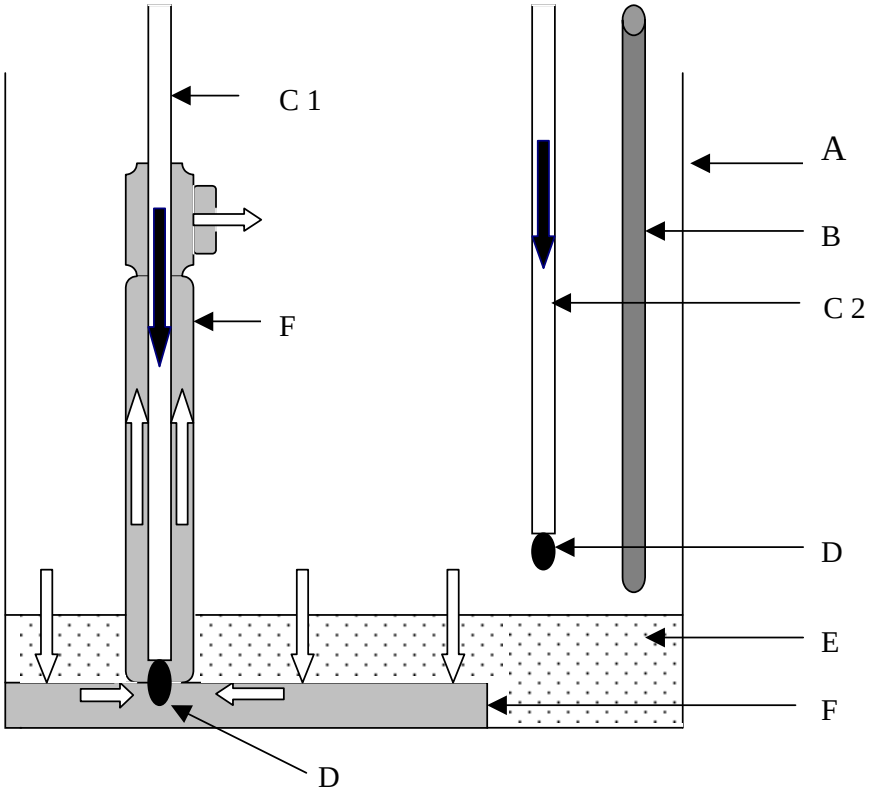


Figure 2.1 The recirculating system used during the short-term study. ⇨ = direction of water flow, ➡ = direction of air flow, A = fish tank, B = water heater, C 1 and C 2 = air delivery tubes, D = air stone, E = filter substrate (a mixture of small stone gravel and broken shells), F = under-gravel filter

2.2.2 Experimental design of the short-term exposure

To simulate the tropical photoperiod, the lighting in the laboratory was set with a timer to come on at 6.00 a.m. and go off at 6.00 p.m. The fish were allowed to acclimatize in the experimental tanks for 15 days before Cu was added to the water. Sixty *Oreochromis mossambicus* yearlings were used for the experiment with ten fish per triplicate exposed to 0.00 mg/l (control) and 0.75 ± 0.20 mg/l of Cu for 96 hours. Before exposure, the fish were anaesthetized using 2-phenoxyethanol (1 ml/l), and weight and length measurements were taken. Only fish weighing above 32g were selected, and the sex ratio was 1:1. The fish were kept in the tank overnight (day zero) before Cu exposure. This was to allow the fish to recover from the stress due to anaesthesia and handling during measurements. The fish were not fed during the experiment.

2.2.3 Measurement of copper in water

The concentration of copper in the water was measured daily spectrophotometrically using a Shimadzu digital double-beam spectrophotometer UV–150–02 (P/N204-25350-02), Japan. The lost Cu was replaced during the daily water change to restore the desired Cu dosages which were kept as constant as possible during the experiment.

Pure copper (Cu^o) powder (assay 99.9%) from Met-U-Ed laboratory suppliers South Africa was used. The required quantity of copper was measured. To this 0.5ml of redistilled water was added followed by 2-3 drops of concentrated nitric acid. After the reaction had slowed, the mixture was warmed gently to complete dissolution of the copper powder and slightly boiled to expel oxides of nitrogen. The bathocuproine method of measuring Cu in water as described by Clesceri *et al*. (1989) was applied.

The bathocuproine method works on the principle that cuprous ions form a water-soluble orange coloured chelate with bathocuproine disulfonate (2,9-dimethyl-4-7-di-phenyl-1,10-phenanthrolinedisulfonic acid, disodium salt). This colour forms over the pH ranges 3.5 to 11.0 but the recommended pH range is between 4 and 5. The sample is buffered at a pH of about 4.3 and reduced with hydroxylamine hydrochloride. The absorbance is measured at 484 nm. This method has a minimum detectable concentration of 20 µg/l and a maximum detectable concentration of 5 mg/l.

To determine the concentration of copper in the water, the following solutions were prepared: stock copper solution, standard copper solution, hydrochloric acid solution, hydroxylamine hydrochloride solution, sodium citrate solution, and disodium bathocuproine disulfonate solution. The stock copper solution was prepared by adding 10 ml redistilled water and 5 ml concentrated HNO_3 to 20 mg polished electrolytic copper wire in a 250-ml flask. After the reaction had slowed, the mixture was warmed gently to complete dissolution of the copper wire and slightly boiled to expel oxides of nitrogen. After the mixture had cooled it was made up to one litre in a one-litre volumetric flask. One ml of the stock copper solution contained 20 µg copper. The standard copper solution was prepared by diluting 250 ml stock copper solution to 1000 ml with redistilled water, 1 ml of this solution contained 5 µg copper. Hydrochloric acid solution (200 ml) was prepared by adding 100 ml concentrated HCl

to 100 ml redistilled water. Hydroxylamine hydrochloride solution was prepared by dissolving 50 g $NH_2OH.HCl$ in 450 ml redistilled water. Sodium citrate solution was prepared by dissolving 300 g $Na_3C_6H_5O_7.2H_2O$ in 1000 ml redistilled water. Disodium bathocuproine disulfonate solution was prepared by dissolving 1 g $C_{12}H_4N_2(CH_3)_2(C_6H_4)_2(SO_3Na)_2$ in 1000 ml redistilled water.

For the standard curve, a series of 50 ml Cu standards, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/l Cu, were prepared from the standard Cu solution. To the standards and blank the following solutions were added, 1ml of hydrochloric acid, 5 ml hydroxylamine hydrochloride solution, 5 ml sodium citrate solution, and 5 ml disodium bathocuproine disulfonate solution. The mixture was transferred to cells and the absorbance against the blank was read at 484 nm. For the calibration curve the absorbance was plotted against the Cu concentration in the standard solutions (figure 2.2).

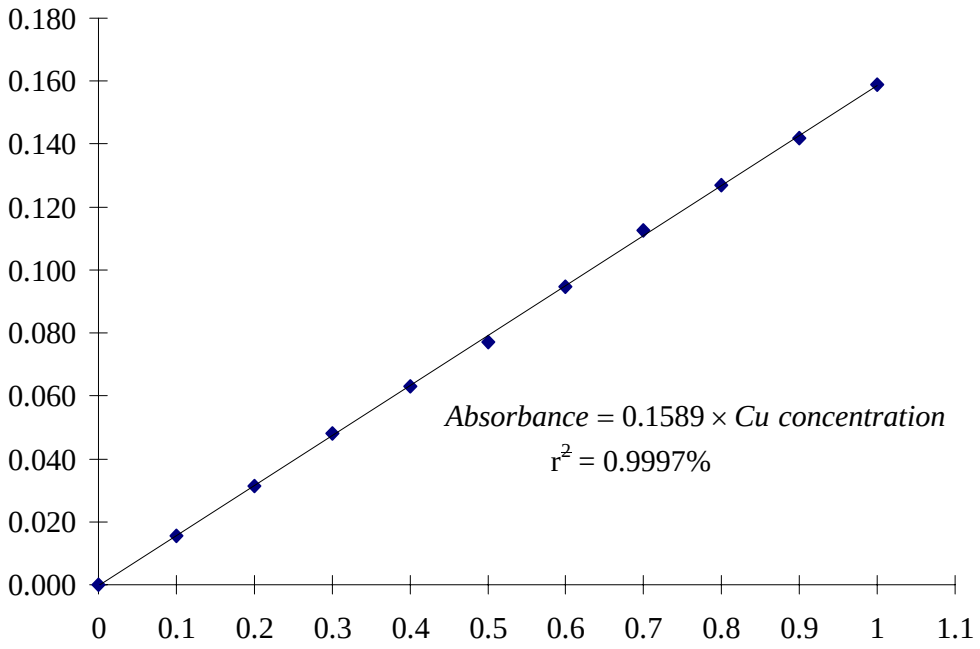


Figure 2.2 The copper calibration curve used for estimating the concentration of copper in the water

2.2.4 Water quality

The pH, temperature, water hardness, and oxygen concentration were measured daily and ammonia was measured on day one and day three of the experiment. pH was measured using a portable pH meter (Hanna Instruments, USA). Temperature and dissolved oxygen were measured using a temperature – oxygen probe (OxyGuard® Mk III). Total water hardness was measured using the complexometric method with Triplex® solution A and indicator buffer powder (Merck 1974). Total water hardness was measured in mmol/l and results were converted to mg/l $CaCO_3$ as described by Merck (1974). Ammonia was measured spectrophotometrically with Nessler's reagent at (425 nm) according to Merck (1974).

After the 96-hour copper exposure, weight, total length and standard length measurements were taken before dissecting the fish. Each fish was killed using a single sharp incision through the vertebral column right behind the head. The gills, gonads, liver and spleen from four fish (sex ratio of 1:1) were preserved in bouin's solution for histopathological analysis. Organs from four fish were put in plastic vials and stored in a deep freezer for copper analysis.

2.3 Long-term study

2.3.1 System design of the long-term exposure

Eight 120-litre glass tanks with a box filter in one corner were used during the long-term study. Details of the system description are presented in figure 2.3. This system did not contain a filter substrate. The corner box filter (D) facilitated the mixing of the water. Water in the tank entered the corner box filter through a hole (G) at the bottom of the tank and it was air-lifted with the aid of air delivered through the air tube (B 1) into the air lift tube (E) to the tank (see direction of air and water flow). The water flow ranged between 2.8 to 3.0 litres per minute.

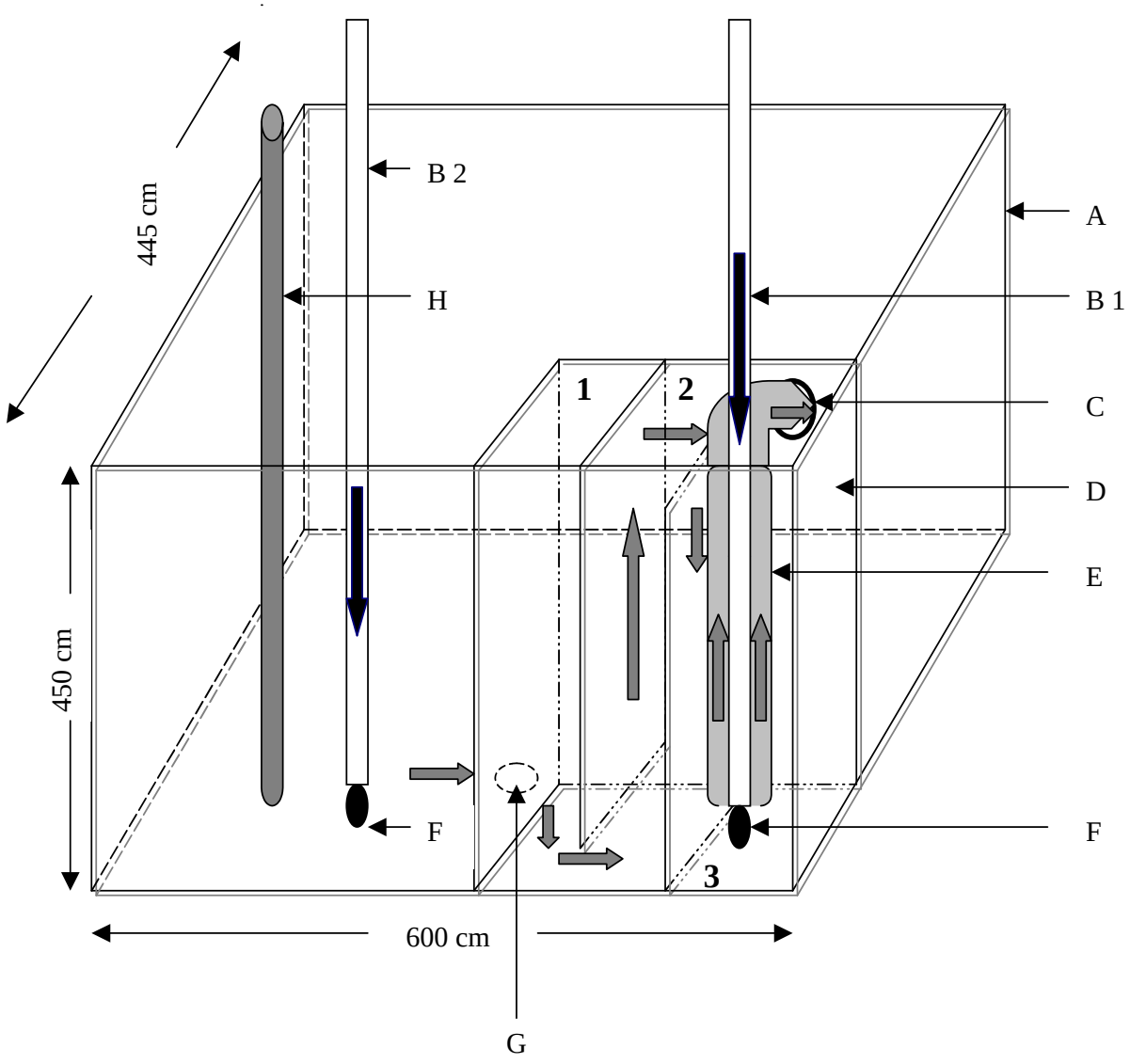


Figure 2.3 The recirculating system used in the long-term study with a corner box filter (compartments 1, 2, and 3). ⟹ = direction of water flow, ➡ = direction of air flow, A = tank, B 1 and B 2 = air tubes C = box filter water outlet, D = corner box filter, E = air lift tube, F = air stone, G = box filter water inlet, H = submersible water heater

2.3.2 Experimental design of the long-term exposure

The study involved exposing fish to three copper levels (means ± standard deviation), including a control with no copper 0.00, 0.11 ± 0.02, 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu. Each treatment was replicated twice. For each treatment, 14 fish (sex ratio 1:1) were used. Only fish weighing above 40g were selected. Cu was measured daily. The lost Cu was replaced during the daily water change in the morning to restore the desired Cu dosages which were kept as constant as possible during the experiment. A water change of 50 litres for every tank was carried out daily in the morning during which faecal matter and uneaten food was siphoned. The fish were fed once daily to satiation (3% body weight) on dry pellets supplied by WPK Aqua Feeds (PTY) Ltd, South Africa, containing 450g protein, 120g moisture, 80g fat, 40g fibre, 7g phosphorous and 30g calcium per kg dry matter.

2.3.3 Water quality

Total water hardness was measured once a day, ammonia was measured every two weeks, and pH, temperature and oxygen were measured twice a day in the morning before the water change and in the afternoon.

2.3.4 Sampling

Weight and length measurements were taken before dissecting the fish. A stainless steel dissecting kit from laboratory and scientific equipment company Pty. Ltd was used for dissecting the fish. Each fish was killed using a single sharp incision through the vertebral column behind the head. Six dissections were carried out after 1, 2, 4, 16, 32 and 64 days during which the gills, liver and spleen were collected and preserved in bouin's solution for histological analysis. The liver, gills and kidney were collected after 1, 32 and 64 days, put in plastic vials and stored in a deep freezer at –10 ^{o}C until used for Cu analysis.

2.3.5 Processing samples for histology

Preserved samples in bouin's solution were processed for histological analysis. Following treatment with formalin and formalin-alcohol mixture, samples were dehydrated in increasing concentrations of alcohol, i.e. 1 hour 10% buffered formalin, 1 hour ½ formalin + ½ 95% ethanol, 1 hour 70% ethanol, 1 hour 94% ethanol, 30 minutes 100% ethanol. Dehydrated samples were immersed in chloroform (clearing

agent) for 1 hour to remove the dehydrating medium. The tissue samples were immersed in a series of melted wax, 30 minutes per each of 3 series to remove the clearing agent at the same time infiltrating the tissues without adding water. Specimens were imbedded in wax. Waxed blocks were cut at ± 2 micron thickness and put on slides. Slides were placed in the oven (60 ^{o}C) for 10 minutes before staining.

Staining procedures involved removing wax from the sections by immersing slides in xylene for 5 minutes followed by re-hydration of the sections in grades of alcohol starting at 100 % to 70%. Hydrated slides were stained with Harris haematoxylin for 5 minutes, approximately 2 minutes in Scotts solution, 30 seconds in 0.5% acid alcohol, and about 2 minutes Scotts solution before washing with tap water. Washed slides were placed in 80 % alcohol solution then stained with eosin for 1 minute. This was followed by taking the slides through grades of alcohol starting at 80% to 100%, followed by rinsing in two changes of xylene then mounting. The slides were mounted in a mounting media (dipex) with cover slip (Luna 1968).

2.3.6 Behavioural observations

Every three days, two separate video recordings of the fish were done per treatment, 5 minutes for respiration and 15 minutes for fish activity. With the aid of a computer programme, data on the video was used to quantify the behavioural activity of the fish through the course of the experiment. Details about the behavioural observations are given in chapter seven.

CHAPTER 3

Bioaccumulation of copper in *O. mossambicus*

3.1 Introduction

Metals are considered hazardous to aquatic life because of their extreme persistence in the environment, high toxicity, tendency to bioaccumulate, and because they are made available through diverse anthropogenic sources to air, water, soil and food (Goyer 1996, Atchison *et al*.1987). Since heavy metals cannot be biologically degraded, they can be bioaccumulated by fish, either directly from the surrounding water or by ingestion of contaminated food (Avenant-Oldewage and Marx 2000). Bioaccumulation is the uptake of a chemical into an organism through one or several environmental pathways such as food, water or air (Leatherland and Woo 1998).

Monitoring the bioaccumulation of metals in the aquatic system is important because it gives an indication of the temporal and spatial extent of metal accumulation, as well as an assessment of the potential impact on human health (fish consumed), and health of organisms if the organisms are exposed to elevated levels of a pollutant or if they are consumed by predators (Kotze *et al*. 1999). The toxic modes of action and the target biological systems affected by bioaccumulated metals involve the inhibition of certain enzymes, and the impairment of locomotion, osmoregulation, respiration and reproduction (Atchison *et al*. 1987). In aquatic ecosystems metals are commonly present at low levels, and any increase in their concentration may lead to accumulation of the metals in organs and/or tissues of aquatic organisms (Nussey *et al*. 1999).

Chemicals absorbed via the gills (Stagg and Shuttleworth 1982), integument or gut (Robinson and Avenant-Oldewage 1997, Nussey *et al*. 1999), are usually bound to a protein and then transported by blood to either a storage point, such as fat, or to the liver for transformation and/or storage (Heath 1995, Olsson 1998, Nussey *et al*. 1999). If transformed by the liver, the new compound may be stored there, excreted in the bile, or passed back into the blood for possible excretion by the kidney or gills, or

stored in extrahepatic tissues such as fat (Goyer 1996, Olsson 1998). Thus, the concentration of metals in different organs depends on several processes. For example, the availability of the metal for absorption depends on its chemical form or speciation (Goyer 1996, Olsson 1998). The cupric ion (Cu^{2+}) is the most important state of copper since it is the oxidation state generally encountered in water (ATSDR 1990). However, cupric ions are adsorbed to sediments, clays, organic particulates and form complexes with several inorganic and organic compounds (ATSDR 1990, Eisler 1998). Due to this complex interaction with the chemical species normally found in natural waters, the amount of copper compounds that actually exist in solution and are available for absorption will depend on the pH, temperature and water hardness (USEPA 1980). It is therefore essential that contamination of water systems by heavy metals be carefully monitored and their effect on aquatic organisms evaluated.

3.2 Literature review

3.2.1 Copper concentration-duration-response

The concentration-duration-response function has either not been established or such studies were field-based where migration behaviour of fish and seasonal variation in environmental factors could not be taken into account. Environmental factors include pollutant concentration, metal interactions, organics, pH, temperature, alkalinity and hardness, sediment and dissolved oxygen levels (see chapter 1).

Most studies concerning metal accumulation in fish tissues did not investigate the concentration – and duration – response relationships and a few studies that investigated these relationships varied in their findings. For example there was a decline in the liver Cu content after 6 weeks of exposure to 1.7, 2.7, or 3.6 mg/l copper sulphate for 2, 4, 6, 8, and 10 weeks in channel catfish, *Ictalurus punctatus*, (Griffin *et al*. 1997). The authors reported an initial increase in the liver Cu content during the first 4 – 6 weeks of exposure in all treatments followed by a decline whereby the liver Cu content was about 50% lower than peak concentrations by the 10^{th} week. A ranking of four metals in the order of Pb > Cr > Cu > Zn and a dose- and time-dependent relationship was observed in *Clarias gariepinus* exposed to sublethal

levels of combined (composite) tannery effluent for 8 weeks (Gbem *et al*. 2001). The difference in the concentration-duration relationships reported by Griffin *et al*. (1997) and Gbem *et al*. (2001) could be attributed to the difference in the fish species worked on, Cu source and time of exposure.

Other studies only investigated the pollutant concentration – accumulation relationship. For example metals in a decreasing order of Pb, Fe, Zn, Cu, Mn, Cr, Ni, and Cd and a metal concentration-accumulation relationship was reported in *Oreochromis niloticus* exposed to treated petroleum refinery effluent for 8 weeks (Onwumere and Oladimeji 1990). There was a Cu concentration-accumulation relationship following exposure of copper nitrate for 42 days to seawater-adapted and for 28 days to freshwater-adapted flounder, *Platichthys flesus* (Stagg and Shuttleworth 1982). The pollutant concentration – accumulation relationships observed by Stagg and Shuttleworth (1982), and Onwumere and Oladimeji (1990) were based on one exposure duration, thus it was not possible to establish a relationship between metal accumulation in the fish and exposure time. Like for Griffin *et al*. (1997) and Gbem *et al*. (2001), the two studies dealt with different fish species, copper source and time of exposure.

3.2.2 Copper accumulation in different organs and tissues

Studies on bioaccumulation showed that the liver, gills and kidney were better indicators of copper exposure than muscle (see review, chapter 1). Some authors (van den Heever and Frey 1994, Avenant-Oldewage and Marx 2000) established indicator organs for metal accumulation but could not provide the exact metal concentrations to which the fish had been exposed and the exposure duration mainly because the studies were field-based whereby seasonal variation in environmental factors could not be evaluated. For example Cu accumulated in the order of liver > gill > skin > muscle in the African sharptooth catfish *Clarias gariepinus* exposed to anthropogenic effluent from the Olifants River, Kruger National Park (Avenant-Oldewage and Marx 2000). In the same species exposed to treated sewage effluent in food fish culture, Cu accumulated in the order of liver > kidney > muscle (van den Heever and Frey 1994). However, the temporal trends of Cu accumulation did not correlate with the temporal trends of the metal concentration in the water. Thus, the Cu concentration in fish tissues was due to exposure of fish to metals before sampling.

3.2.3 Conclusions and objective

Metal bioaccumulation in fish correlates to the duration of exposure of fish to metal in their environment and water samples can only indicate the metal concentration at the time of sampling (Kotze *et al*. 1999). A concentration-duration-response function could be used to predict the concentration and duration of exposure that constitutes a hazard to aquatic organisms. A precise dose-duration-response function can only be established under controlled environmental conditions when the concentration of metals and exposure time are known. Under field conditions, the effects of individual contaminants may be worsened as a result of their interactions with each other or with normally inert factors in the environment (see review, chapter 1). Thus, to effectively monitor and define environmental factors, exposure time and bioavailability of the chemical, laboratory tests ought to be conducted. Such tests allow maximum control over external variables and water quality parameters are monitored and recorded regularly (Kendall *et al*. 1996). The objective of this trial was to establish a relationship between exposure and copper accumulation in the liver, gills, and kidney using elementary copper under experimental conditions in the laboratory.

3.3 Materials and Methods

This study was conducted in two parts, a short-term acute study (4-day copper exposure) and a long-term sub-lethal copper exposure (64-day copper exposure). For the short term acute study sixty *Oreochromis mossambicus* yearlings were used with ten fish per treatment exposed to 0 mg/l (control) and 0.75 ± 0.20 mg/l copper for 96 hours. Dissections were carried out after 96 hours of exposure. The long term study involved exposing fish to three copper levels, including a control with no addition of copper i.e. 0, 0.11 ± 0.02, 0.29 ± 0.02 and 0.47 ± 0.04 mg/l copper. Each treatment was replicated twice. For each treatment, 14 fish above 40 g body mass (sex ratio of 1:1) were used. Tissue samples of the liver, gills and kidney were collected after 1, 32 and 64 days, respectively. Details of the aquarium system and the experimental design are given in the general methods (chapter 2). The pH, temperature, water hardness, dissolved oxygen and ammonia were monitored as described in the general methods (chapter 2).

3.3.1 Laboratory analysis

All organ samples (liver, gills and kidney) were weighed (wet weight) using a Mettler AE 240 weighing balance with a sensitivity of 0.0001 g. Weighed samples were put into plastic vials and stored in a deep freezer (-10 ^{o}C) until determination of metal concentration. Frozen samples were later freeze-dried using an Edwards EF4 Modulyo freeze dryer at – 45 ^{o}C for 96 hours. Freeze dried samples were weighed to obtain the dry weight before being chemically digested. Before digestion, the freeze-dried samples were thawed overnight at 40^{o}C. Approximately 1 g of a thawed sample was digested in 1 ml of 67% nitric acid to which 9 ml of double-distilled (deionised) water was added. The digested solution was filtered using a 0.45 micron nylon membrane filter (MSI). The copper content in the digested sample was measured using an Atomic Absorption Spectrophotometer - AAS (GBS 909AA linked to a GBC integrator) with a sensitivity of 1 µmole. Standard curves of 2, 5, 10, 20 and 50 µmole/l and 100, 200, 300, 400 and 500 µmole/l were prepared (r^2 = 0.999%). The samples were read at a wavelength of 324.7 nm, lamp current of 3.0 mA and slit width of 0.5 nm. Readings on the AAS were obtained in µg/l and were later converted to µg/dry sample mass using equation 3.1.

$$C1 = \frac{R \times 10 \times 63.55}{1000} \qquad \text{(Equation 3.1)}$$

Where:

$C1$	=	copper concentration per dry sample mass (µg)
R	=	AAS reading (µg/l)
10	=	dilution
63.55 g	=	1 molar mass of copper

After the loss of moisture during freeze drying sample mass was below 1 g. To obtain a standard unit of copper concentration per g of dry sample mass (µg/g dry mass), equation 3.2 was used.

$$C2 = \frac{C1}{D} \qquad \text{(Equation 3.2)}$$

Where:

$C2$	=	copper concentration per g of the dry mass (µg/g dry mass)
$C1$	=	copper concentration per dry sample mass (µg)
D	=	sample dry mass (g)

The wet mass of the kidneys of the fish was below 1 g. Thus, after the loss of moisture during freeze drying the sample mass of these organs were below 1 g making Cu concentration in the kidney samples fall below the minimum sensitivity of the atomic absorption spectrophotometer.

3.3.2 Bioconcentration factor

Bioconcentration factor values were obtained using equation 3.3.

$$BF = \frac{Co}{Cw} \qquad \text{(Equation 3.3)}$$

Where:

BF	=	biological concentration factor (bioconcentration factor)
Co	=	average concentration of Cu in the organ (µg/g dry mass)
Cw	=	average concentration of Cu in the water (mg/l)

3.3.3 Statistical analysis

The mean Cu accumulation values and standard deviations for each organ at different exposure periods were determined using descriptive statistics. Regression analysis was used to establish a relationship between Cu accumulation in the organs, exposure concentration and exposure duration. Descriptive statistics was used to derive the means, standard deviations, and ranges of water quality (temperature, oxygen and ammonia) values. The Kruskal-Wallis nonparametric test was used to compare differences between median water quality values for different treatments. If significant differences were indicated the Mann-Whitney U test was used to compare medians, after Bon-Ferroni corrections of the p – value for pre-planned pair-wise comparisons.

3.4 Results

3.4.1 Short-term study

The pH ranged between 7.6 – 8.0 (n = 12) for the control and 7.4 – 8.0 (n = 12) for the copper treatments. The average temperature, oxygen and NH_4^+ values were not significantly different ($p > 0.05$) between the control and the copper-treated tanks. Temperature averaged 24.4 ± 0.6 ^oC (23.5 – 25.5 ^oC, n = 12) for the control and 24.8

± 0.9 ^{o}C (23.5 – 26.5 ^{o}C, n = 12) for the copper treatments. Dissolved oxygen averaged 7.2 ± 0.8 mg/l (6.0 – 8.1 mg/l, n = 12) for the control and 7.9 ± 0.2 mg/l (7.7 – 8.3 mg/l, n = 12) for the Cu treatments. NH_4^+ averaged 0.018 ± 0.004 mg/l (0.012 – 0.024 mg/l, n = 6) for the control and 0.018 ± 0.006 mg/l (0.011 – 0.027 mg/l, n = 6) for the copper treatments. Total water hardness averaged 290 ± 11 mg/l $CaCO_3$ (270 – 300 mg/l $CaCO_3$, n = 12) for the control and was significantly different ($p < 0.05$) from that of the copper treated tanks, 140 ± 29 mg/l $CaCO_3$ (100 – 190 mg/l $CaCO_3$, n = 12). The average copper concentration of the exposed replicates was 0.75 ± 0.20 mg/l (0.4 – 1.0 mg/l, n = 12).

3.4.2 Long-term study

3.4.2.1 Copper treatments used during the long-term study

During the long-term study, the fish were exposed to three Cu levels (table 3.1).

Table 3.1 Means ± standard deviation, minimum and maximum Cu concentration for the three Cu treatments during the long-term study (64 days). Fish in treatment 3 only survived up to day 32.

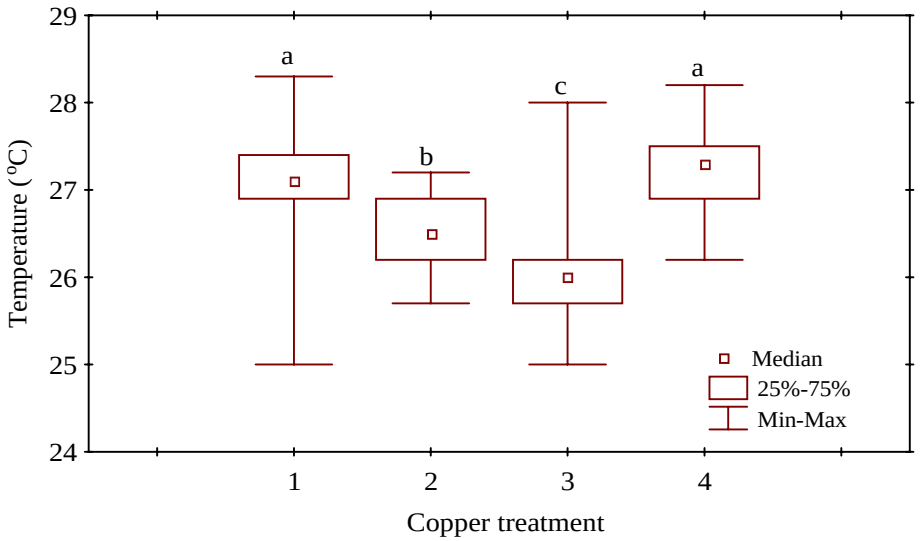
Treatments	Cu concentration in the water (mg/l)			Sample size
	Mean ± Standard deviation	Minimum	Maximum	
Treatment 1	0.11 ± 0.02	0.07	0.2	128
Treatment 2	0.29 ± 0.02	0.24	0.33	128
Treatment 3	0.47 ± 0.04	0.36	0.52	64

3.4.2.2 Water quality

(i) Temperature

Average temperature between treatments was significantly different ($p < 0.05$) between measurements done before and after the water change except between the control and the 0.47 ± 0.02 mg/l copper treatment ($p > 0.05$), figure 3.1 a and b.

a) Before water change



b) After water change

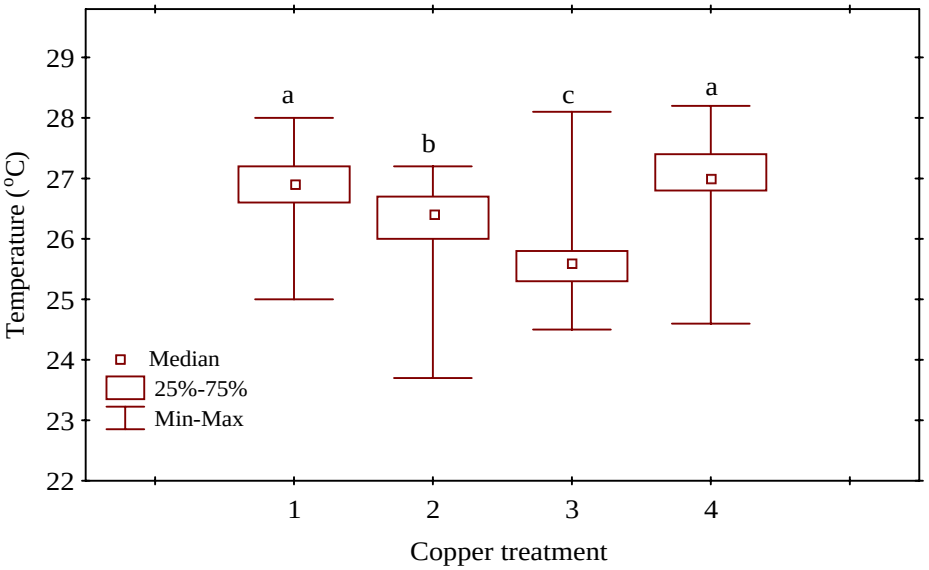


Figure 3.1 Box plots of temperature ranges, lower and upper quartiles, and medians for the different Cu treatments during the long-term study. Different letters denote significant differences. Treatments 1, 2, 3 and 4 = 0, 0.11 ± 0.02, 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu, respectively.

(ii) pH

The minimum pH was maintained above pH 6 in all treatments before and after water change. The highest pH of 8.9 was recorded in 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels before water change (table 3.2).

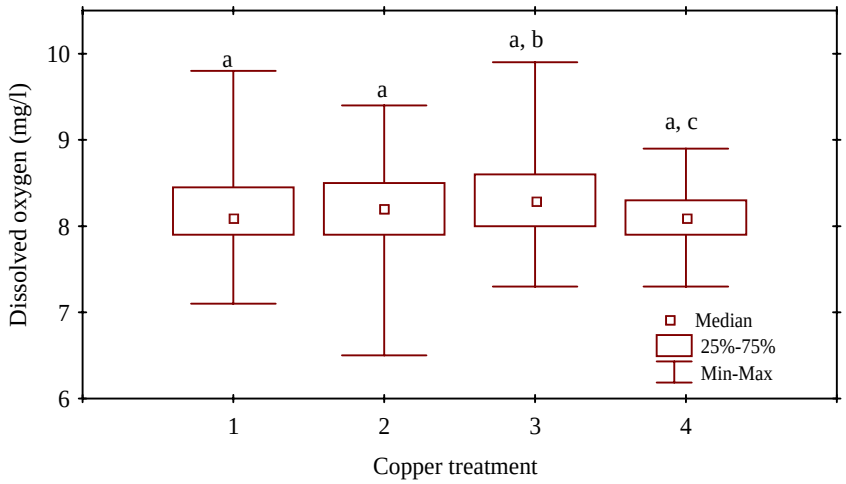
Table 3.2 Minimum and maximum pH values before and after water change for the different Cu treatments during the long-term study (64 days).

	Treatments (mg/l copper)	pH values		Sample size
		Minimum	Maximum	
Before water change	0 (control)	6.6	7.9	128
	0.11 ± 0.02	6.2	8.8	128
	0.29 ± 0.02	7.0	8.9	128
	0.47 ± 0.04	6.8	8.9	64
After water change	0 (control)	6.8	8.3	126
	0.11 ± 0.02	6.1	8.4	126
	0.29 ± 0.02	6.8	8.0	126
	0.47 ± 0.04	6.8	8.3	64

(iii) Oxygen

The average oxygen level for the control groups was not significantly different from that of the Cu treated groups before and after the water change ($p > 0.05$). There was a significant difference ($p < 0.05$) in average oxygen levels between 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu treatments before the water change (figure 3.2 a). There were significant differences in average oxygen levels between Cu treated groups ($p < 0.05$), except between the 0.11 ± 0.02 and the 0.47 ± 0.04 mg/l Cu treatment (figure 3.2 b).

a) Before water change



b) After water change

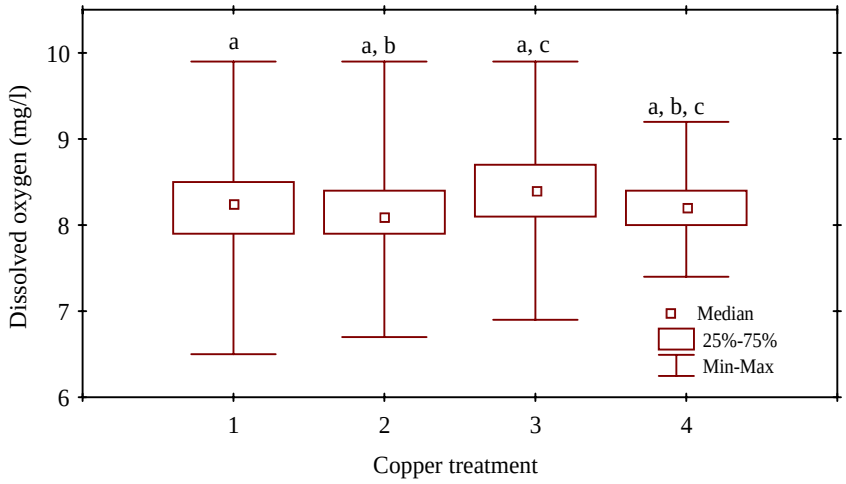


Figure 3.2 Box plots of oxygen ranges, lower and upper quartiles, and medians before and after changing the water for the different copper treatments during the long-term study. Different letters denote significant differences. Treatments 1, 2, 3 and 4 = 0, 0.11 ± 0.02, 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu, respectively.

(iv) Ammonia

There was no significant difference ($p > 0.05$) between mean NH_4^+ values between treatments (table 3.3).

Table 3.3 Means ± standard deviation, minimum and maximum ammonia values (mg/l NH_4^+) per treatment during the long-term study (64 days).

Treatments (mg/l copper)	Ammonia (mg/l NH_4^+)			Sample size
	Mean ± Standard deviation	Minimum	Maximum	
0 (control)	0.013 ± 0.002	0.010	0.016	12
0.11 ± 0.02	0.013 ± 0.002	0.010	0.016	12
0.29 ± 0.02	0.013 ± 0.002	0.010	0.016	12
0.47 ± 0.04	0.013 ± 0.002	0.010	0.015	6

(v) Water hardness

There was a significant difference in average water hardness between the control and each of the Cu treatments ($p < 0.05$). There was no significant difference in average water hardness between the Cu treatments ($p > 0.05$), table 3.4.

Table 3.4 Means ± standard deviation, minimum and maximum water hardness values (mg/l $CaCO_3$) per treatment during the long-term study (64 days). Different superscripts denote significant differences between treatments

Treatments (mg/l Copper)	Water hardness (mg/l $CaCO_3$)			Sample size
	Mean ± Standard deviation	Minimum	Maximum	
0.0 (control)	122.8 ± 10.8^{a}	100.00	150.00	128
0.11 ± 0.02	64.8 ± 9.4^{b}	50.00	110.00	128
0.29 ± 0.02	64.7 ± 12.8^{b}	50.00	150.00	128
0.47 ± 0.04	67.5 ± 12.9^{b}	55.00	120.00	64

3.4.3 Cu concentration in the liver and gills of *O. mossambicus* after the short-term study

Cu levels in the kidney for both the control and the Cu treated groups fell below the sensitivity of the atomic absorption spectrophotometer hence only the gills and liver

were considered for statistical analysis. There were significant differences ($p < 0.05$) between the mean Cu accumulation values in the organs between the control and the Cu exposed fish (table 3.5).

Table 3.5 Means ± standard deviation, minimum and maximum Cu concentrations (μg/g dry weight) in the gills and liver of *O. mossambicus* after the short-term Cu exposure. Sample size per treatment = 6

Organ	***Treatments (mg/l copper)***	***Cu concentration in organs (μg/g dry weight)***		
		Mean ± Standard deviation	***Minimum***	***Maximum***
Gills	Control	3.94 ± 2.10	0.00	5.91
	0.75 ± 0.20	18.36 ± 6.66	11.12	30.81
Liver	Control	77.12 ± 25.33	37.05	99.90
	0.75 ± 0.20	248.85 ± 72.90	161.61	326.85

3.4.4 Copper concentration in the liver and gills of *O. mossambicus* during the long-term study

Cu concentration in the kidney in more than three quarters of the samples for both the control and the Cu treated fish fell below the sensitivity of the atomic absorption spectrophotometer. Thus, only the gills and the liver were considered for statistical analysis.

With the exception of the control, the mean Cu concentration in the liver and gills for the three Cu treatments increased with increase in exposure time. However, the rate of Cu accumulation was slow during the first 32 days of exposure and faster between 32 and 64 days of exposure (figures 3.3 and 3.4).

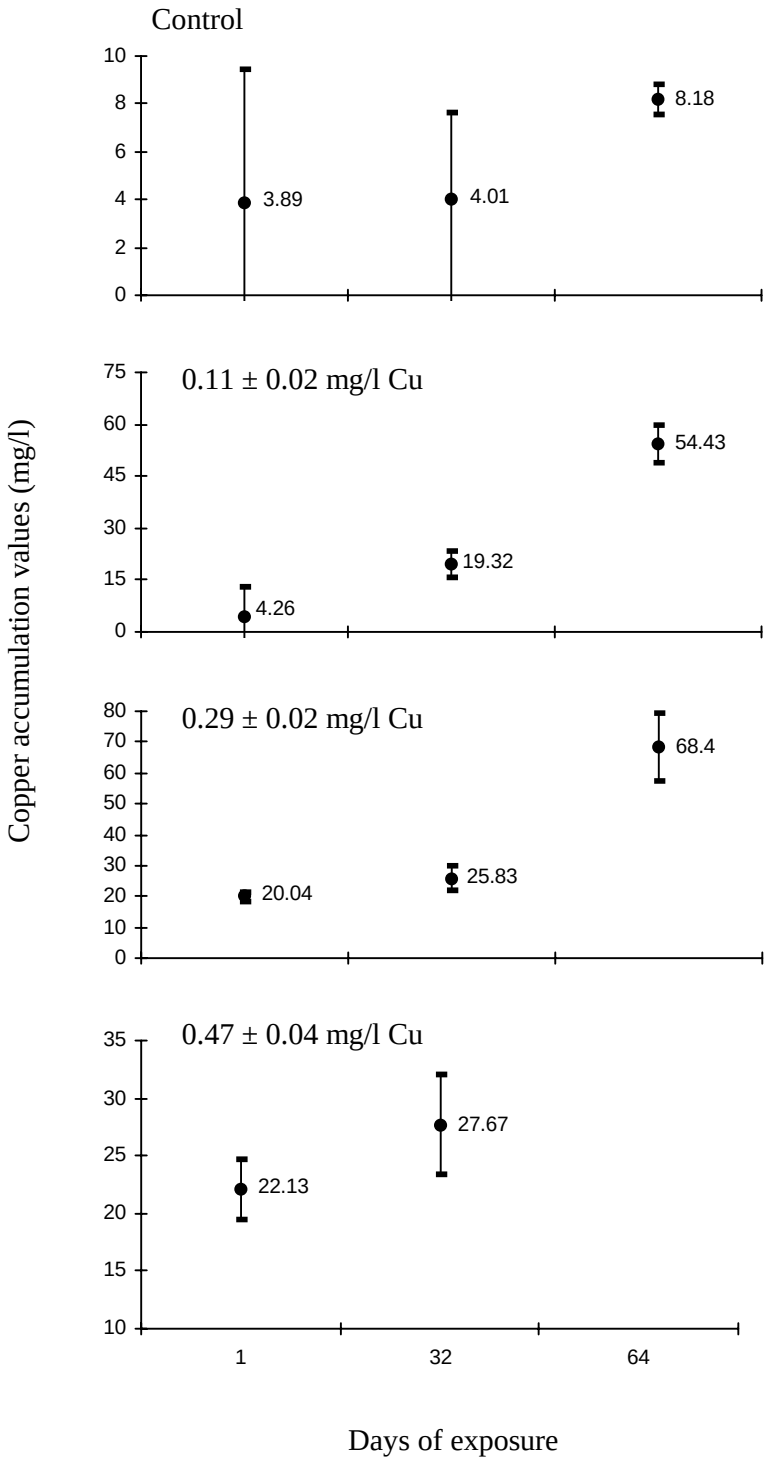


Figure 3.3 Mean Cu accumulation values ± standard deviation (µg/g dry mass) in the gills of *O. mossambicus* exposed to Cu during the long-term study (64 days).

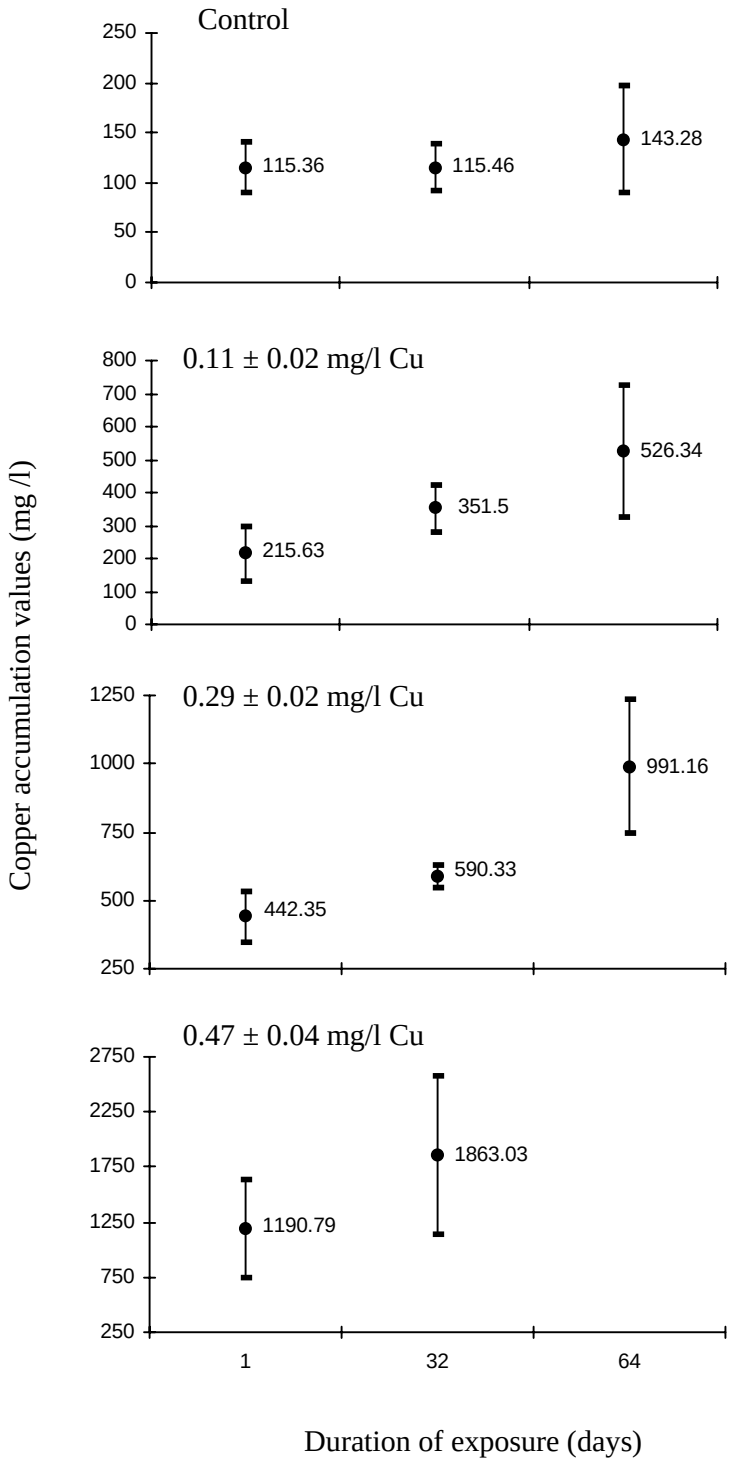


Figure 3.4 Mean Cu accumulation values ± standard deviation (µg/g dry mass) in the liver of *O. mossambicus* exposed to Cu during the long-term study (64 days).

3.4.4.1 Relationship between Cu accumulation in the gills and liver, exposure concentration and duration of exposure

The concentration of Cu in the water and the duration of exposure had a significant impact on the Cu accumulation levels in the gills ($r^2 = 0.63$, $p < 0.05$) and the liver ($r^2 = 0.89$, $p < 0.05$). Thus, accumulation of Cu in these organs was a function of Cu concentration in the water and duration of exposure. A relationship between Cu accumulation levels in the organs, Cu concentrations in the water and duration of exposure was summarized in models (equation 4 and 5 for the gills and the liver, respectively).

$$G = -35.09 + 10.58W + 17.58T \quad (p < 0.05, r^2 = 0.632) \qquad \text{(Equation 4)}$$

Where:

G = copper accumulation in the gills (µg/g dry mass)

W = copper concentration in the water (mg/l)

T = exposure time (days)

$$Log\ L = 3.35 + 0.85W + 0.31T \quad (p < 0.05, r^2 = 0.892) \qquad \text{(Equation 5)}$$

Where:

L = copper accumulation in the liver (µg/g dry mass)

W = copper concentration in the water (mg/l)

T = exposure time (days)

3.4.4.2 Bioconcentration factors for copper in the gills and liver

The bioconcentration factors for copper during the long-term study were higher in the liver than in the gills, implying that the liver had a higher copper accumulation capacity than the gills (table 3.6).

Table 3.6 Bioconcentration factors for copper in the gills and liver of *O. mossambicus* exposed to different Cu concentrations for 1, 32 and 64 days. * = no fish left by the time of dissection.

Organ	Cu treatments (mg/l copper)	Bioconcentration factors at different days of exposure		
		Day 1	Day 32	Day 64
Gills	0.11 ± 0.02	38.73	175.64	494.82
	0.29 ± 0.02	69.10	89.07	235.86
	0.47 ± 0.04	48.11	60.15	*
Liver	0.11 ± 0.02	1960.27	3195.46	4784.91
	0.29 ± 0.02	1525.34	2035.62	3417.79
	0.47 ± 0.04	2588.67	4050.07	*

3.5 Discussion

Copper accumulation in the liver and gills increased with increase in Cu concentration in the water and duration of exposure. These findings agree with those of Gbem e*t al*. (2001) who observed a Cu accumulation-exposure concentration-duration relationship in the liver and gills of *Clarias gariepinus* exposed to sublethal dilutions of tannery effluent containing Cr, Cu, Pb and Zn. Considering that Gbem e*t al*. (2001) used effluent and the present study used elementary Cu it could be that when the concentration of Cu exposed to fish is maintained, accumulation of this metal in the liver and gills may depend on the Cu concentration in the water and the duration of exposure with maximum accumulation occurring in the highest treatment within the longest time of exposure irrespective of the Cu source.

However, this hypothesis disagrees with Griffin *et al*. (1997) who observed the highest liver Cu content in channel catfish exposed to copper sulphate after 6 weeks and a more than 50% decline after 10 weeks. Griffin *et al*. (1997) looked at liver and muscle and this trend was only observed in the liver. This difference could not be explained. It is possible that the liver was able to regulate Cu and probably histological observations of the liver could have given an insight into the cause for the

decline. The present study also agreed with Stagg and Shuttleworth (1982) and Onwumere and Oladimeji (1990) who observed a dose-dependent metal accumulation in the gills, liver and kidney. As, these findings were based on one exposure period one cannot establish an exposure duration-accumulation relationship.

The highest Cu accumulation capacity was observed in the liver. This reinforces the fact that the liver plays a major role in Cu homeostasis and detoxification as well as storage by producing metallothioneins, the main cellular heavy metal binding and detoxifying proteins in fish (Heath 1995, Olsson 1998). This was supported by the degenerative changes that were observed in the hepatocytes, for example by vacuolar degeneration that reflected an increase in colloidal proteins in the cytoplasm (see chapter 4), although this study could not tell whether a high proportion of the cytoplasmic proteins were metallothioneins. This explanation was also supported by Felts and Heath (1984) who observed an elevation in the liver oxygen utilization (elevated liver energy consumption) that reflected an increase in the detoxification activity associated with increased protein synthesis in the liver. Perkins *et al*. (1997) observed a similar pattern in metallothionein content and hepatic Cu accumulation (correlation coefficient of 78%) in channel catfish following a 10-week exposure to copper sulphate. Schlenk *et al*. (1999) observed a time dependent increase in hepatic metallothionein expression and hepatic Cu content after acute exposure to therapeutic concentrations of copper sulphate. The high accumulation of Cu in the liver relative to the gills agrees with bioaccumulation studies summarized in table 1.2.

Given that waterborne Cu was used in this study, the gills could have played a big role in the absorption of this metal. This could explain the accumulation of Cu in this organ. The gills serve as areas of absorption of dissolved metals, respiratory uptake: hence this organ is the main port of entry for dissolved substances (Heath 1995, Olsson 1998). The gills also possess a countercurrent blood / water flow system, very thin epithelial membranes, and a large surface area, features that facilitate the uptake of materials from the water and their transfer to blood (Heath 1995). Thus Cu accumulation in the gills could be attributed to the large surface area available for adsorption and the large volume of water that passes through the gills (Robinson and Avenant-Oldewage 1997).

The slow increase in Cu accumulation in the gills during the first 32 days of exposure could imply that by this time, the fish were still able to regulate the metal uptake through the gills and hypersecretion of mucous could have played a role in this regulation. This was supported by the mucous cell hypertrophy and hyperplasia and the hypersecretion of mucous that were observed until 32 days of exposure and not after 64 days (chapter 6). The mucous layer controls the permeability of the integument to toxic substances in the water by concentrating trace elements from the water (Jones 1964). Thus, the slow rise in Cu accumulation levels in the gills and liver between 1 and 32 days of exposure could be attributed to the homeostatic control of this metal by the liver and the mucous-mediated control of metals by the gills. These organs also play a role in the excretion of metals (Olsson 1998). It is therefore possible that fish exposed to metal pollution are capable of homeostatically regulating metal absorption for a specific time. A similar trend was observed by Gbem *et al*. (2001) who observed a relatively stable uptake pattern of metals for the first 28 days followed by an increased uptake in *C. gariepinus* exposed to sub-lethal dilutions of tannery effluent containing Cr, Cu, Pb, and Zn for 56 days. Given that the proteins in the liver play a role in the detoxification and homeostasis of metals by sequestering the metals and preventing them from binding and inhibiting critical homeostatic proteins (Kägi and Schäffer 1988). It could be that within the early days of exposure, the liver proteins detoxify metals by inhibition of metal binding and as the exposure increases, the system gets overwhelmed and detoxify metals by sequestering the metals instead hence the rise in Cu accumulation after 32 days of exposure.

Trace Cu concentrations (baseline values) were detected in the control in both the liver and gills. The baseline values reinforce the fact that Cu is an essential trace element for all eucaryotic cells since the metal plays a critical role in biochemical processes (Urani *et al*. 2001) and is required for the normal growth and metabolism in all living organisms (International Copper Association [ICA] 1995, Eisler 1998). Requirements for Cu in living organisms include cell maturation, structure and function, normal formation of connective tissue, normal structure and respiration in mitochondria, and its role as a factor in formation of new capillaries (ICA 1995). Hepatic oxidative enzymes, and liver proteins (haemocuprein and hepatocuprein) need Cu as an important component to function (ATSDR 1990, Goyer 1996, Eisler 1998, Avenant-Oldewage and Marx, 2000). Cu is also required for the normal growth

and function of pancreatic acinar cells (ICA 1995), which in *O. mossambicus* are interspersed in the liver.

Water hardness exerted a uniform effect on the bioavailability of Cu in all the Cu treatments given that there was no significant difference in the mean water hardness values between Cu treatments. Temperature, oxygen and NH_4^+ never influenced the results of the short-term study, given that there were no significant differences between the control means of these variables and those of the Cu treated tanks. However, during the long-term study, except for ammonia, there were significant differences in temperature and oxygen levels between treatments. The influence of these parameters and pH on Cu availability could not be investigated but it is possible that these parameters never influenced the overall results considering that there was no evidence of visual damage and Cu accumulation in organs from fish in the control treatments.

From the findings of this study, it is concluded that the accumulation of Cu in the liver and gills of O. *mossambicus* was concentration and time-dependent with the highest concentrations in the highest Cu treatments at the longest exposure time. The liver accumulated Cu to a higher capacity than the gills as reflected in the higher bioconcentration factor. Using the models established during this study, exposure duration can be predicted when exposure concentration and Cu accumulation in the organ is known and Cu accumulation in organs can be estimated when exposure concentration and duration is known. However, results may need to be corrected for fluctuations of Cu concentration in the water. Considering that exposure time may not be known for field-collected fish, when the organ concentrations and the water concentrations are known, this model can be used to give an idea on how long the fish have been exposed.

CHAPTER 4

The histopathology of copper in the liver of *Oreochromis mossambicus*

4. 1 Introduction

Knowledge of the histopathological effects of pollutants on different organs of fishes is an important tool leading to the understanding of the impact of pollutants on both, individual species and the ecosystem (Mount 1968, Gardner and LaRoche 1973). Pathological changes are the net effects of biochemical and physiological changes, and they indicate target organs, tissues, cells, and organelles (Hinton and Laurén 1990). Given that histopathology is the only method that preserves biochemical and physiological changes, histopathological approaches have been recommended as obligatory components of environmental assessments (Hinton and Laurén 1990).

The liver develops from pocket-like anlage evaginating from the archenterons (Hibiya *et al*. 1982). It is composed of parenchymal cells, and hepatic cells (Hibiya *et al*. 1982, Hinton and Laurén 1990) supported by lattice fibres (Hibiya *et al*. 1982). Residing between the intestinal tract and the rest of the body, the liver maintains the body's metabolic homeostasis (Heath 1995, Moslen 1996). Venous blood from the stomach and or intestines flows into the portal vein and then through the liver before entering the systemic circulation (Moslen 1996). Thus, the liver is the first organ to encounter absorbed nutrients, vitamins, metals, drugs and environmental toxicants as well as waste products of bacteria that enter the portal blood (Moslen 1996). Efficient scavenging or uptake processes extract these absorbed materials from the blood for catabolism, storage, and and/or excretion into bile (Moslen 1996). Thus, the liver is the primary organ for biotransformations of xenobiotics and excretion of harmful trace metals. The poor blood perfusion in the liver of fish as compared to mammals may lead to the stasis of chemical compounds thus making it an important organ when considering the action of toxic chemicals on fish (Hinton and Laurén 1990, Heath 1995, Metcalfe 1998). All major functions of the liver can be detrimentally altered by liver injury resulting from acute or chronic exposure to toxicants (Moslen 1996). Chemical pollutants induce toxicopathic lesions in the livers of fishes (Gardner and

LaRoche 1973, Kumar and Pant 1981, Sultan and Khan 1983, and Benedetti *et al*. 1989). Such lesions include blood vessel engorgement and congestion, vacuolar and fatty degeneration, nuclei pycnosis and karyorhexis, and necrosis (Hibiya *et al*. 1982, Hinton and Laurén 1990).

Lesions observed in the livers following acute or long-term exposure to Cu were consistent in many fish species. However, the severity of the lesions was not indicated in many studies making it impossible to relate the intensity of the lesions to exposure time and Cu concentration. For example, destruction of the cytoplasmic material and partial and complete vacuolation of hepatocytes were observed in goldfish, *Carassius auratus* following 24-hour exposure to 1 mg/l and 25-day exposure to 0.1 mg/l copper sulphate (Sultan and Khan 1983). Vacuolation and necrosis was reported in the liver of *Puntius concornius* exposed to 0.6 mg/l copper sulphate for 24 and 48 hours. (Kumar and Pant 1981). Areas of patchy degeneration and isolated degenerated elements located within normal hepatocytes were manifested in brown bullhead, *Ictalurus nebulosus* exposed to 5 mg/l and 0.3 mg/l cupuric chloride for 24 hours and 40 days, respectively (Benedetti *et al*. 1989). However, it is not known whether all exposed fish exhibited the same degree of these lesions considering that the liver within a single fish may manifest more than one stage of the same lesion.

Increase in intensity of the lesions with time of exposure followed by acclimation was reported in *O. mossambicus* exposed to cadmium chloride. Blood vessel engorgement and congestion, vacuolar degeneration of hepatocytes, necrosis of pancreatic cells, and fatty changes in the peripancreatic hepatocytes were observed in *O. mossambicus* exposed to 5 mg/l cadmium chloride for 1, 7, 15 and 30 days (Usha Rani and Ramamurthi 1989). Lesions gradually increased with time of exposure but remained constant from 15 days of exposure onwards. However, only one sublethal concentration of 5 mg/l CdCl was used. Thus, no trend was available for histopatholgical lesions with concentrations below and above this level.

These studies used metal salts for example copper sulphate and copper chloride to investigate the histopathological effects of Cu on fish. The acidity of the solution resulting from the hydrolysis of these metal salts may not be sufficiently high for Cu

to be lethal to the fish but its potential influence on the results can not be accounted for. This makes it difficult to give a definite Cu concentration – response relationship.

Most of the pathological changes in the liver are non-specific. Different chemical pollutants can induce them. However, there is little knowledge on the magnitude of the changes caused by these pollutants (dose-response relationship). In mammals, the hepatic toxicity of many organic chemicals can be classified according to the zone of the lobular liver in which histological alterations are seen, such as centrilobular, midzonal or periportal zones (Metcalfe 1998). Because the teleost liver is not lobular but tubular, this classification does not apply to fish (Droy and Hinton 1988). Reimschuessel *et al.* (1992) presented a system for classifying and coding histological data from fish and invertebrate species. This study attempts to quantify the changes that were manifested in the liver using elementary Cu. The objective was to investigate the effect of elementary Cu on liver histology of *O. mossambicus* following a short and long-term exposure and to relate Cu concentration and exposure duration to the extent and severity of histological damage.

4.2 Materials and Methods

The study was conducted in two parts (see general methods chapter 2). For the short-term acute study, 60 *O. mossambicus* yearlings were used with 10 fish per treatment exposed to 0 mg/l (control) and 0.75 ± 0.20 mg/l copper for 96 hours. Dissections were carried out after 96 hours of exposure. The long-term study involved exposing fish to three Cu levels, including a control with no addition of Cu, and three treatments with 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l Cu. Each treatment was replicated twice. For each treatment, 14 fish above 40 g body mass were used (sex ratio of 1:1). Tissue samples of the liver, gills, and spleen were collected after 1, 2, 4, 16, 32 and 64 days, respectively and preserved in bouin's solution for histological analysis. Details of the experimental system and design, and processing of samples for histology are given in the general methods (chapter 2).

4.2.1 Microscopic observation and data presentation

Histologically, the liver of *O. mossambicus* appeared as a compound organ in the form of a hepatopancreas. A lesion was classified according to the extent, depending on the area of the tissue affected. Lesions were either focal where a lesion occurred in less than two localized areas, multi-focal where a lesion occurred in several localized areas and diffuse where a lesion was manifested in the whole tissue preparation. The extent of the lesion was classified according to severity as minimal, moderate, marked and severe. “Minimal” represented lesions that affected the tissue to a very low degree and “severe” represented the highest degree of the lesion. Micrographs of the tissues were taken using a JVC digital still camera (GC-X3).

4.3 Results

The liver of the control fish showed a relatively normal structural arrangement with hepatocytes located among the sinusoids forming cord-like structures (hepatic cell cords). In longitudinal sections the liver cords appeared one to two cell layers thick and sinusoids had a tubulo-sinusoidal pattern. Pancreatic acinar tissue and branches of the portal vein appeared normal. The highly basophilic nucleus appeared eccentrically located along the sinusoids. The cytoplasm stained uniformly and was more acidophilic than the nucleus (Plate 4.1).

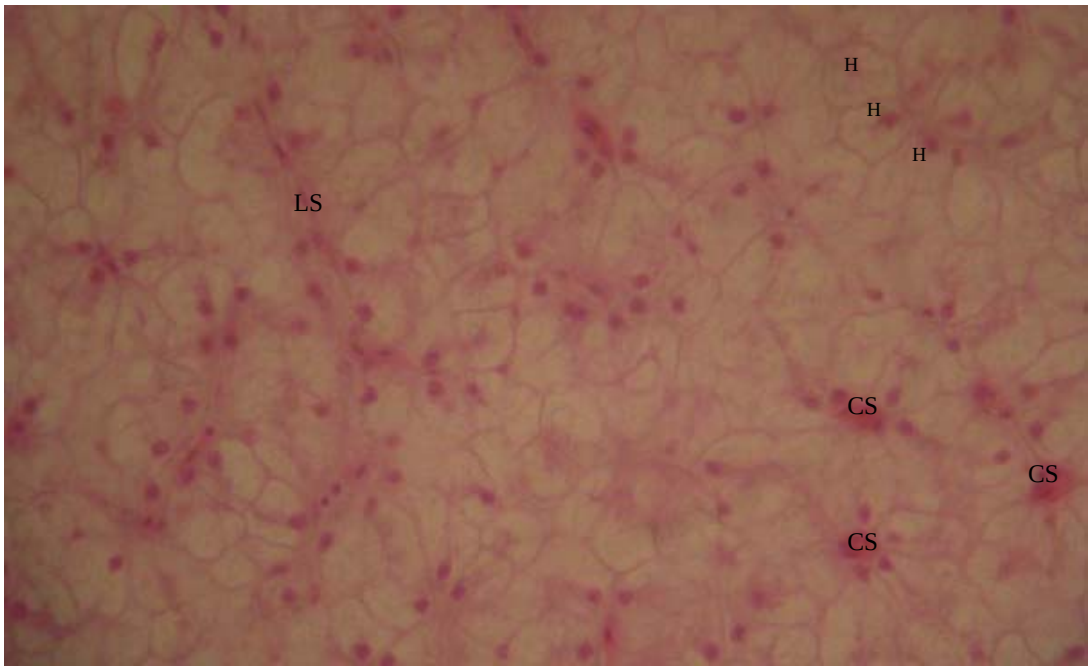


Plate 4.1 Section of a control liver from the control treatment showing hepatocytes (H) with their eccentrically located nucleus along the sinusoids, longitudinal section (LS) and cross section (CS) of the sinusoids ($\times 400$).

Lesions were observed in the hepatocytes and pancreatic acinar cells in all fish from the Cu treatments regardless of time of exposure or concentration of Cu in the water. Hepatic cells displayed various degrees of degenerative changes ranging from minimal focal vacuolar degeneration of hepatocytes to severe diffuse necrosis. The severity and progression of the lesion was related to length of exposure and was most clear at 0.29 ± 0.02 to 0.47 ± 0.04 mg/l Cu.

4.3.1 Lesions observed after the short-term exposure

After short-term exposure the liver had hepatocytes with diffuse vacuolar and fatty degeneration that had also affected the pancreatic acinar cell masses (Plate 4.2). In many areas pancreatic cords had degenerated to an amorphous mass. No necrosis was observed in fish after short-term exposure.

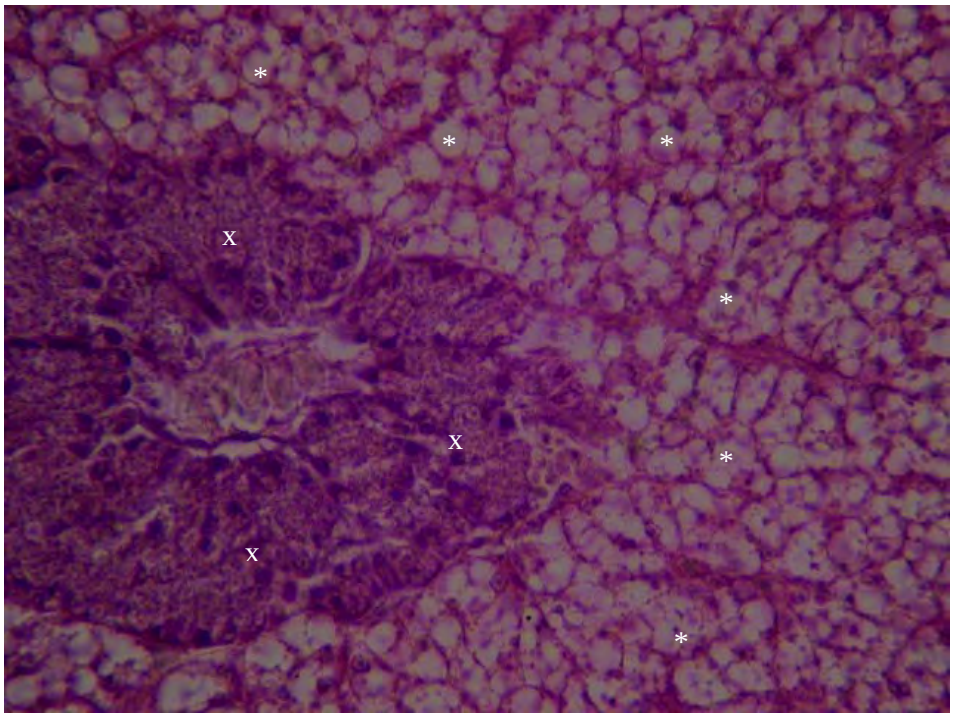


Plate 4.2 Liver from a fish ($\times 400$) exposed to 0.75 ± 0.20 mg/l Cu during the short-term study showing diffuse vacuolar and fatty degeneration of hepatocytes (*) and pancreatic tissue (x) respectively.

4.3.2 Lesions observed during the long-term exposure

Lesions resulting from long-term exposure comprised hepatic and pancreatic vacuolar and fatty degeneration, atrophy and necrosis, and the liver from one fish had more than one lesion. The extent and severity of these lesions increased with increasing exposure time and Cu concentration.

4.3.2.1 Hepatic vacuolar degeneration

Hepatic vacuolar degeneration was observed in livers from all Cu-exposed fish. After 1- and 4-day exposure vacuoles were mainly focal, multi-focal and minimal but became multi-focally marked and diffuse (Plate 4.3) with increase in exposure time (table 4.1).

Table 4.1 Number of *O. mossambicus* showing the extent and severity of hepatic vacuolar degeneration during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions and blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity								
		Focal	Multi-focal				Diffuse			
		Minimal	Minimal	Moderate	Marked	Severe	Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02	1	1	2						
	0.29 ± 0.02		3	1						
	0.47 ± 0.04		3				1	1		
2	0.11 ± 0.02		1	1				1		
	0.29 ± 0.02				4					
	0.47 ± 0.04								3	1
4	0.11 ± 0.02							4		
	0.29 ± 0.02				4					
	0.47 ± 0.04								4	
16	0.11 ± 0.02								4	
	0.29 ± 0.02								1	3
	0.47 ± 0.04									
32	0.11 ± 0.02							3	1	
	0.29 ± 0.02							2		
	0.47 ± 0.04					3				1
64	0.11 ± 0.02								4	
	0.29 ± 0.02								1	3

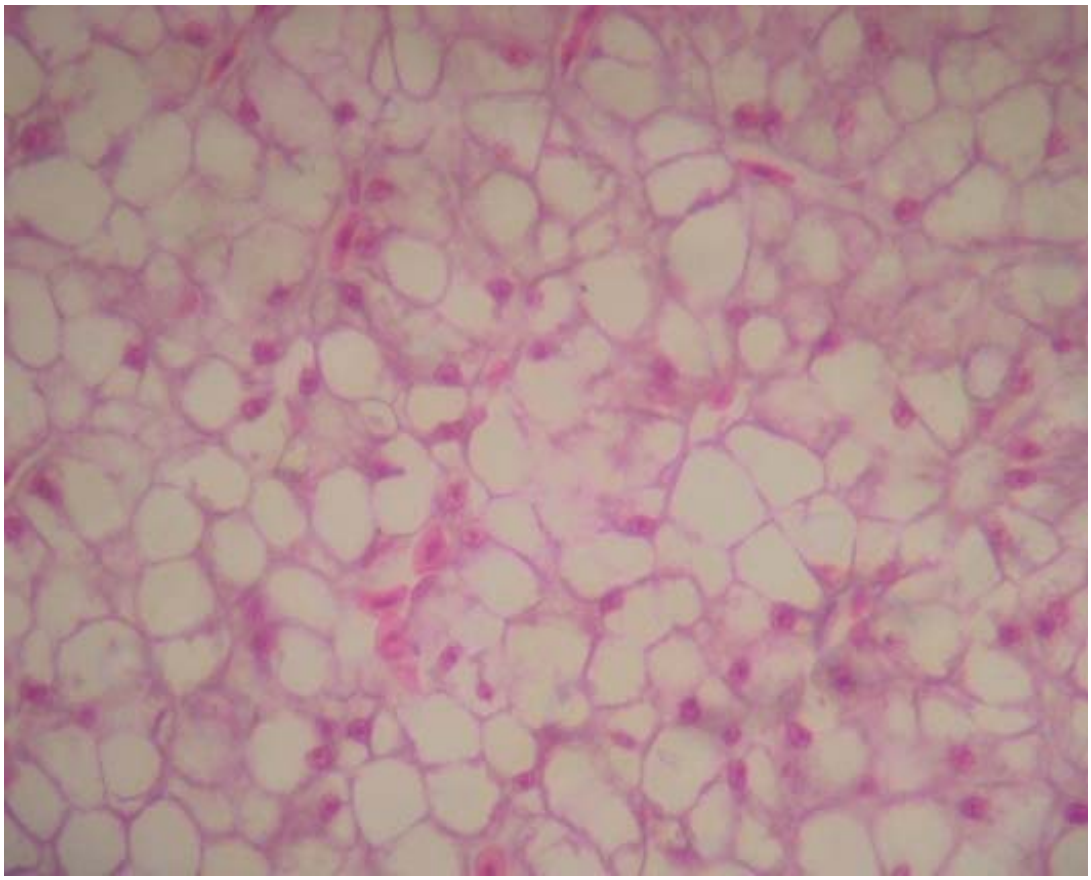


Plate 4.3 Section of a liver from a fish exposed to 0.11 ± 0.02 mg/l Cu for 64 days showing diffuse vacuolar degeneration of hepatocytes (× 400).

4.3.2.2 Hepatic fatty degeneration

Hepatic fatty degeneration was first observed after 4 days of exposure in fish exposed to the highest Cu level (0.47 ± 0.04 mg/l Cu) where it was diffuse (table 4.2). This lesion was also found after 16 and 32 days of exposure at 0.29 ± 0.02 (Plate 4.4) and 0.47 ± 0.04 mg/l Cu and at 0.11 ± 0.02 mg/l Cu treatment after 32 and 64 days of exposure (table 4.2).

Table 4.2 Number of *O. mossambicus* showing the extent and severity of hepatic fatty degeneration during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity					
		Multi-focal			Diffuse		
		Minimal	Moderate	Marked	Minimal	Moderate	Marked
4	0.11 ± 0.02						
	0.29 ± 0.02						
	0.47 ± 0.04					4	
16	0.11 ± 0.02						
	0.29 ± 0.02	2			2		
	0.47 ± 0.04					3	1
32	0.11 ± 0.02	4	1				
	0.29 ± 0.02		1	1	1	2	
	0.47 ± 0.04						4
64	0.11 ± 0.02	4	3				
	0.29 ± 0.02					1	3

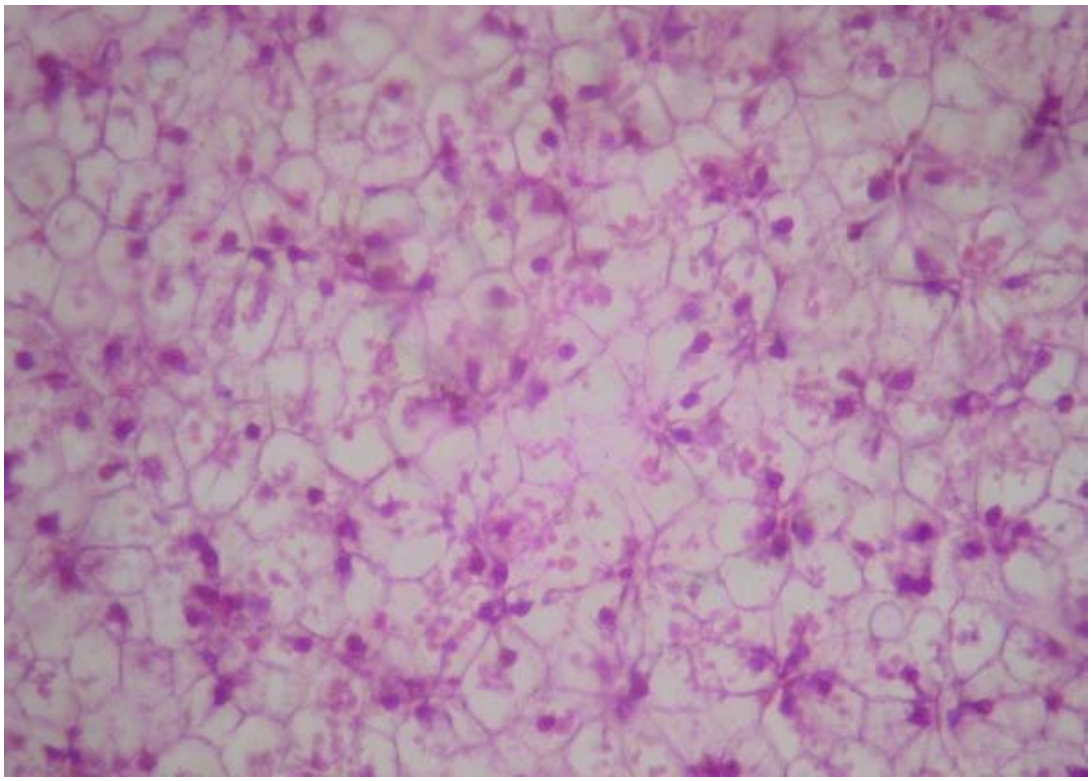


Plate 4.4 Section of a liver from a fish exposed to 0.29 ± 0.02 mg/l Cu for 32 days showing diffuse fatty degeneration of hepatocytes ($\times 400$).

4.3.2.3 Hepatic necrosis

Hepatic necrosis was first observed in fish from 0.47 ± 0.04 mg/l Cu treatment after 4 days of exposure when it was diffuse and minimal but became diffuse and marked after 16 days of exposure (Plate 4.5) and diffuse and severe after 32 days of exposure (Plate 4.6). Hepatic necrosis was only manifested in fish exposed to 0.29 ± 0.02 mg/l Cu after 32 and 64 days. This lesion was not observed after exposure to 0.11 ± 0.02 mg/l Cu throughout the exposure period (table 4.3).

Table 4.3 Number of *O. mossambicus* showing the extent and severity of hepatic necrosis during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity						
		Multi-focal			Diffuse			
		Minimal	Moderate	Severe	Minimal	Moderate	Marked	Severe
4	0.11 ± 0.02							
	0.29 ± 0.02							
	0.47 ± 0.04				3		1	
16	0.11 ± 0.02							
	0.29 ± 0.02							
	0.47 ± 0.04					1	3	
32	0.11 ± 0.02							
	0.29 ± 0.02	1	1	1			2	
	0.47 ± 0.04							4
64	0.11 ± 0.02							
	0.29 ± 0.02						1	3

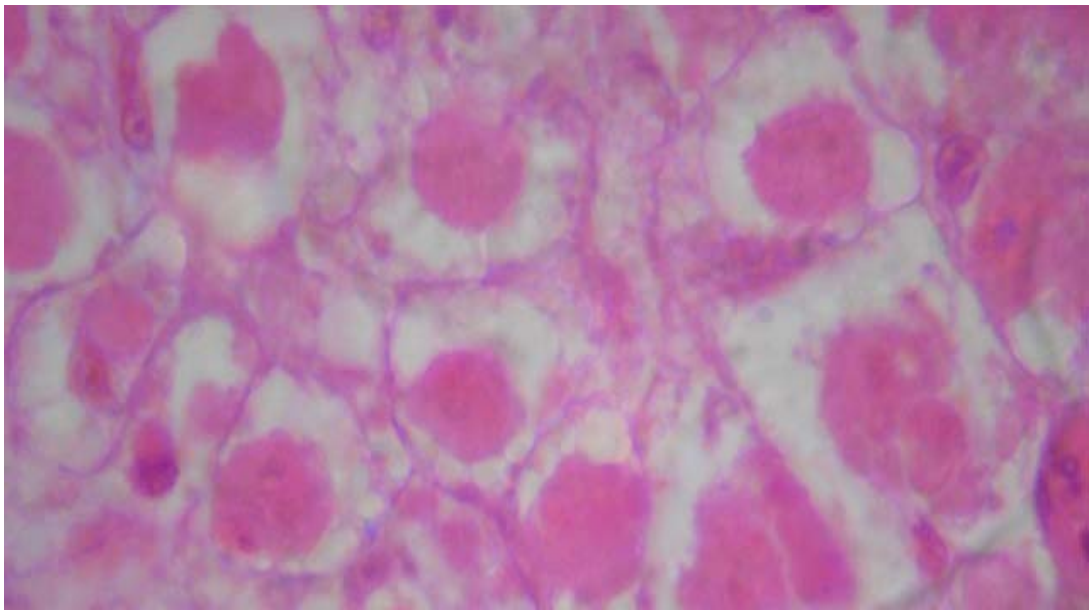


Plate 4.5 Section of a liver from a fish exposed to 0.47 ± 0.04 mg/l Cu for 16 days showing diffuse and marked necrosis of hepatocytes ($\times 400$).

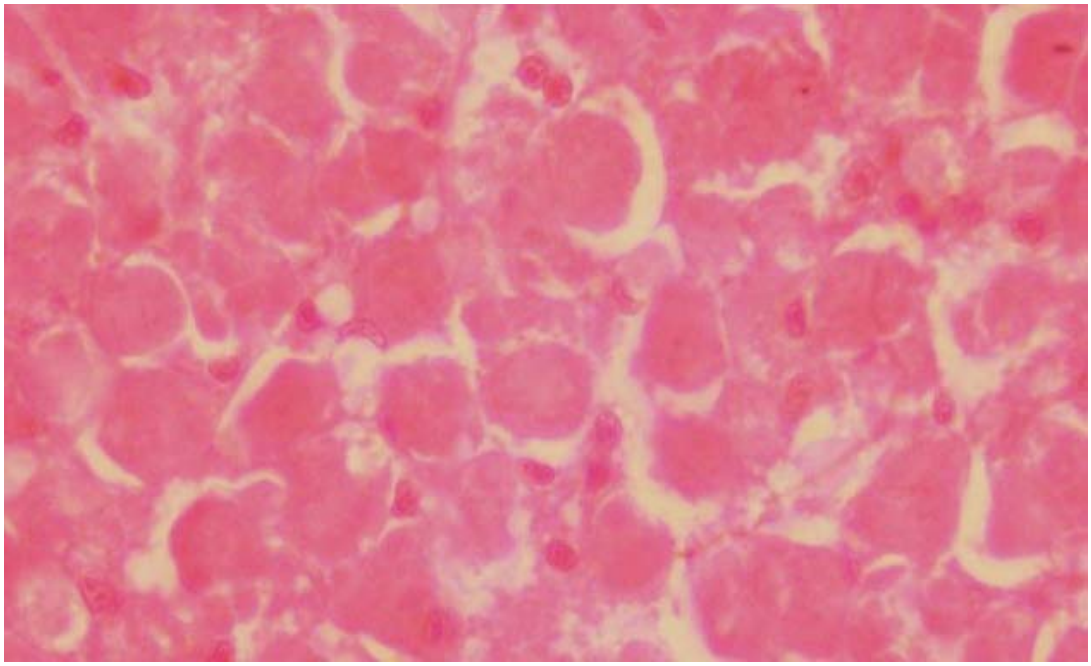


Plate 4.6 Section of a liver from a fish exposed to 0.47 ± 0.04 mg/l Cu for 32 days showing diffuse and severe necrosis of hepatocytes ($\times 400$).

4.3.2.4 Pancreatic and other hepatic changes

Diffuse and minimal hepatic atrophy was observed in two fish i.e. one fish after 2 days and another after 16 days of exposure. Aggregates of macrophages, venous congestion, pycnosis and karyorhexis of the nucleus were observed. Aggregates of macrophages and venous congestion occurred intermittently and pycnosis and karyorrhexis could only be appreciated at $\times 1000$ magnification when a small section was in view, thus these lesions were not quantified.

The pancreatic tissue appeared around the hepatic portal vein. In the control, pancreatic acinar cells were prominent with a dark basophilic cytoplasm containing secretory granules. Pancreatic fatty degeneration and necrosis were first observed after 2 days and 16 days of exposure, respectively. The extent and severity of these lesions increased with increase in exposure time and Cu concentration in the water (tables 4.4 and 4.5).

Table 4.4 Number of *O. mossambicus* showing pancreatic necrosis during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity		
		Diffuse		
		Moderate	Marked	Severe
16	0.11 ± 0.02			
	0.29 ± 0.02			
	0.47 ± 0.04	1	3	
32	0.11 ± 0.02			
	0.29 ± 0.02	2		2
	0.47 ± 0.04			4
64	0.11 ± 0.02			
	0.29 ± 0.02			4

Table 4.5 Number of *O. mossambicus* showing pancreatic fatty degenerative changes during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions and blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity						
		Multi-focal			Diffuse			
		Minimal	Marked	Severe	Minimal	Moderate	Marked	Severe
2	0.11 ± 0.02							
	0.29 ± 0.02							
	0.47 ± 0.04		1					
4	0.11 ± 0.02	3						
	0.29 ± 0.02		1	2			1	
	0.47 ± 0.04				1		3	
16	0.11 ± 0.02							
	0.29 ± 0.02					2	2	
	0.47 ± 0.04						4	
32	0.11 ± 0.02					2	1	
	0.29 ± 0.02							4
	0.47 ± 0.04							
64	0.11 ± 0.02					1	3	
	0.29 ± 0.02			1			1	1

4.4 Discussion

Lesions were observed in the livers of all fish exposed to Cu irrespective of the Cu level. However, the sequential appearance of lesions in the order of hepatic vacuolar degeneration, fatty degeneration and necrosis indicated a gradual increase in damage with larger duration of exposure time and increasing Cu concentration. These findings agree with Usha Rani and Ramamurthi (1989) who observed an increase in pathological changes of the liver with time for the first 15 days into the study in *O. mossambicus* exposed to cadmium chloride. As only one CdCl treatment was used the

study did not produce a concentration response relationship. Unlike in the present study, Usha Rani and Ramamurthi (1989) observed acclimation of the fish to CdCl by observing similar lesion in the fish exposed for 15 and 30 days. As the same species was tested, different pollutants manifested differently.

The initial lesions observed during the present study demonstrate the physiological changes that took place in the liver tissue in the process of trying to homeostatically regulate and detoxify the metal during continuous exposure. The mechanism by which Cu brought about these changes is beyond the scope of this study. The first lesion in all Cu treatments from 1-day-exposure onwards was vacuolar degeneration, suggesting liver hyperfunction as an initial response to Cu poisoning. Increase in liver colloidal proteins is important in the detoxification and homeostasis of metals by sequestering the metals and preventing them from binding and inhibiting critical homeostatic proteins (Kägi and Schäffer 1988). This could explain the slow increase in Cu accumulation in the liver after 1 and 32 days of exposure (chapter 3). These findings agree with Sultan and Khan (1983) who observed partial and complete vacuolation of hepatocytes in goldfish, *Carassius auratus* after acute (24 hour exposure to 1 mg/l) and chronic (25 day exposure to 0.1 mg/l) exposure to copper sulphate. Accumulation of glycogen in fish exposed to organic toxicants can also cause liver cell vacuolation (Metcalfe 1998).

As the exposure continued, the homeostatic mechanism appears to have failed to cope with the increasing metal burden. The liver became hypofunctional and hepatocytes eventually became necrotic. Hypofunction demonstrated by fatty degeneration and early stages of necrosis could be related to the damage to cellular organelles like mitochondria. For example, Benedetti *et al*. (1989) showed an increase in cytoplasmic granules and succinatedehydrogenase activity, a mitochondrial enzyme, after a 40-day exposure of bullhead, *Ictalurus nebulosus* to 0.3 mg/l Cu. The authors attributed the mobilization of this enzyme to damage to the mitochondrial membrane.

Considering that the liver serves a number of functions related to different physiological activities for example vitellogenin synthesis, the reticulo-endothelial and imunoglobulin system, bile secretion, and storage of lipids and carbohydrates, one can speculate that these functions are likely to be affected by liver hypofunction and

cell death. The pancreatic degenerative changes may lead to pancreatic insufficiency of both the exocrine and endocrine system consequently impacting on the liver lesions. For example, the conversion of glycogen to glucose, which occurs in the liver and is stimulated by glucagon from the endocrine pancreas is likely to be affected. The digestive function is likely to be affected given that the acinar pancreatic tissue is a highly active exocrine organ producing the active digestive enzymes lipase and amylase, and trypsinogen and chymotrypsinogen which are activated in the intestines (Roberts 1989).

The presence of macrophage aggregates in the liver is considered a generalized non-specific marker of environmental stress (Metcalfe 1998), which includes exposure to organic chemicals, thus explaining why macrophage centres were observed in the liver of *O. mossambicus* exposed to Cu.

In conclusion, lesions were observed in the liver in all Cu treatments regardless of exposure time. The incidence and degree of alteration was related to the concentration of Cu in the water and exposure time. Sequential histopathological changes indicated an initial liver hyperfunction followed by hypofunction and consequently cell death. The hyperfunction explains the liver's ability to homeostatically regulate and detoxify Cu. However, the lesions observed during this study were non-specific thus it would be difficult to derive a conclusive diagnosis in cases of Cu toxicity to fish using liver histological procedures unless the lesions are compared to Cu accumulation in the tissues and history of Cu exposure.

CHAPTER 5

The histopathology of copper in the spleen of *Oreochromis mossambicus*

5.1 Introduction

The spleen is a primary haemopoietic organ and serves as a temporary blood bank (Takashima 1982, Fänge 1992). This organ is the main site of destruction of senescent erythrocytes and other blood cells (Ashley 1975, Fänge and Nilsson 1985). The spleen is also an important immune organ containing plasma cells and phagocytes and has a blood filtering, antigen-capturing function (Fänge 1992). Macrophages have been reported to digest phagocytosed erythrocytes leading to the deposition of haemosiderin with melanomacrophages (Fänge and Nilsson 1985). Under physiological conditions, the amount of haemosiderin is usually small but when large quantities are deposited they lead to a condition known as haemosiderosis (Takashima 1982).

Pollutants have been associated with suppression of the immune system of fishes by acting on critical physiological pathways (Anderson *et al*. 1989, Holladay *et al*. 1996). For example Cu suppressed antibody-producing cells in rainbow trout (*Oncorhynchus mykiss*) spleen sections exposed to CuCl *in vitro* (Anderson *et al*. 1989). The host defence mechanism against pathogens in fish and other animals involves humoral and cell-mediated immunity (Fänge 1992). Macrophages are an important part of the cellular immune system of fish as they protect the host by phagocytosing foreign material and are frequently found in discrete groups within the haematopoietic and some other soft tissues of teleost fish (Ellis 1989, Ellis *et al*. 1989, Fänge 1992). The density of splenic macrophage aggregates in fish positively correlated with high concentrations of sediment contaminants (Fournie *et al*. 2001). Similar findings were observed in the pronephros of fish (Holladay *et al*. 1996). A decrease in pronephros macrophage chemotactic response has been related to pollutant exposure (Weeks *et al*. 1986, Holladay *et al*. 1996).

In teleosts the spleen consists of the erythropoietic red pulp and the lymphoid white pulp (Fänge 1992). The boundaries of these tissues are diffuse (Fänge 1992). Increase in size and number of melanomacrophage centres, expansion of the splenic white pulp and a decrease in lymphocytes were observed in *Oreochromis niloticus* dosed intraperitoneally with 50.0 mg/kg azathioprine, an anti-neoplastic drug and therapeutic immunosupressant (Gogal *et al*. 1999). However, a normal appearance of the spleen was observed in fish dosed with 10 mg/kg of this drug.

The histopathology of the spleen due to exposure to chemicals is not as well investigated as that of the gills and liver. The spleen being a haemopoietic and important immune organ is bound to be affected by chemical pollutants absorbed into the blood stream. The aim of this study was to investigate the effect of Cu on the histology of this organ. Findings can be used as a reference for further studies. The objective of this study was to investigate the effect of elementary Cu on the histology of the spleen in *O. mossambicus* following a short and long-term exposure and to relate Cu concentration and exposure duration to the extent and severity of histological damage.

5.2 Materials and methods

Details of the experimental system and experimental design are given in the general methods (chapter 2). In the short-term acute study, the fish were exposed to 0 mg/l (control) and 0.75 ± 0.20 mg/l Cu for 96 hours. The long-term study involved exposing fish to three Cu levels, including a control with no addition of Cu, and three treatments with 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l Cu. The spleens were collected after 1, 2, 4, 16, 32 and 64 days, respectively and preserved in bouin's solution for histological analysis. Details of histological analysis are given in materials and methods (chapter 4). At the highest Cu concentration there were no samples at day 64 as all the fish had died.

For microscopic observation and data presentation, a lesion/condition was classified according to the extent, depending on the area of the tissue affected. Lesions were either multi-focal where a lesion occurred in several localized areas and diffuse where a lesion was manifested in the whole tissue. The extent of the lesion was classified

according to severity as minimal, moderate, marked and severe whereby minimal represented lesions that affected the tissue to a very low degree and severe represented the highest degree of the lesion. The severity of macrophage centers and haemosiderosis involved assessment of the relative density of these lesions in the whole tissue section. Micrographs of the tissues were taken using a JVC digital still camera (GC-X3).

5.3 Results

The spleen from the control fish displayed a relatively normal histological structure with readily distinguishable red and white pulp scattered through the tissue sections (Plate 5.1). A common feature of the red pulp was the presence of capillary ellipsoids and erythrocytes. The white pulp consisted mainly of lymphocytes and stromal elements. A few scattered macrophages and organized macrophage centers were observed. Minimal haemosiderin also occurred in some parts of the red pulp.

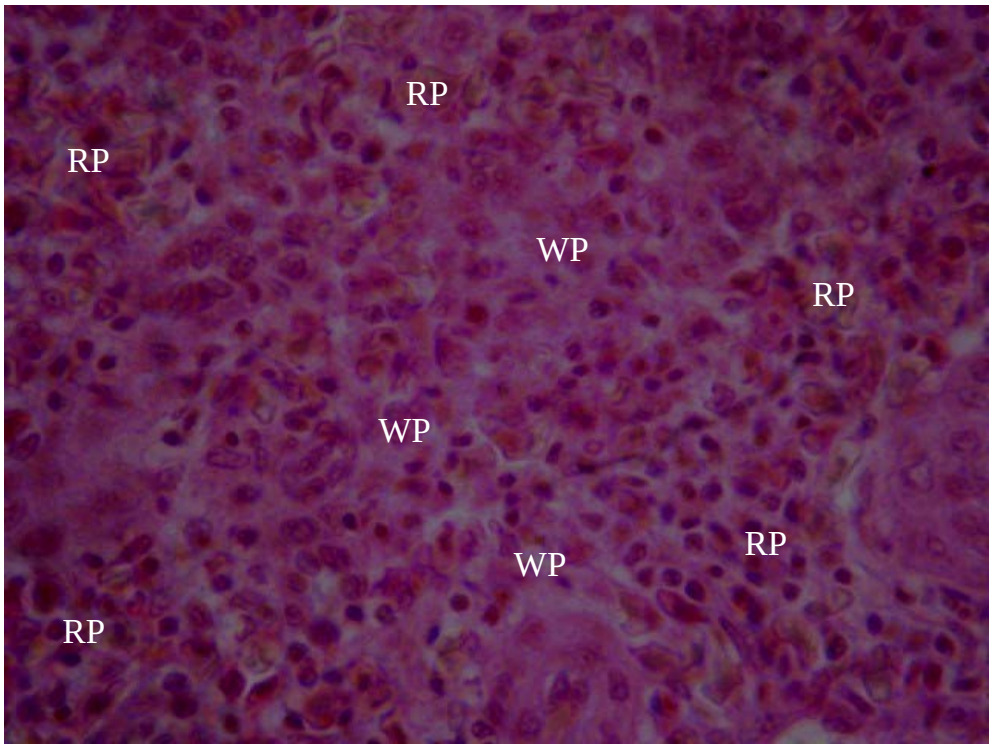


Plate 5.1 Section of a control spleen (×1000) from the control treatment showing the red pulp (RP) and the white pulp (WP).

5.3.1 Lesions observed after the short-term study

After the short-term Cu exposure, the spleen showed severe haemosiderosis and fatty infiltration of the parenchyma. There were moderate to severe necrotic eosinophilic cells (Plate 5.2) and necrosis of the splenic capsule.

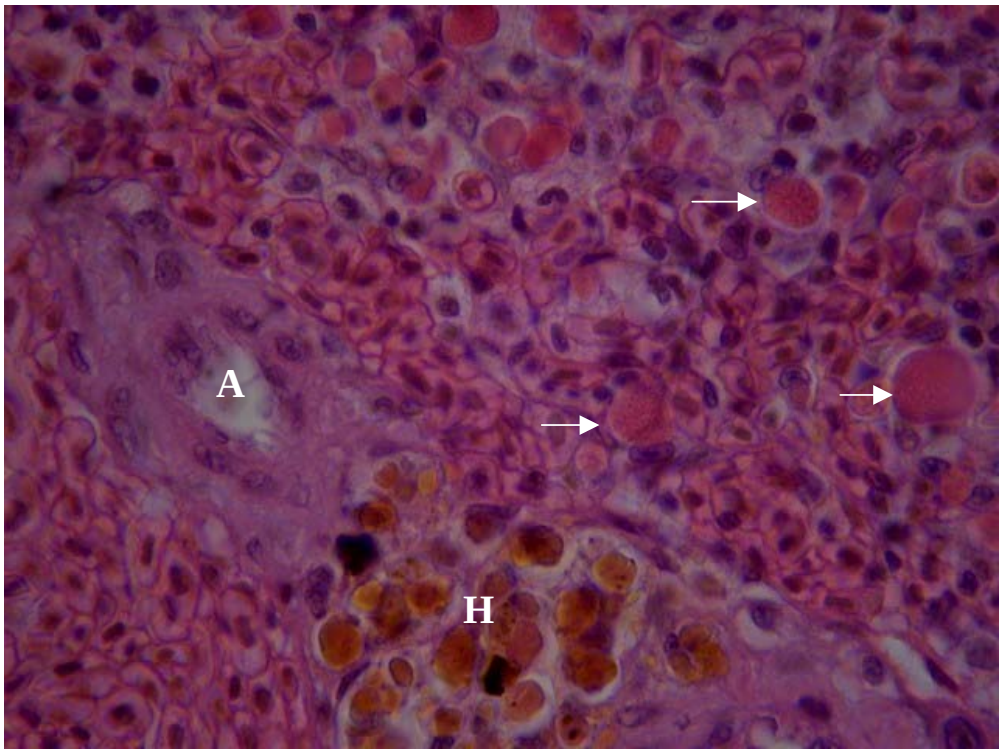


Plate 5.2 Section of a spleen (×1000) from a fish exposed to 0.75 ± 0.20 mg/l Cu for 96 hours showing eosinophilic necrosis (arrows). A = splenic artery and H = haemosiderosis.

5.3.2 Lesions observed during the long-term exposure

Changes resulting from long-term exposure comprised minimal to severe haemosiderosis and macrophage centers, expansion of the white pulp, decrease in the red pulp, venous congestion, vacuolation and necrosis. The extent and severity of these lesions increased with increasing exposure time and Cu concentration.

5.3.2.1 Haemosiderosis and macrophage centers

Haemosiderosis and macrophage centers appeared multi-focally in spleens of all Cu-exposed fish. In the 0.11 ± 0.02 Cu treatment haemosiderosis was marked after 4 days

of exposure and became moderate in some samples thereafter (table 5.1). This condition became more severe with an increase in Cu concentration and duration of exposure at 0.29 ± 0.02 (Plate 5.3) and 0.47 ± 0.04 mg/l Cu, respectively (table 5.1).

Table 5.1 Number of *O. mossambicus* showing the extent and severity of splenic haemosiderosis during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity			
		Multi-focal			
		Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02	4			
	0.29 ± 0.02	1	3		
	0.47 ± 0.04	1		3	
2	0.11 ± 0.02	4			
	0.29 ± 0.02	2		2	
	0.47 ± 0.04	2		2	
4	0.11 ± 0.02			4	
	0.29 ± 0.02			4	
	0.47 ± 0.04			1	3
16	0.11 ± 0.02		2	1	
	0.29 ± 0.02				4
	0.47 ± 0.04				4
32	0.11 ± 0.02		3	1	
	0.29 ± 0.02			3	1
	0.47 ± 0.04			1	3
64	0.11 ± 0.02		3		1
	0.29 ± 0.02			1	3

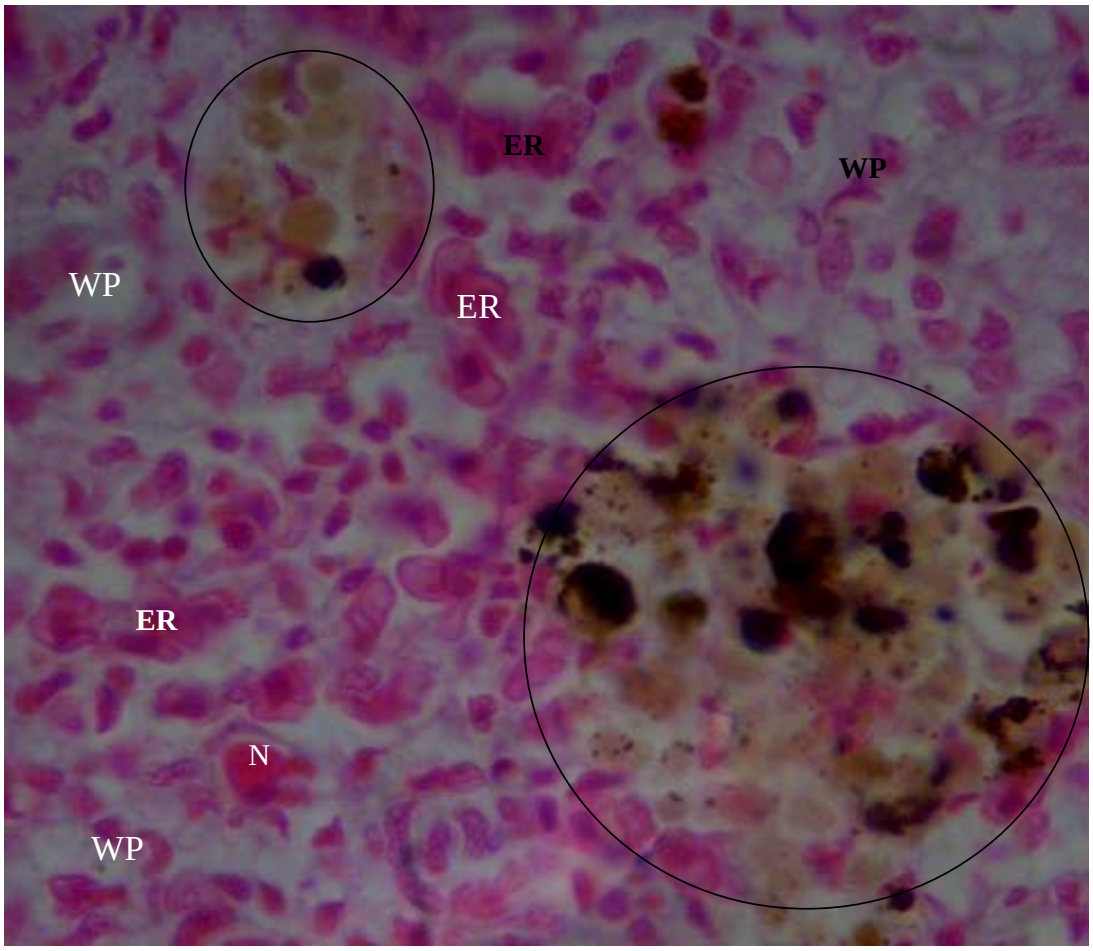


Plate 5.3 Section of a spleen (×1000) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 32 days showing macrophage centers with haemosiderin deposits (circles). Note the increase in the white pulp (WP) with few red pulp pockets of erythrocytes (ER), N = Necrotic cell.

Macrophage centers were larger and more numerous with increase in Cu concentration and exposure time. In the 0.11 ± 0.02 Cu treatment the centers were marked after 4 and 16 days of exposure and became moderate and minimal thereafter (table 5.2). Macrophage centers were in many cases closely attached to blood vessels and were found in association with clusters of lymphocytes.

Table 5.2 Number of *O. mossambicus* showing the extent and severity of macrophage centres in the spleen during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions and blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity			
		Multi-focal			
		Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02	4			
	0.29 ± 0.02	1	3		
	0.47 ± 0.04	1		3	
2	0.11 ± 0.02	4			
	0.29 ± 0.02	2		2	
	0.47 ± 0.04	2		2	
4	0.11 ± 0.02			4	
	0.29 ± 0.02			4	
	0.47 ± 0.04			1	3
16	0.11 ± 0.02	1	1	2	
	0.29 ± 0.02			2	2
	0.47 ± 0.04			2	2
32	0.11 ± 0.02	1	3		
	0.29 ± 0.02	1		2	1
	0.47 ± 0.04		1		3
64	0.11 ± 0.02	3		1	
	0.29 ± 0.02			1	3

5.3.2.2 Expansion of the white pulp

With increasing concentration the white pulp was greatly expanded and the red pulp decreased (Plate 5.4). Expansion of the white pulp was observed after 2 days at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu exposure but was only noticed after 4 days at 0.11 ± 0.02 Cu exposure and in this treatment the white and red pulp were more evenly distributed after 16 days of exposure (table 5.3). However in all the exposed treatments the number of mature lymphocytes did not seem to increase. In some cases mature lymphocytes were greatly reduced in number. Increase in exposure time did not seem to affect the expansion of the white pulp (table 5.3).

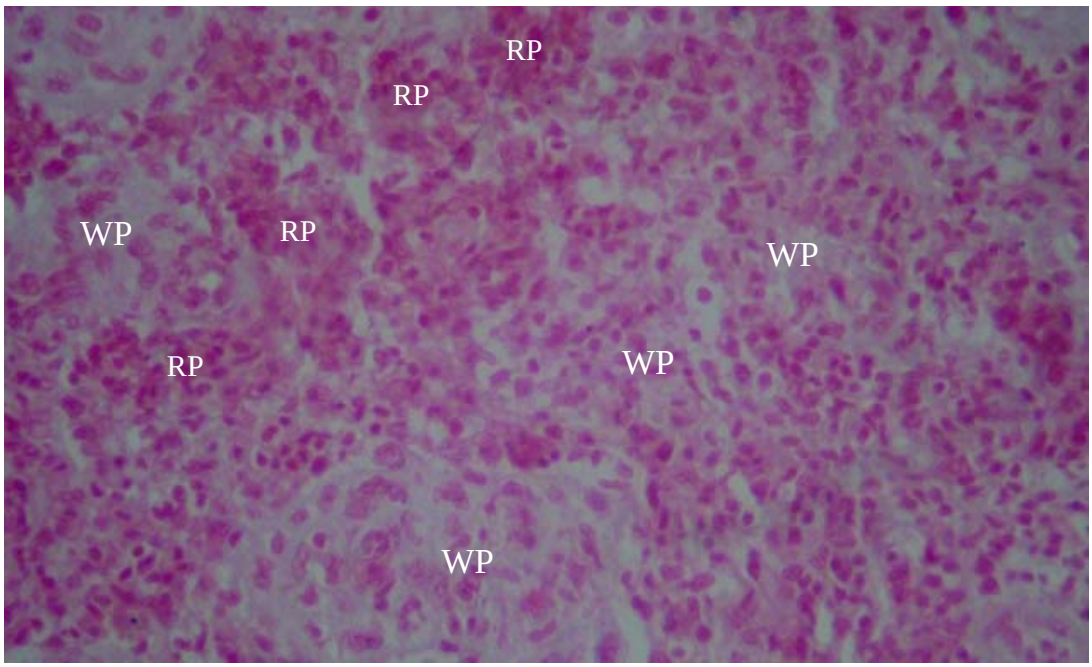


Plate 5.4 Section of a spleen (× 400) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 4 days showing expansion of the white pulp (WP) and a decrease in the red pulp (RP).

Table 5.3 Number of *O. mossambicus* showing the extent and severity of expansion of the splenic white pulp during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity					
		Multi-focal		Diffuse			
		Minimal	Moderate	Minimal	Moderate	Marked	Severe
2	0.11 ± 0.02						
	0.29 ± 0.02				3		
	0.47 ± 0.04				3		
4	0.11 ± 0.02	2	2				
	0.29 ± 0.02			4			
	0.47 ± 0.04					4	
16	0.11 ± 0.02						
	0.29 ± 0.02					4	
	0.47 ± 0.04			1			3
32	0.11 ± 0.02		2				
	0.29 ± 0.02				1	1	
	0.47 ± 0.04				2	2	
64	0.11 ± 0.02						
	0.29 ± 0.02					3	

5.3.2.3 Decrease in the red pulp

Decrease in the red pulp was first noticed after 4 days at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu exposure levels where it was diffuse. There was no trend of a decrease in the red pulp at the 0.11 ± 0.02 Cu exposure level over time (table 5.4).

Table 5.4 Number of *O. mossambicus* showing the extent and severity of reduction in the splenic red pulp during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity			
		Diffuse			
		Minimal	Moderate	Marked	Severe
4	0.11 ± 0.02				
	0.29 ± 0.02		4		
	0.47 ± 0.04			4	
16	0.11 ± 0.02				
	0.29 ± 0.02			4	
	0.47 ± 0.04	1		2	1
32	0.11 ± 0.02				
	0.29 ± 0.02			2	
	0.47 ± 0.04		2	2	
64	0.11 ± 0.02				
	0.29 ± 0.02			3	

5.3.2.4 Venous congestion and ellipsoid vacuolation

Venous congestion occurred irregularly in the control and the Cu exposed groups. Vacuolation occurred multi-focally in the ellipsoid sheath cells and the white pulp at 0.47 ± 0.04 mg/l Cu exposure level after 32 days of exposure when it was severe (Plate 5.5) and at 0.29 ± 0.02 mg/l Cu exposure after 32 and 64 days of exposure when it was marked.

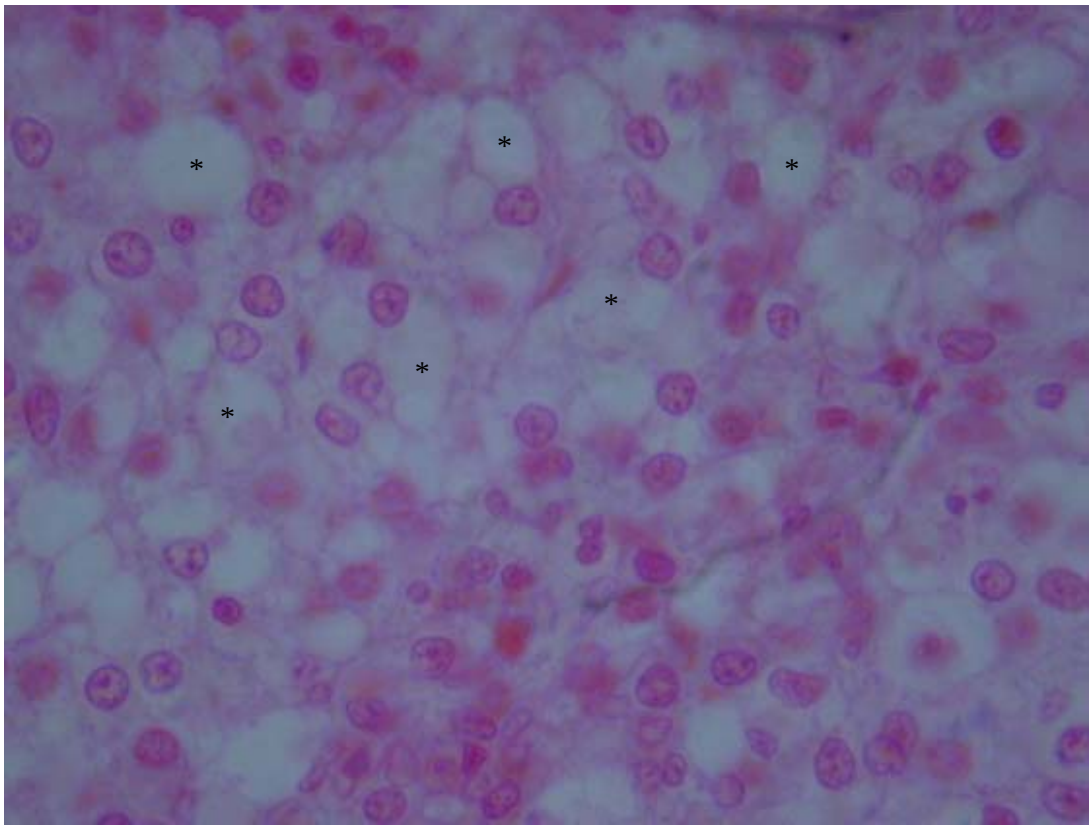


Plate 5.5 Section of a spleen (×1000) from a fish exposed to 0.47 ± 0.04 mg/l Cu for 32 days showing vacuolation (*).

5.3.2.5 Splenic necrosis

Necrosis was first observed in fish from the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu treatment after 16 days of exposure and became more severe with increase in Cu concentration and duration of exposure (Plates 5.6 and 5.7). This lesion was not manifested in the 0.11 ± 0.02 mg/l Cu treatment (table 5.5).

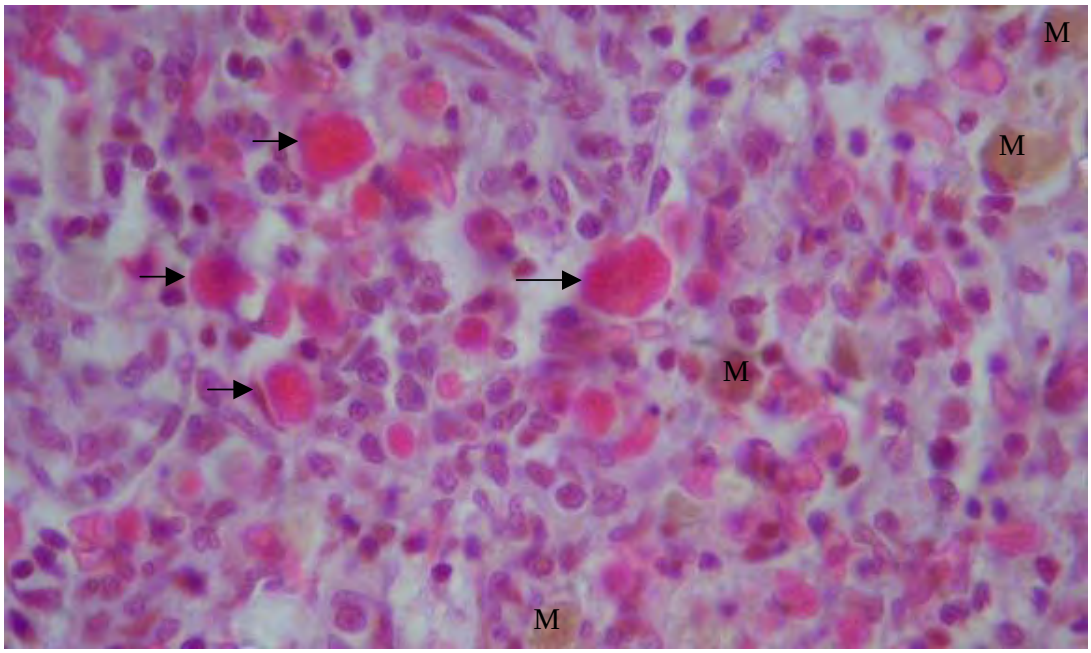


Plate 5.6 Section of a spleen (×1000) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 64 days showing eosinophilic necrosis (arrows). M = macrophages.

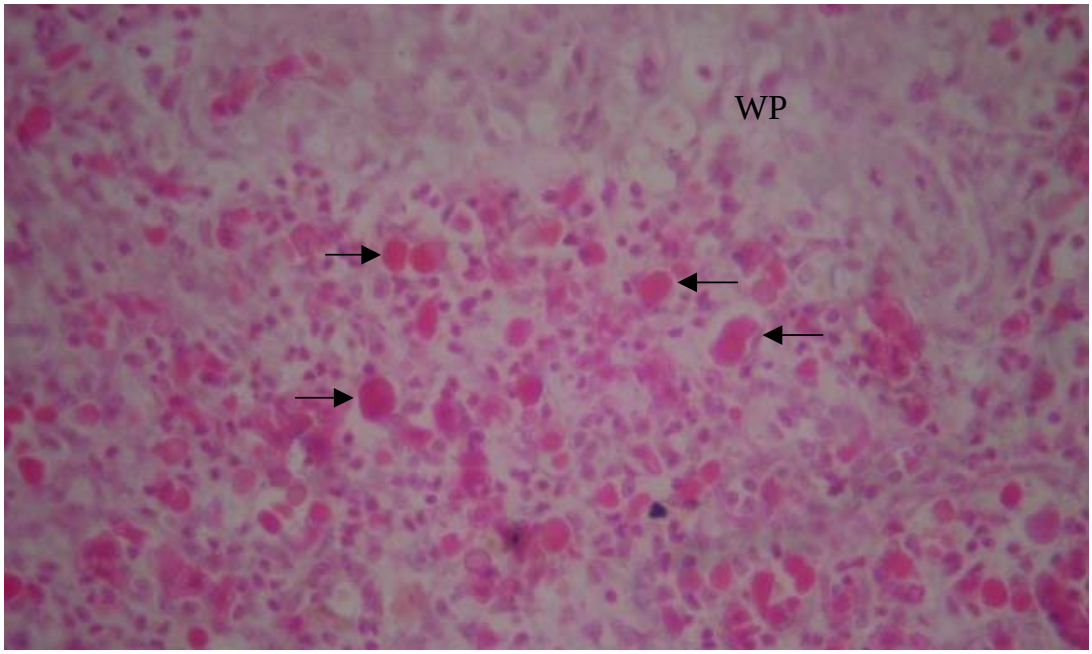


Plate 5.7 Section of a spleen from a fish exposed to 0.47 ± 0.04 mg/l Cu for 16 days showing eosinophilic necrosis (arrows), increase in the white pulp (WP) with few lymphocytes (×400).

Table 5.5 Number of *O. mossambicus* showing the extent and severity of splenic necrosis during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion extent and severity represent the number of fish with lesions and blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Lesion extent and severity				
		Multi-focal			Diffuse	
		Minimal	Moderate	Marked	Moderate	Marked
16	0.11 ± 0.02					
	0.29 ± 0.02	2				
	0.47 ± 0.04		1	2		1
32	0.11 ± 0.02					
	0.29 ± 0.02				4	
	0.47 ± 0.04				1	3
64	0.11 ± 0.02					
	0.29 ± 0.02				2	2

5.4 Discussion

Haemosiderosis was observed in the spleens of all fish exposed to copper and the severity of this condition increased with increasing copper concentration and longer exposure. This explains the decrease in the red pulp and may result in a decrease in the number of mature erythrocytes in the circulating blood leading to anaemia. Expansion of the white pulp with reduced mature lymphocytes reflected an increase in the white pulp stromal cells. Reduction in the number of mature lymphocytes may result in immunosuppression. This is supported by Anderson *et al*. (1989) who demonstrated immunosuppression in sections of rainbow trout (*Oncorhynchus mykiss*) spleens immunised *in vitro* and exposed in culture to different concentrations of copper chloride. The number of antibody producing cells in the immunized spleen sections was correlated with the concentration of Cu in the nutrient media. At the highest Cu concentration of 100 µg/ml no antibody producing cells were demonstrated. Anderson *et al*. (1989) could not relate their findings to histopathology. Gogal *et al*. (1999) observed an increase in splenic total cellularity and a decrease in lymphocytes and erythrocytes in *Oreochromis niloticus* dosed intraperitoneally with

50.0 mg/kg azathioprine. The present study showed acclimation in the lowest Cu level of 0.11 ± 0.02 mg/l Cu where histopathological changes were minimal to moderate even after 64 days of exposure. Gogal *et al*. (1999) observed normal pattern of the spleen in fish dosed with 10 mg/kg of azathioprine. These findings suggest that only fish exposed to environmentally relevant levels of pollutants may be histopathologically and immunologically affected. Mild haemosiderosis observed in the control represented granules produced after the degradation of senescent erythrocytes.

Increased severity in the macrophage centers indicate a homeostatic mechanism of the fish spleen to phagocytose the increasing deposits of haemosiderin and other debris resulting from the destruction of tissues as a result of exposure to Cu. This agrees with findings by Fournie *et al*. (2001) who associated the density of splenic macrophage aggregates in estuarine fishes to exposure to degraded environments. These findings further agree with Holladay *et al*. (1996) who observed an increased diameter of melanomacrophage centers in some sections of pronephros of *Oreochromis niloticus* exposed to 1 µg/l of chlorpyrifos, an organophosphate insecticide. As the present study dealt with the spleen and Holladay *et al*. (1996) worked on the pronephros, this comparison takes into account the fact that both organs have haematopoietic functions. Given that findings by Holladay *et al*. (1996), Fournie *et al*. (2001) and the present study are in agreement yet different chemical pollutants, fish species and organ tissues were involved, one can conclude that splenic macrophage aggregates are potentially effective biotic indicators of fish health and environmental degradation but that this condition does not define the cause of the degradation.

Macrophages and the white pulp rich in lymphocytes are important parts of the cellular immune system of fish (Ellis *et al*. 1989). Expansion of the white pulp and increase in the density of macrophage centers due to environmental degradation should be interpreted with caution because it may not imply a boost in the cellular immune system. Chemical pollutants may reduce the phagocytic activity of macrophages even when the cells are intact. For example, Holladay *et al*. (1996) observed depressed phagocytic function in macrophages isolated from the pronephros. Similar findings were observed by Weeks *et al*. (1986) who observed a decrease in pronephros macrophage chemotactic response related to pollutant exposure in fish

from Elizabeth river, a river whose sediments contained high levels of polynuclear aromatic hydrocarbons. The density of melanomacrophage centers in the pronephros could not be established since histopathology was not considered in this study. A differential leucocyte cell and Massons' trichrome stain revealed that the increase in the white pulp in *O. niloticus* dosed intraperitoneally with 50.0 mg/kg azathioprine was a result of fibrotic changes observed in the stromal cell compartment of the fish spleen rather than an increase in immune cell size or number (Gogal *et al*. 1999). Although expansion of the white pulp and an increase in the density of macrophage centers was observed in the present study immunosuppression could not be ruled out thus predisposing the fish to pathogenic and opportunistic organisms.

Vacuolation after 32 and 64 days of Cu exposure mainly in the ellipsoid sheath cells could imply increased activity of the ellipsoids. Ellipsoids function as valves or filters removing senescent blood cells and they are capable of trapping large quantities of particulate matter from circulation (Ellis 1989). Fänge and Nilsson (1985) reported that blood from the splenic arteries passes through the ellipsoids into the red pulp in two ways: through the vascular lumen or through the loose-texture ellipsoid sheath. With increased Cu concentration and duration of exposure the splenic cells started undergoing necrosis consequently reducing the number of active cells both in the spleen and in circulation which may lead to anaemia and / or immunosuppression.

In conclusion, these findings suggest that fish exposed to environmentally relevant levels of copper may be histopathologically and immunologically affected leading to anaemia and immunossupression. Thus, parameters such as blood cell differentials and the splenic ability to elicit immune responses after copper exposure need to be investigated. These criteria combined with histopathology may serve as better indicators of environmental exposure. Massons' trichrome stain should be combined with haematoxylin and eosin in order to appreciate the cellular and fibrotic changes observed in the stromal cell compartment.

CHAPTER 6

The histopathology of copper in the gills of *Oreochromis mossambicus*

6.1 Introduction

The gill is a multifunctional organ that engages in homeostatic activities such as osmoregulation, excretion of nitrogenous waste products, acid-base balance and respiration (Randall 1970, Hinton and Laurén 1990, Heath 1995, Iwama and Farrell 1998, Metcalfe 1998). The large surface area of the gill, its delicate structure and thin tissue barrier between the water and the fish's blood makes this organ a prime target for interaction with pollutants (Ellis *et al*. 1989, Iwama and Farrell 1998). The large surface area of the gills facilitates the movement of oxygen, carbon dioxide, electrolytes, ammonia and hydrogen ions between the blood and the water. Histological alterations in fish gills associated with a chemical and physical irritant include epithelial cell lifting, necrosis, hypertrophy, hyperplasia, and rupture of lamellar epithelium, lamellar fusion, clevate-globate lamellar (haemorrhage, aneurisms, telangiectasis), hypersecretion of mucous and proliferation of mucous cell and chloride cells and gill vasculature (Mallatt 1985). Cu has been reported to induce alterations in gill histology after acute (Kumar and Pant 1981, Sultan and Khan 1983, Daoust *et al*. 1984) and chronic (Sultan and Khan 1983, Benedetti *et al*. 1989, Nelson *et al*. 1999) exposure.

Histological alterations observed with light microscopy following acute and chronic exposure to Cu were consistent across many fish species. For example, enlarged lamellar epithelial cells and lamellar fusion were reported in rainbow trout, *Oncorhynchus mykiss* (Daoust *et al*. 1984) and *Puntius conchonius* (Kumar and Pant 1981) following acute exposure to $CuSO_4$. Increase in mucous excretion (Kumar and Pant 1981) and accumulation of cellular debris in the epithelium of lamellar and interlamellar regions and apoptotic bodies (Daoust *et al*. 1984) were also observed after acute exposure to $CuSO_4$. Vacuolation and necrosis of gill tissue, mucous cell hypertrophy and collapse of blood capillaries occurred in the gills of goldfish

Carassius auratus following acute exposure to $CuSO_4$ (Sultan and Khan 1983). Necrosis and disaggregation of the epithelium in the gills of brown bullhead, *Ictalurus nebulosus*, occurred following acute exposure to $CuSO_4$ (Benedetti *et al.* 1989). Chronic effects of Cu on the gills include necrosis of gill filaments, hyperplasia of the epithelium and lamellar fusion (Sultan and Khan 1983, Nelson *et al.* 1999). Derangement of the gill epithelium with focal necrosis was observed in brown bullhead *Ictalurus nebulosus* following long-term exposure to $CuCl_2$ (Benedetti *et al.* 1989).

However, despite the similarity of the lesions observed, under any given set of exposure conditions, each kind of gill lesion tends to vary in intensity (Mallatt 1985). The lamellae within a single fish may show different severity of alterations and different fish species may be affected to varying degrees (Mallatt 1985). Alterations are influenced by type and concentration of the irritant and exposure time (Mallatt 1985, Heath 1995). In most studies regarding Cu toxicity, the authors used Cu salts, $CuSO_4$ in particular and most of the histological lesions were induced by acute Cu exposure. No study was found that considered the effect of elementary Cu on fish histopathology. The objective of this chapter was to investigate the effect of elementary Cu on gill histology of *O. mossambicus* following a short and long-term exposure and to relate Cu concentration and exposure duration to the severity of histological damage.

6.2 Materials and methods

Details of the experimental system and experimental design are given in the general methods (chapter 2). For the short-term acute study, the fish were exposed to 0 mg/l (control) and 0.75 ± 0.20 mg/l copper for 96 hours. The long-term study involved exposing fish to three copper levels, including a control with no addition of copper, and three treatments with 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l copper. The gills were collected after 1, 2, 4, 16, 32 and 64 days, respectively and preserved in bouin's solution for histological analysis. Details of histological analysis are given in materials and methods (chapter 4). At the highest Cu concentration there were no samples at day 64 as all the fish had died.

For microscopic observation and data presentation, a lesion/condition was classified according to the severity as minimal, moderate, marked and severe. This involved assessment of the intensity of the lesions in the tissue section whereby minimal represented lesions that affected the tissue to a very low degree and severe represented the highest intensity of the lesion that could be observed. Micrographs of the tissues were taken using a JVC digital still camera (GC-X3).

6.3 Results

6.3.1 Control group

The gills of the control fish showed a relatively normal structural arrangement of the lamellae. The primary lamellae appeared normal with a central core of cartilage and chondrocytes extending from base to tip in variable thickness. The cores of the primary lamellae were filled with erythrocytes in pockets. The secondary lamellae were lined by simple squamous epithelial cells and they were filled with erythrocytes and pillar cells (Plates 6.1 and 6.2). The gill rakers appeared normal with stratified squamous epithelium and a few cuboidal mucous cells.

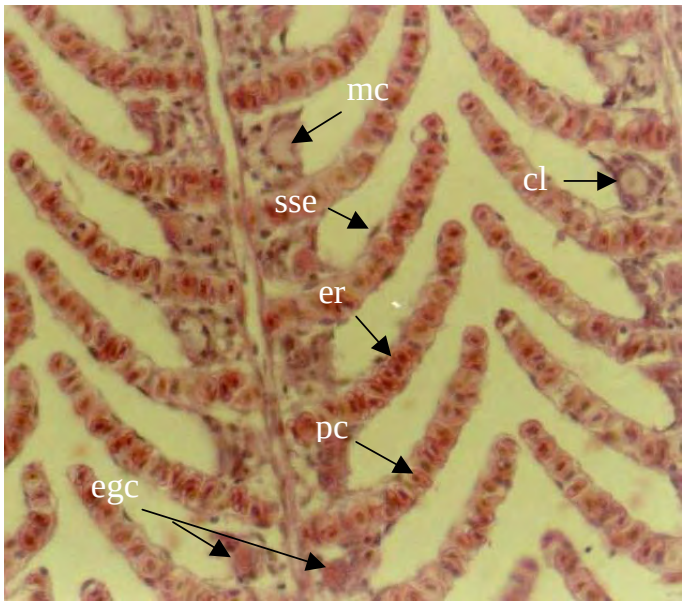


Plate 6.1 Section of a control gill from the control treatment showing the primary lamellae (× 400). pc = pillar cell, er = erythrocyte, sse = simple squamous epithelium, cl = chloride cell, mc = mucous cell, egc = eosinophilic granule cells

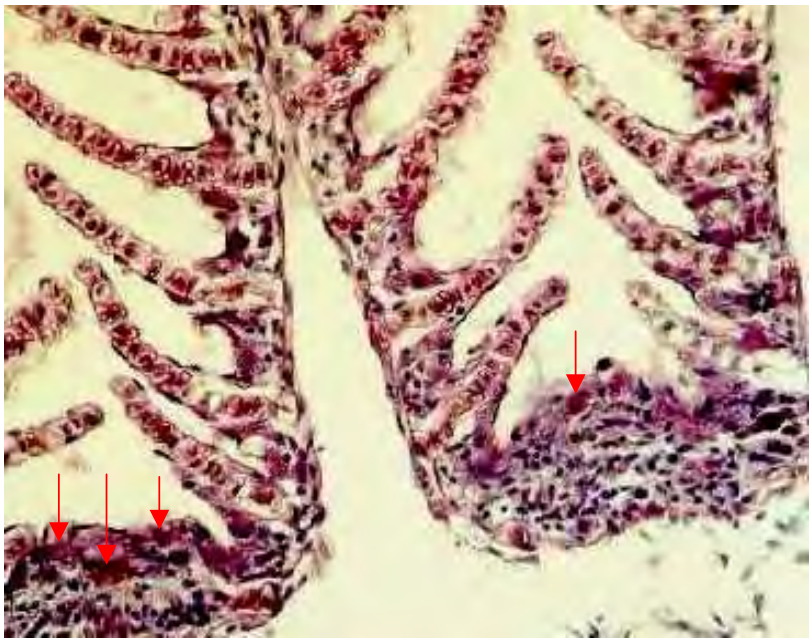


Plate 6.2 Section of a control gill from the control treatment showing the base of the primary lamellae with a few eosinophilic granule cells (arrows), $\times 400$.

6.3.2 Short-term study

After the short-term study the gills had their primary lamellae fused to one another either partially or completely as a result of hyperplasia of the epithelium and interlamellar cells. In some areas the secondary lamellae had atrophied. Mucous exudate had coated the tips of the secondary lamellae welding them together (Plate 6.3). There was intense infiltration of eosinophilic granule cells at the base of the primary lamellae and less intense along the length of the primary lamellae. The stratified squamous epithelium of the gill rakers had degenerated and showed intracellular oedema (Plate 6.4).

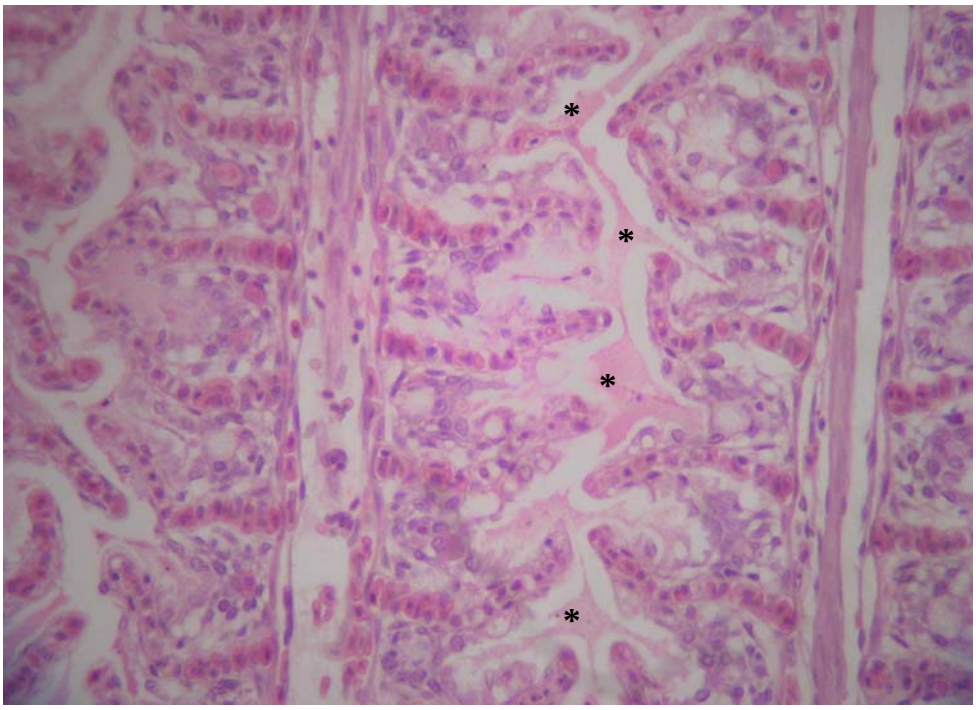


Plate 6.3 Section of a gill from a fish exposed to 0.75 ± 0.20 mg/l Cu for 96 hours (× 400) showing hyperplasia of the epithelium and interlamellar cells as well as mucous exudate (*).

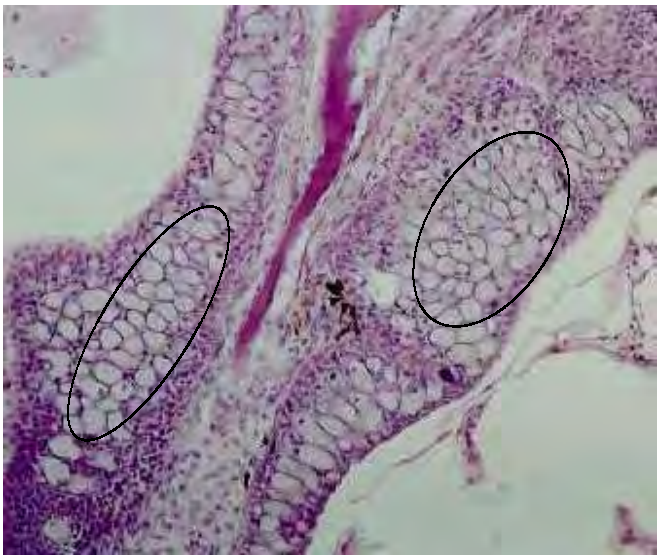


Plate 6.4 Section of a gill (× 400) from a fish exposed to 0.75 ± 0.20 mg/l Cu for 96 hours showing intracellular oedema in the stratified squamous epithelial cells of a gill raker (circles).

6.3.3 Long-term study

Histological changes following long-term exposure for up to 64 days comprised hypertrophy and hyperplasia of mucous cells, hypersecretion of mucous, hyperplasia of eosinophilic granule cells, hypertrophy and hyperplasia of the simple squamous epithelial cells, hyperplasia of interlamellar cells, lamellar oedema, epithelial cell lifting and telangiectasis. The severity and progression of the changes was most pronounced at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels.

6.3.3.1 Hyperplasia and hypertrophy of mucous cells

Hyperplasia and hypertrophy of mucous cells (Plate 6.5) appeared moderately in all treatments within the first 4 days of exposure and they were not observed after 64 days of exposure (tables 6.1 and 6.2).

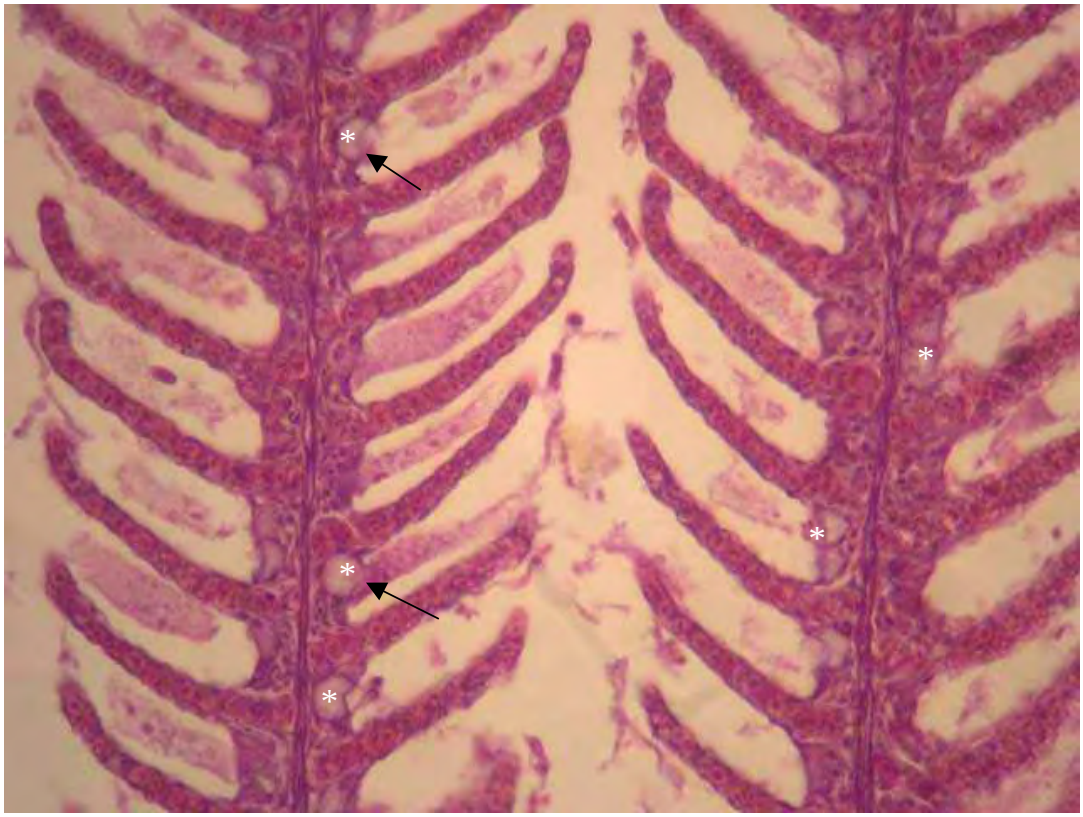


Plate 6.5 Section of a gill (× 400) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 1 day during the long-term study showing hypertrophied mucous cells (*****). Some of the mucous cells are discharging mucous (arrows).

Table 6.1 Number of *O. mossambicus* showing the severity of mucous cell hypertrophy in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions and blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Severity of the lesion		
		Minimal	Moderate	Marked
1	0.11 ± 0.02	2	2	
	0.29 ± 0.02		2	
	0.47 ± 0.04	2	2	
2	0.11 ± 0.02		4	
	0.29 ± 0.02	1	3	
	0.47 ± 0.04		4	
4	0.11 ± 0.02		3	1
	0.29 ± 0.02		4	
	0.47 ± 0.04		4	
16	0.11 ± 0.02			
	0.29 ± 0.02		4	
	0.47 ± 0.04			
32	0.11 ± 0.02			
	0.29 ± 0.02			2
	0.47 ± 0.04			

Table 6.2 Number of *O. mossambicus* showing the severity of mucous cell hyperplasia in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Severity of the lesion		
		Minimal	Moderate	Marked
2	0.11 ± 0.02			
	0.29 ± 0.02		1	
	0.47 ± 0.04		4	
4	0.11 ± 0.02		4	
	0.29 ± 0.02		4	
	0.47 ± 0.04		4	
16	0.11 ± 0.02			
	0.29 ± 0.02		4	
	0.47 ± 0.04			
32	0.11 ± 0.02			
	0.29 ± 0.02			
	0.47 ± 0.04			1

6.3.3.2 Mucous exudate

Mucous exudate appeared moderately in all Cu treatments within the first four days of exposure (table 6.3). This condition was minimal at 0.11 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels, and severe at 0.29 ± 0.02 mg/l Cu level (Plate 6.6) after 16 days of exposure and was not observed after 64 days of exposure (table 6.3).

Table 6.3 Number of *O. mossambicus* showing the severity of mucous exudate in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment (mg/l)	Severity of the lesion		
		Minimal	Moderate	Marked
1	0.11 ± 0.02	1	3	
	0.29 ± 0.02		3	2
	0.47 ± 0.04	2	2	
2	0.11 ± 0.02		4	
	0.29 ± 0.02		2	2
	0.47 ± 0.04		4	
4	0.11 ± 0.02		3	1
	0.29 ± 0.02		4	
	0.47 ± 0.04		4	
16	0.11 ± 0.02	4		
	0.29 ± 0.02		1	3
	0.47 ± 0.04	1		
32	0.11 ± 0.02			
	0.29 ± 0.02		2	
	0.47 ± 0.04			

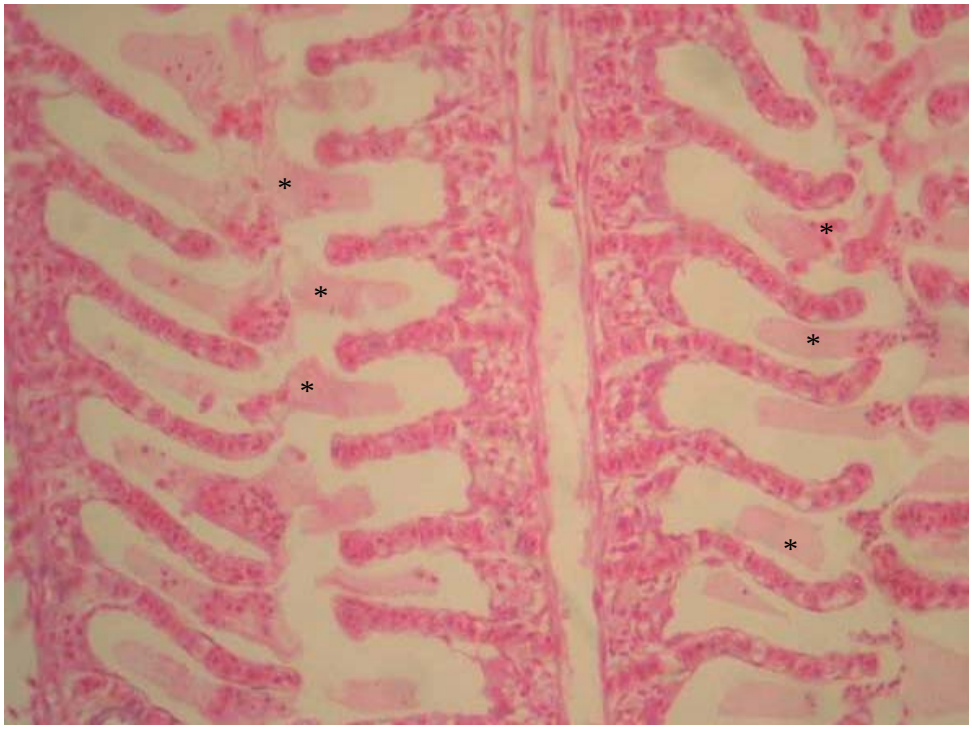


Plate 6.6 Section of the gill ($\times 400$) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 16 days showing mucous exudate (*) and moderate interlamellar hyperplasia.

6.3.3.3 Hyperplasia of the eosinophilic granule cells

Hyperplasia of the eosinophilic granule cells was mainly observed at the base of the primary lamellae and to a lesser extent along the length of the primary lamellae. Hyperplasia of these cells was observed at 0.11 ± 0.02 and 0.29 ± 0.02 mg/l Cu throughout the entire exposure duration (table 6.4). This condition was marked after 16 days of exposure at 0.29 ± 0.02 mg/l Cu (Plate 6.7). The eosinophilic granule cells at the 0.47 ± 0.04 mg/l Cu level were necrotic after 16 days of exposure. The severity of changes in these cells increased with increase in Cu concentration and duration of exposure.

Table 6.4 Number of *O. mossambicus* showing the severity of hyperplasia of the eosinophilic granule cells in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment	Severity of the lesion			
		Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02	2	1		
	0.29 ± 0.02	2	1	1	
	0.47 ± 0.04		4		
2	0.11 ± 0.02	2	1		
	0.29 ± 0.02		2	1	
	0.47 ± 0.04		2	2	
4	0.11 ± 0.02		2		
	0.29 ± 0.02		2	2	
	0.47 ± 0.04		2	2	
16	0.11 ± 0.02	2			
	0.29 ± 0.02		1	3	
	0.47 ± 0.04				
32	0.11 ± 0.02		2	1	
	0.29 ± 0.02			3	1
	0.47 ± 0.04				
64	0.11 ± 0.02		2		
	0.29 ± 0.02				4

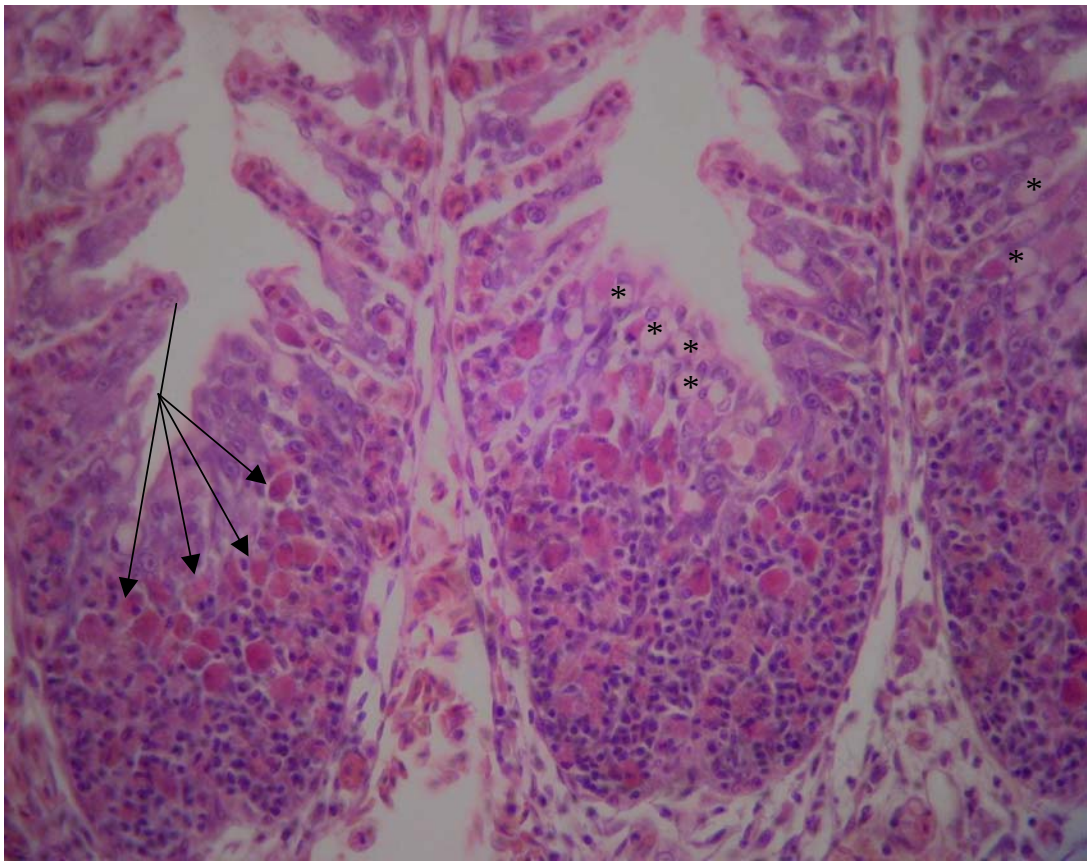


Plate 6.7 Section of a gill (×400) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 16 days during the long-term study showing the base of the primary lamellae with intense infiltration of eosinophilic granule cells (arrows) and hyperplasia of mucous cells (*).

6.3.3.4 Epithelial cell hypertrophy

Epithelial cell hypertrophy was related to Cu concentration and duration of exposure as it was more pronounced at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu where it was observed after 1 day of exposure (table 6.5, Plate 6.8). The severity increased with an increase in exposure duration. At the highest Cu level, the epithelial cells had started undergoing necrosis by day 32. In some fish, the lamellae manifested different severity of this lesion.

Table 6.5 Number of *O. mossambicus* showing the severity of gill epithelial cell hypertrophy during the long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment	Severity of the lesion			
		Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02				
	0.29 ± 0.02		4		
	0.47 ± 0.04		4		
2	0.11 ± 0.02	3			
	0.29 ± 0.02		4		
	0.47 ± 0.04		4		
4	0.11 ± 0.02	4			
	0.29 ± 0.02		4		
	0.47 ± 0.04		4	2	
16	0.11 ± 0.02		2	2	
	0.29 ± 0.02		1	3	
	0.47 ± 0.04		4	4	
32	0.11 ± 0.02	2	2		
	0.29 ± 0.02			4	
	0.47 ± 0.04				4
64	0.11 ± 0.02	4			
	0.29 ± 0.02				4

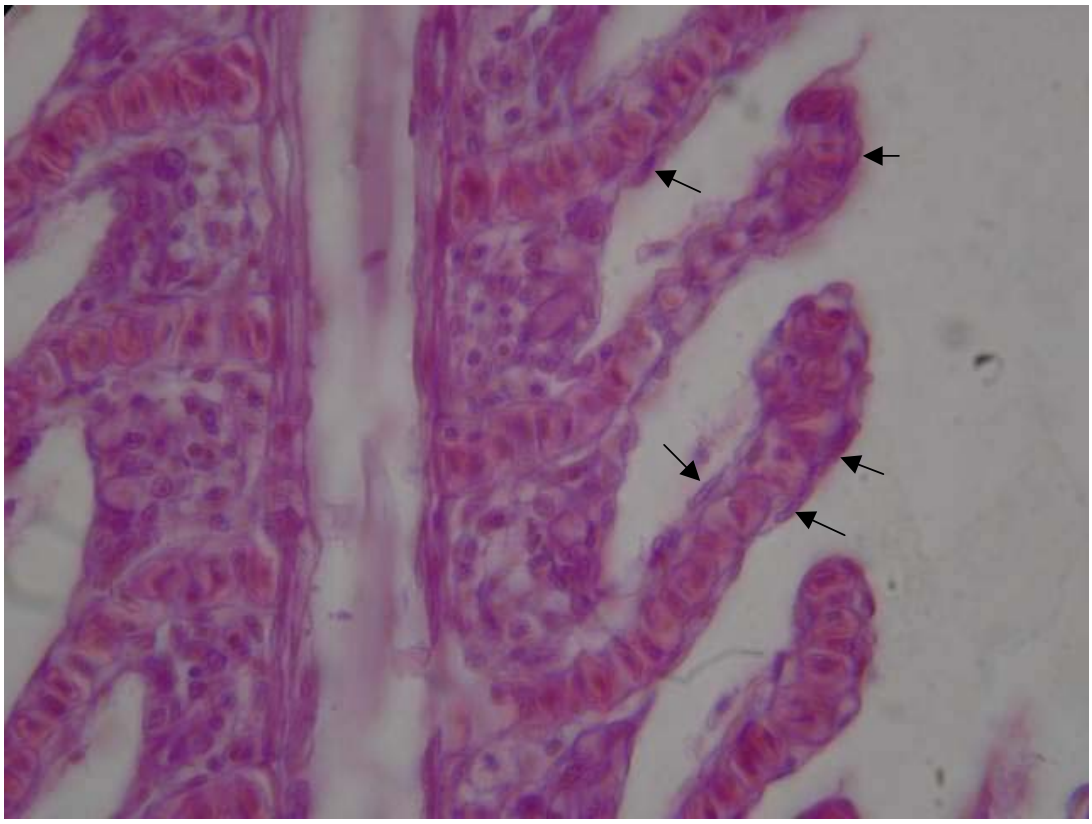


Plate 6.8 Section of a gill (×1000) from a fish exposed to 0.47 ± 0.04 mg/l Cu for 1 day during the long-term study showing epithelial cell hypertrophy (arrows).

6.3.3.5 Lamellar hyperplasia

Lamellar hyperplasia comprised of interlamellar cell hyperplasia and epithelial cell hyperplasia thereby increasing the thickness of the primary lamellae. Lamellar hyperplasia was Cu concentration and exposure duration dependent becoming marked and severe with increasing exposure time at the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels (Plates 6.9 and 6.10). At the lowest Cu level (0.11 ± 0.02 mg/l Cu) lamellar hyperplasia was first observed after 4 days of exposure and ranged between minimal and moderate until the end of the experiment. In some fish, the lamellae manifested different severity of hyperplasia (table 6.6).

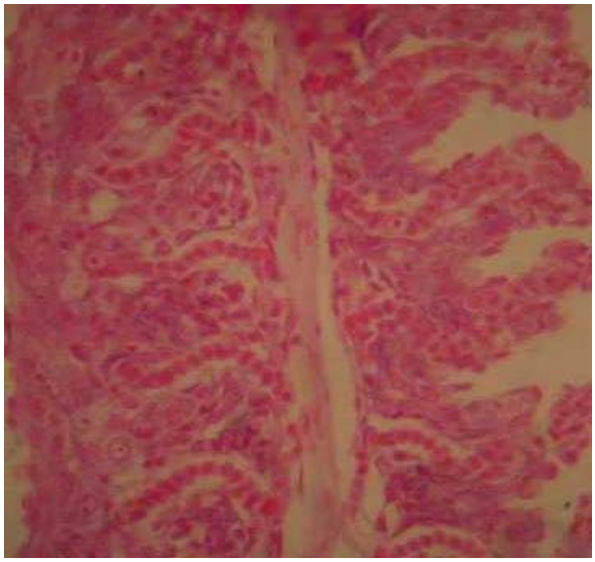


Plate 6.9 Sections of gills from a fish exposed to 0.29 ± 0.02 mg/l Cu for 64 days showing lamellar hyperplasia (× 400).

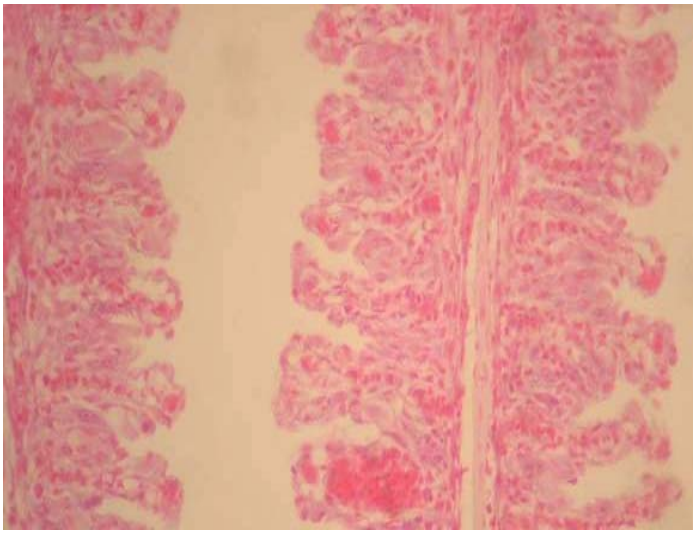


Plate 6.10 Sections of a gill from a fish exposed to 0.47 ± 0.04 mg/l Cu for 32 days showing lamellar hyperplasia (× 400)

Table 6.6 Number of *O. mossambicus* showing the severity of gill lamellar hyperplasia during the long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment	Severity of the lesion			
		Minimal	Moderate	Marked	Severe
1	0.11 ± 0.02				
	0.29 ± 0.02				
	0.47 ± 0.04	4			
2	0.11 ± 0.02				
	0.29 ± 0.02	4			
	0.47 ± 0.04	4			
4	0.11 ± 0.02	4			
	0.29 ± 0.02	4			
	0.47 ± 0.04		2	2	
16	0.11 ± 0.02	4			
	0.29 ± 0.02		4		
	0.47 ± 0.04			4	
32	0.11 ± 0.02	2	2	1	
	0.29 ± 0.02			4	
	0.47 ± 0.04				4
64	0.11 ± 0.02		4		
	0.29 ± 0.02				4

6.3.3.6 Lamellar oedema

Lamellar oedema was first observed at the highest Cu level after 4 days of exposure where it was minimal to moderate. After 32 days of exposure it was severe (table 6.7). At 0.29 ± 0.02 mg/l Cu this condition was first observed after 16 days of exposure (Plate 6.11). Here it was moderate becoming severe after 64 days of exposure (table 6.7). Lamellar oedema was also observed in the lowest Cu treatment (0.11 ± 0.02 mg/l

Cu) after 32 and 64 days of exposure where it was minimal to moderate, respectively. In some fish, the lamellae manifested different severity of oedema (table 6.7).

Table 6.7 Number of *O. mossambicus* showing the severity of lamellar oedema in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment	Severity of the lesion			
		Minimal	Moderate	Marked	Severe
4	0.11 ± 0.02				
	0.29 ± 0.02				
	0.47 ± 0.04	3	1		
16	0.11 ± 0.02				
	0.29 ± 0.02		4		
	0.47 ± 0.04			4	
32	0.11 ± 0.02	4			
	0.29 ± 0.02		4		
	0.47 ± 0.04			2	2
64	0.11 ± 0.02	2	2		
	0.29 ± 0.02			3	2

6.3.3.6 Desquamation of epithelial cells

Desquamation of epithelial cells (epithelial cell lifting) occurred mainly at the base of the secondary lamellae and was first observed after 16 days of exposure to 0.29 ± 0.02 (Plate 6.11) and 0.47 ± 0.04 mg/l Cu were it was moderate and marked, respectively (table 6.8). The lifting of the epithelial cells led to exposure of the pillar cells and blood capillaries.

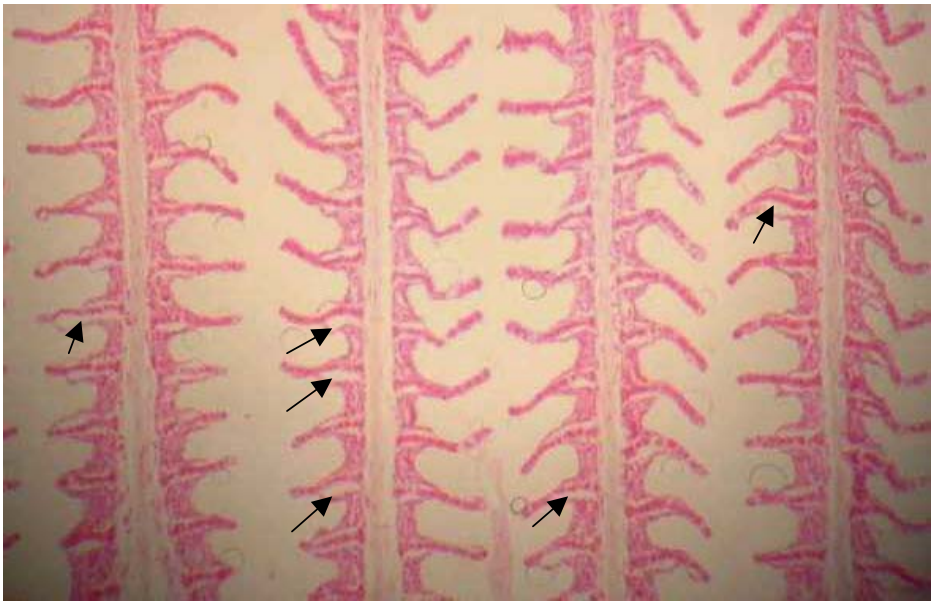


Plate 6.11 Section of a gill (×100) from a fish exposed to 0.29 ± 0.02 mg/l Cu for 16 days showing lamellar oedema and epithelial cell lifting mainly at the base of the secondary lamellae (arrows).

Table 6.8 Number of *O. mossambicus* showing the severity of epithelial cell desquamation in the gills during long-term Cu exposure. Every treatment had 4 fish. The numbers under lesion severity represent the number of fish with lesions. Blank cells imply absence of lesions.

Days of exposure	Cu Treatment	Severity of the lesion		
		Minimal	Moderate	Marked
16	0.11 ± 0.02			
	0.29 ± 0.02		4	
	0.47 ± 0.04			4
32	0.11 ± 0.02	4		
	0.29 ± 0.02		4	
	0.47 ± 0.04			4
64	0.11 ± 0.02			
	0.29 ± 0.02		4	4

6.3.3.7 Lamellar fusion and telangiectasis

Lamellar fusion and telangiectasis were noticed after 32 and 64 days of exposure to 0.47 ± 0.04 (Plate 6.12) and 0.29 ± 0.02 mg/l Cu, respectively.

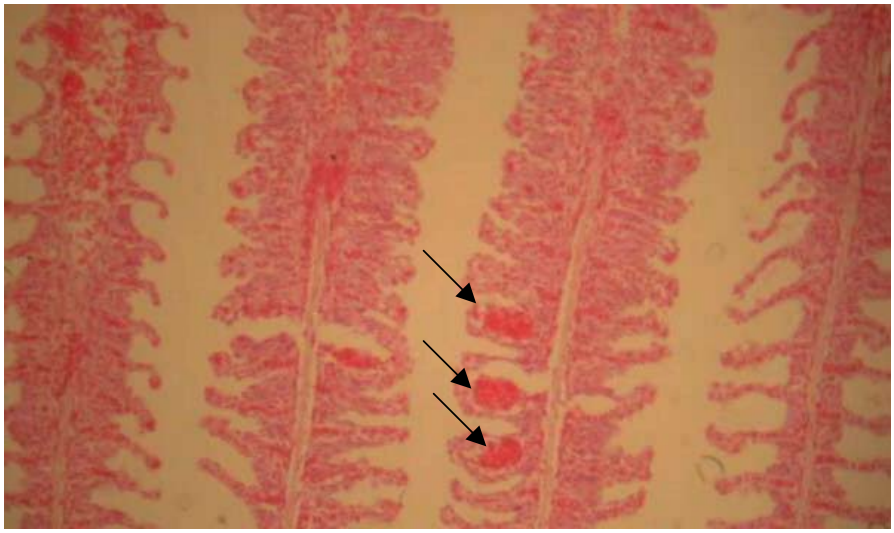


Plate 6.12 Section of a gill (×100) from a fish exposed to 0.47 ± 0.04 mg/l Cu for 32 days showing telangiectasis (arrows) and lamellar fusion.

6.4 Discussion

The chronology of lesions observed following long-term Cu exposure indicated an initial defence mechanism of the fish against copper toxicity followed by advanced histological changes. The initial defence mechanism was manifested as hypertrophy and hyperplasia of the gill epithelium causing increase in the thickness of the secondary lamellae, mucous cell hypertrophy and proliferation, mucous hypersecretion, proliferation of eosinophilic granule cells and hyperplasia of interlamellar cells. Mucous exudate was observed in all treatments after 16 days of exposure but mucous cell hypertrophy and hyperplasia were only prominent within the first four days of exposure in all Cu treatments. Only at the 0.29 ± 0.02 mg/l Cu level were these lesions observed after 16 and 32 days of exposure. These findings agree with those of Sultan and Khan (1983) who observed hyperplasia of the gill epithelium in *Carassius auratus* exposed to 0.1 mg/l Cu for 25 days. These authors did not report any alterations in the mucous secretion and mucous cells, probably

because histological observations were carried out after 25 days of exposure and these changes could have been missed at the 0.1 mg/l Cu level. Thus, hypersecretion of mucous appears to be pronounced within the first three weeks of exposure to Cu.

Hyperplasia of the gill epithelium is a measure to protect the gill filaments from any irritation in the surrounding water (Eller 1975). According to Daoust (1984) epithelial hypertrophy may indicate an increased cellular metabolism directed towards repair of subcellular damages or detoxification, such as synthesis of metallothionein-like metal-binding proteins. Interlamellar cells are involved in the regeneration of the secondary lamellae epithelium (Conte and Lin 1967). This could explain the proliferation of these cells observed in the present study. Hypertrophy and hyperplasia of mucous cells could have increased mucous secretion thus the hypersecretion of mucous exudates. The mucous layer controls the permeability of the integument to salts and other substances in the water by creating a microenvironment which may act as an ion trap, concentrating trace elements from the water (Jones 1964). According to Ellis (1989) mucous production and a highly responsive epithelium resulting in hyperplasia protects the gills from irritations. However, metals have been reported to interact with mucous leading to the formation of a film of coagulating mucous which together with damage to the gills brings about death by asphyxiation (Jones 1964). According to Roberts (1989) coagulating mucous may form a respiratory exchange obstruction and may act as a substrate for the rapid growth of bacteria such as cytophagan bacteria or pseudomonads causing bacterial gill disease.

Given that the eosinophilic granule cells are part of the lymphoid tissue (Ellis *et al*. 1989), proliferation of these cells following exposure to Cu may indicate activity of the immunological defence system. Proliferation of eosinophilic granule cells was predominant at the base of the primary lamellae and to a lesser extent along the length and tips of the primary lamellae. This alteration has not been reported in Cu toxicity studies of the gills probably because these cells do not occur in many fish species (Ellis *et al.* 1989). According to these findings, increase in mucous secretion, hypertrophy and hyperplasia of the epithelium and mucous cells, and proliferation of eosinophilic granule cells were the earliest physiological responses of *O. mossambicus* to Cu exposure.

With an increase in exposure time, more severe histological changes were observed. These included necrosis of the eosinophilic granule cells, lamellar oedema, epithelial desquamation leading to development of non-cellular gaps especially at the base of the secondary lamellae and an increase in severity of lamellar hyperplasia as a result of hypertrophy of epithelial cells and hyperplasia of epithelial and interlamellar cells. Unlike the earliest lesions representing the defence mechanism of the fish against Cu toxicity, the intensity of the advanced histological changes were related to Cu concentration and exposure duration. This reinforces the concept that these lesions are indicators of direct toxic effects of the chemical (Metcalfe 1998). Although lamellar oedema was not reported in many Cu toxicity studies of the gills, occurrence of this lesion in the present study is in agreement with Roberts (1989) that lamellar oedema is most frequent following exposure to chemical pollutants such as heavy metals, pesticides and therapeutic formalin overdose. This lesion was not observed during the short-term study and during the long-term study it was first observed in the gills of fish exposed to the highest Cu level (0.47 ± 0.04 mg/l Cu) after 4 days of exposure. In the gills of fish exposed to the lowest Cu level (0.11 ± 0.02 mg/l Cu) lamellar oedema was not observed before 32 days of exposure. Thus, this lesion could serve as an indicator of chronic or long-term Cu exposure. This could explain why this lesion was not frequent in many of the Cu toxicity studies of the gills considering that the majority of these studies dealt with acute Cu exposure.

Desquamation of the epithelium could have been a consequence of the pressure exerted on the epithelium as a result of lamellar oedema. This condition could have led to telangiectasis as a result of exposure of the pillar cells and blood capillaries causing them to weaken and disintegrate. It affects blood circulation and causes respiratory impairment (Roberts 1989). A similar condition was reported by Sultan and Khan (1983) who observed desquamation of some of the gill filaments causing gradual separation of epithelial cells from pillar cells and consequent disintegration, following chronic exposure of goldfish, *Carassius auratus* to 0.1 mg/l Cu for 25 days. During the present study desquamation of the epithelium was first observed at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels after 16 days of exposure and at 0.11 ± 0.02 mg/l Cu level after 32 days. Telangiectasis was not observed in the present study at 0.11 ± 0.02 mg/l Cu and Sultan and Khan (1983) did not report this condition after exposing

C. auratus to 0.1 mg/l Cu for 25 days. Telangiectasis may follow epithelial cell desquamation at Cu levels above 0.1 mg/l.

Lamellar hyperplasia caused partial or complete fusion of the lamellae thereby reducing the functional surface area for oxygen uptake. This condition was severe after acute Cu exposure during the short-term study. Following sub-lethal Cu exposure during the long-term study lamellar hyperplasia was related to Cu concentration and duration of exposure. This lesion could be induced within a short time under acute conditions as compared to chronic conditions. Similar changes were reported in *Puntius conchonius* (Kumar and Pant 1981) and rainbow trout, *O. mykiss* (Daoust *et al*. 1984) following acute exposure to $CuSO_4$. These authors observed lamellar fusion either as a result of simple apposition of adjacent lamellae or through epithelial hypertrophy and hyperplasia. Kumar and Pant (1981) exposed the fish to 0.6 mg/l Cu for 24 and 48 hours while Daoust *et al*. (1984) exposed the fish for 96 hours to 0.135 mg/l Cu. Thus, the effect of Cu was species specific.

There was some form of acclimation to Cu at the 0.11 ± 0.02 mg/l Cu level as the intensity of the lesions did not increase further with an increase in duration of exposure. Lamellar fusion was not observed at this exposure level. According to Daoust (1984) it is possible for the affected lamellae to return to normal structure in chronic injuries that have resulted in epithelial hyperplasia. However, epithelial hypertrophy increases the diffusion distance for oxygen transfer and this reduces oxygen uptake and carbon dioxide excretion (Evans *et al*. 1988). This also hinders the secretory and excretory function of the gills (Eller 1975). Hypertrophy and hyperplasia of epithelial cells could have affected the gaseous exchange at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu. This could partly explain the decrease in the frequency of opercular movements observed at these exposure levels (see chapter 7).

In conclusion, the lesions observed in the gills in this study were not specific to Cu toxicity, which reinforced the concept that irritant-induced gill alterations are not always irritant specific. This study further agreed with the concept that chemically induced gill damage is divided into indicators of direct toxic effects such as necrosis and epithelial desquamation, and indicators of defence responses to toxic effects such as mucous hypersecretion, lamellar fusion and hyperplasia of the gill epithelium (Mallatt 1985, Metcalfe 1998).

CHAPTER 7

The effects of copper on behaviour of *O. mossambicus* with reference to general activity and ventilation

7.1 Introduction

Fish behaviour has been described by patterns of swimming, preferred resting positions, activity, feeding, respiration, learning, schooling, migration, predator avoidance, reproductive, and non-reproductive interactions of one fish with another (Adler 1975, Atchison *et al*. 1987). Fish detect chemical stimuli through at least two different channels of chemoreception, i.e. olfaction for smell, and gustation for taste (Hara 1986). Behaviours such as feeding, defence, schooling, spawning, orientation and migration are largely dependent on olfactory cues and changes in olfactory function induced by a toxicant may affect the normal adaptive response of fish (Hara 1986). For example, imprinting odours serve as a stimulus to release a stereotyped behaviour pattern but continuous excitation of the olfactory system induces sensory adaptation or fatigue until the odour is no longer responded to (Hara 1986).

Histopathological studies revealed that Cu induces lesions in the olfactory organ and lateral line (Gardner and LaRoche 1973 and Saucier *et al*. 1990) the key organs in the mediation of fish behaviour (Hara 1996). Cu exerts a neurotoxic effect and consequent irritation to the perceptive system, and can destroy both chemoreceptors and mechanoreceptors, thus rendering the fish incapable of avoidance behaviour (Gardner and LaRoche 1973). Depending on the Cu concentration, some fish species will either avoid or show a preference for Cu (Kleerekoper *et al*. 1972, Westlake *et al*. 1974) and this was attributed to damage of the chemoreceptors and mechanoreceptors in the lateral line (Olsson 1998). The lateral line is important for detection and localization of prey, enemy avoidance, schooling, and intraspecific communication (Bleckmann 1986). Thus, any substance that causes irritation or lesions to the lateral line may adversely affect the normal behavioural pattern of fish. Although a brief encounter with Cu may not cause immediate death, it is apparent it could indirectly promote mortality by disrupting a species ability to perceive peripheral events.

Copper affects locomotion by inducing hyperactivity or hypoactivity (Drummond *et al*. 1973, Steele 1983, Ellgaard and Guillot 1988). However, these effects are not consistent, as some studies have demonstrated acclimation even at concentrations that could cause mortalities. The effect of Cu on locomotion appears to be species specific (Heath 1995). For example brook trout, *Salvelinus fontinalis*, exposed to sub-lethal concentrations of Cu (0.006 – 0.060 mg/l) for 24 hours showed an initial stimulation of locomotor activity within the first two hours followed by a return to normal activity patterns (Drummond *et al*. 1973). Cu elicited a concentration and exposure duration dependent decrease in the locomotor activity of the bluegill, *Lepomis macrochirus* exposed to 0.04, 0.08 and 0.4 mg/l Cu for 8 days (Ellgaard and Guillot 1988). Cu induced hypoactivity in sea catfish, *Arius felis* after exposure to low concentrations (0.005, 0.01 and 0.05 mg/l) for 72 hours whereas high concentrations (0.1 and 0.2 mg/l Cu) induced hyperactivity (Steele 1983).

Copper affects the respiratory system by modifying the breathing / ventilation frequency. The teleost respiratory system consists of the gills, and the buccal and paired opercular cavities (Heath 1995). Coordinated expansion and contraction of these cavities facilitates ventilation (Heath 1995). Olsson (1998) showed that Cu caused increased coughing frequency, ventilation rate and oxygen consumption. While other studies reported a decrease in the ventilation and oxygen consumption rate. For example an initial increase followed by a decrease in oxygen consumption and metabolism was observed following chronic exposure to waterborne Cu in bluegill, *Lepomis macrochirus* (O'Hara 1971, Felts and Heath 1984). Cu caused elevated oxygen consumption in rainbow trout following short-term exposure (Waiwood and Beamish 1978). However, as these authors did not look at the ventilation frequency of the fish it is not possible to relate oxygen consumption to ventilation.

Subjective observations were used to describe the extent of bodily activities of fish as restless, excitable, lethargic or dashing wildly (Heath 1995). Researchers that quantified the effect of Cu on bodily activities carried out short-term studies (Drummond *et al*. 1973, Steele 1983 and Ellgaard and Guillot 1988) and these authors used Cu salts ($CuSO_4$ and $CuCl_2$). The objective of this chapter was to examine and

quantify the effect of elementary Cu on *O. mossambicus* with reference to fish activity and ventilation following long-term Cu exposure.

7.2 Materials and methods

The behavioural study was conducted during the long-term exposure to Cu which involved exposing fish to three Cu levels, including a control with no addition of Cu, and three treatments with 0.11 ± 0.02, 0.29 ± 0.02, and 0.47 ± 0.04 mg/l Cu. Details of the experimental system and experimental design are given in the general methods (chapter 2).

7.2.1 Observation procedure

Ventilation was quantified by counting the number of opercular movements per minute and general activity was defined by the frequency of interactions between fish. These were denoted by a fish: pecking at another in an aggressive encounter (attack), being pecked at (attacked), pecking at any debris in the tank other than food, and swimming. The frequencies for activities were added to arrive at a value for total activity.

Every three days, two separate video recordings of the fish were done, 5 minutes for respiration and 15 minutes for all other activities. For total activities a video camera (Sony, VM-PS12) was positioned in front of the tank chosen for the observation, making the interior of the tank visible on a television screen. The fish were fed 5 minutes into a recording session, using a feeder secured above the tanks. To the feeder a 3 m string was tied and food was delivered into a tank by tilting the feeder on pulling the string. The feeder was operated from a distance to minimise interfering with fish activity. For ventilation the camera zoom lever was set to telephoto to take a close up of one fish at a time making the opercular movements visible on the television screen. Sixteen recording sessions were carried out per treatment.

7.2.2 Observations

The videotapes were played back and viewed on a television screen (Sony, Trinitron KV – 1440SA). An individual fish was watched for two minutes before and after feeding during which time the frequency of the activities defined above was recorded. Observations before feeding were carried out within the first four minutes of every recorded session, and observations after feeding were carried out seven minutes after introducing food into the tanks. Ventilation was quantified by counting the opercular movements per minute in duplicate for each treatment.

7.2.3 Statistical analysis

For each treatment regression analysis was used to quantify the relationship between the total activity of the fish, and duration of exposure. Analysis of covariance (ANCOVA) was used to test for the difference in slopes between treatments taking exposure time as a covariate. Analysis of variance (ANOVA) was used to test for difference between the mean opercular movements between treatments. Tukey's test was used to compare the means.

7.3 Results

7.3.1 Total fish activity

7.3.1.1 Activity before introducing food into the tanks

There was a decline in fish activity before feeding with increase in exposure time. The decline in activity was related to the concentration of Cu with the highest decline at the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels (figure 7.1). There was no significant difference ($p > 0.05$) between slopes of the control and 0.11 ± 0.02 mg/l Cu, and between 0.29 ± 0.02 and 47 ± 0.04 mg/l Cu. The slopes of both the control and 0.11 ± 0.02 mg/l Cu were significantly different from those of 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu ($p < 0.05$).

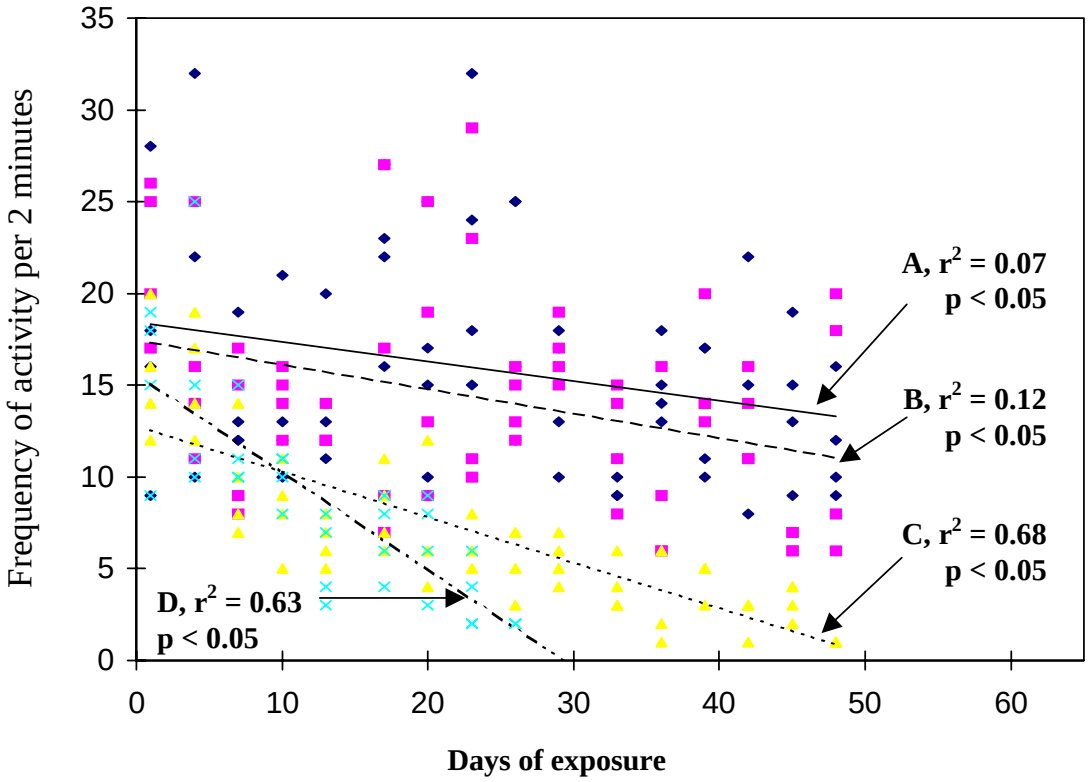


Figure 7.1 Change in frequency of activity over time of *O. mossambicus* before introducing food into the tanks. A = control, B = 0.11 ± 0.02 mg/l Cu, C = 0.29 ± 0.02 mg/l Cu and D = 0.47 ± 0.04 mg/l Cu. Lines are regression lines. r^2 = coefficient of determination.

7.3.1.2 Activity after introducing food into the tanks

There was constant activity seven minutes after introducing food into the control and 0.11 ± 0.02 mg/l Cu tanks. The activity remained steady in these treatments irrespective of increase in exposure time i.e. $r^2 = 0.00$ and $p > 0.05$ in the control and $r^2 = 0.03$ and $p > 0.05$ in the 0.11 ± 0.02 mg/l Cu treatment (figure 7.2). There was a significant decline in fish activities with increase in exposure time at exposure levels of 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu ($r^2 = 0.45$, $p < 0.05$) and ($r^2 = 0.65$, $p < 0.05$), respectively (figure 7.2). There was no significant difference ($p > 0.05$) between slopes of the control and 0.11 ± 0.02 mg/l Cu, and between 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu ($p < 0.05$). The slopes of both the control and 0.11 ± 0.02 mg/l Cu were

significantly different from those of the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu treatments ($p < 0.05$).

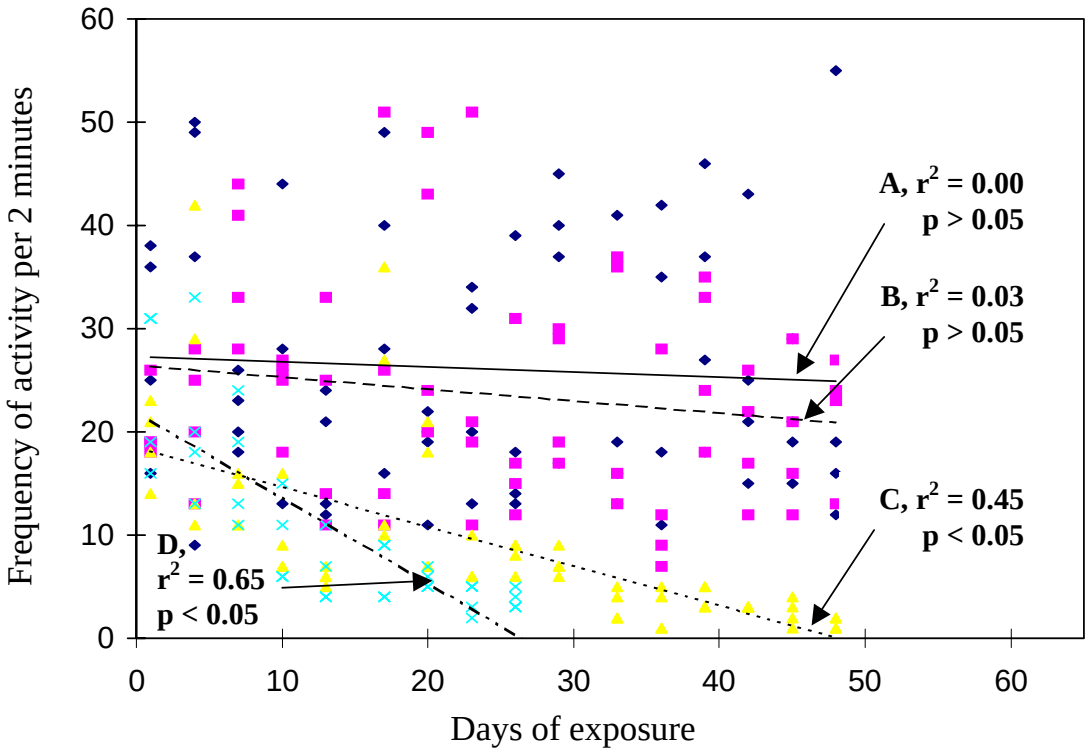


Figure 7.2 Change in frequency of activity over time of *O. mossambicus* after introducing food into the tanks. A = control, B = 0.11 ± 0.02 mg/l Cu, C = 0.29 ± 0.02 mg/l Cu and D = 0.47 ± 0.04 mg/l Cu. Lines are regression lines. r^2 = coefficient of determination.

7.3.1.3 Feeding activity event

There was a constant feeding activity at the control ($r^2 = 0.02$, $p > 0.05$) and 0.11 ± 0.02 mg/l Cu levels ($r^2 = 0.01$, $p > 0.05$) throughout the exposure period (figure 7.3). There was reduced feeding activity at the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels with increase in duration of exposure ($r^2 = 0.13$, $p < 0.05$) and ($r^2 = 0.30$, $p < 0.05$), respectively (figure 7.3). There was no significant difference ($p > 0.05$) between slopes of the control and the 0.11 ± 0.02 mg/l Cu treatment, and between the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu treatment. The slopes of both the control and the 0.11 ±

0.02 mg/l Cu treatment were significantly different from those of the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu treatment.

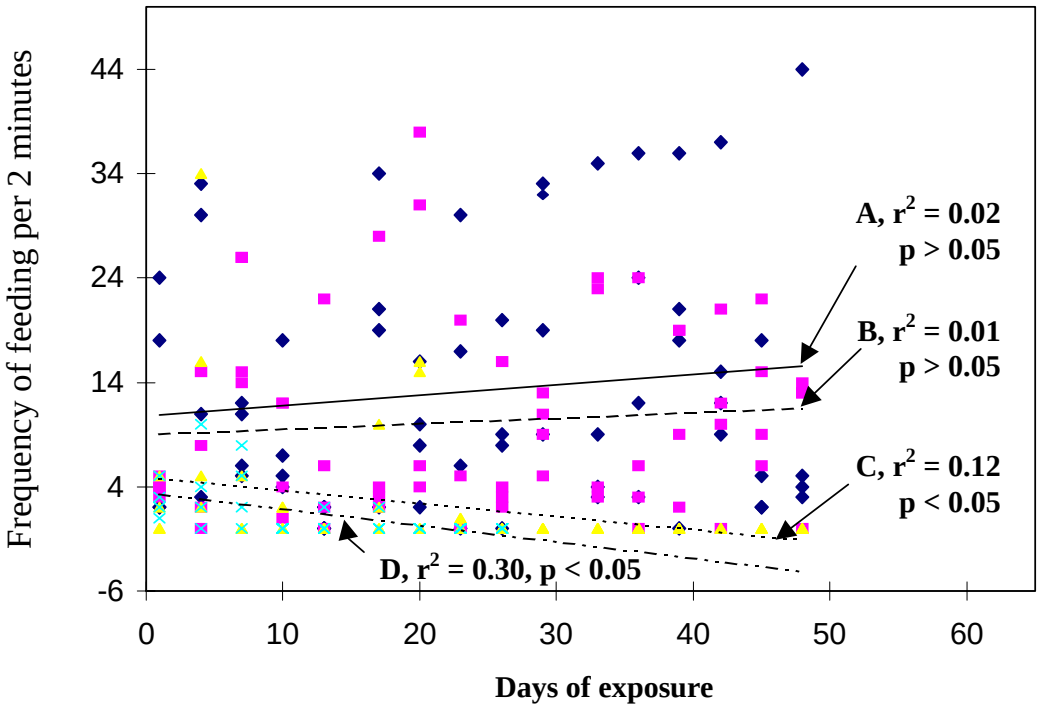


Figure 7.3 Changes in feeding frequency of *O. mossambicus* during long-term Cu exposure. A = control, B = 0.11 ± 0.02 mg/l Cu, C = 0.29 ± 0.02 mg/l Cu and D = 0.47 ± 0.04 mg/l Cu. Lines are regression lines. r^2 = coefficient of determination.

7.3.2 Ventilation

There were significant differences in the mean opercular movements per minute between treatments ($p < 0.05$). There was an increase in the mean opercular movements per minute (hyperventilation) at 0.11 ± 0.02 mg/l Cu (87 ± 18 opercular movements per minute) and a decrease (hypoventilation) at 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu i.e. 37 ± 34 and 13 ± 6 opercular movements per minute compared to the control (table 7.1).

Table 7.1 Means ± standard deviation, minimum and maximum opercular movements per minute in *O. mossambicus* at different Cu exposure levels during long-term exposure (64 days). Different superscripts denote significant differences in the mean opercular movements per minute between treatments ($p < 0.05$)

Treatments (mg/l Cu)	Opercular movements per minute			Sample size
	Mean ± Standard Deviation	Minimum	Maximum	
Control	67 ± 5^{a}	57	79	64
0.11 ± 0.02	87 ± 18^{b}	50	131	64
0.29 ± 0.02	37 ± 34^{c}	7	150	64
0.47 ± 0.04	13 ± 6^{d}	6	33	32

7.3.3 Other behavioural responses

Other behavioural responses that were noticed but not quantified include twitching which was observed as sudden jerky movements of the fish and erratic swimming, especially before a fish died. These parameters were spontaneous and short-lived and were sometimes observed on days when there was no video recording session as such they could not be quantified.

7.4 Discussion

7.4.1 Fish activity

There was a gradual decline in fish activity related to Cu concentration and duration of exposure. The decrease in fish activity may imply reduced metabolism as the concentration of Cu and duration of exposure increases. Hypoactivity has also been attributed to a decrease in exploratory behaviour of the animal due to some type of "memory" or "retention" process after getting familiar with the environment (Steele 1983). However, results of the present study are not in agreement with those of Steele (1983) who observed Cu induced hypoactivity in sea catfish, *Arius felis* following a 72 hour exposure to low concentrations (0.005, 0.01 and 0.05 mg/l) and hyperactivity

at high concentrations (0.1 and 0.2 mg/l Cu). The difference could be attributed to species difference, difference in exposure duration and due to the fact that Cu reacts and acts different in seawater. Conclusions of the present study agree with those of Ellgaard and Guillot (1988) who observed a Cu concentration and exposure duration dependent decrease in the locomotor activity of the bluegill, *Lepomis macrochirus* exposed to 0.04, 0.08 and 0.4 mg/l Cu. These authors based their findings on 8 days of observations and it is not known if this response would have been different after longer exposure. Heath (1995) reported that metals affect the locomotor activities of fish by alterations in free locomotor activity, manifested as hypoactivity or hyperactivity. This effect of Cu on the locomotor activity could have been manifested as hypoactivity in the present study.

There was constant activity after introducing food in the tanks at the control and 0.11 ± 0.02 mg/l Cu exposure levels irrespective of exposure time. The constant activity was attributed to the constant feeding activity at these exposure levels. This is comparable to findings by Lett *et al*. (1976) who observed cessation of feeding upon initial exposure of *Oncorhynchus mykiss* to 0.1 to 0.3 mg/l Cu. After 5 to 15 days of exposure the fish's appetite returned to normal. Waiwood and Beamish (1978) observed a depressed appetite followed by a complete or partial recovery in *O. mykiss* exposed to 0.01 – 0.2 mg/l Cu. Findings of the present study did not reflect fish adaptation to normal appetite as was observed by Waiwood and Beamish (1978) and Lett *et al*. (1976) but instead reflected a steady appetite of the fish in the control and 0.11 ± 0.02 mg/l Cu treatments. Since the Cu concentrations used by Waiwood and Beamish (1978) and Lett *et al*. (1976) were within the same range as the lowest Cu concentration used in the present study, the difference in findings appear to be species specific.

There was a reduction in feeding activity (reduced appetite) after introducing food into the tanks at the 0.29 ± 0.02 mg/l Cu and 0.47 ± 0.04 mg/l Cu levels compared to the control. A similar trend was observed in the total activity (hypoactivity) of the fish in these treatments. The hypoactivity even after introducing food into the tanks implies that the feeding activity did not influence the total fish activity at these exposure levels as in the control and the 0.11 ± 0.02 mg/l Cu treatments. Heath (1995) reported that metals affect the locomotor activities of fish by altering the sensory

perception thus reducing the response to normal olfactory cues associated with such activities as feeding. Fish exposed to higher levels of Cu may become inappetent whereas at low Cu levels, fish appetite may remain constant or may be temporarily reduced depending on the species. Suppressed food consumption was attributed to damage to the taste receptors (Waiwood and Beamish 1978) and hormonal changes resulting into higher blood glucose level (Heath 1995).

7.4.2 Ventilation

The hyperventilation at the 0.11 ± 0.02 mg/l Cu level could be attributed to the histopathological changes observed in the gills at this exposure level. These gills showed minimal to moderate epithelial hypertrophy and lamellae hyperplasia (chapter 6). These lesions could have caused an increased diffusion distance for oxygen from the water to the blood reducing gas exchange in the lamellae. The fish may have been able to maintain their metabolic demands at the 0.11 ± 0.02 mg/l Cu exposure level but at a higher effort than the control. These findings are similar to those of Waiwood and Beamish (1978) who measured the oxygen consumption of fish swimming at controlled speeds while being exposed to 0.01 to 0.2 mg/l Cu for 5 days. Cu caused elevation of oxygen consumption at all swimming speeds suggesting an increased cost of maintenance even at low Cu levels. Under mild Cu concentrations, the oxygen partial pressure of the arterial blood may be maintained at physiological levels through increased ventilation frequency. Thus, an increase in ventilation frequency may not mean increased oxygen consumption but may be caused by damage to the gills which reduces the oxygen perfusion through the epithelial membrane such that extra energy is required to maintain the metabolic demand.

The hypoventilation observed at the 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu levels could be attributed to greater damage to the gills. Lamellar oedema, epithelial desquamation and marked epithelial hyperplasia could have caused a reduced ventilatory rate, lower oxygen flow from the water to the blood, and a decrease in neuromuscular activity. This was supported by the decrease in activity with increased Cu concentration. O'Hara (1971) observed a dose-dependent increase in oxygen consumption within the first 2 hours followed by a dose-dependent depression in oxygen consumption 2 to 18 hours later in bluegill, *Lepomis macrochirus* exposed to 0.3 to 5 mg/l Cu for 24 hours.

Thus, higher Cu levels and prolonged exposures may cause a depression in muscular activity reducing metabolism.

At higher copper levels, the possibility of anaemia reflected by a decrease of erythrocytes in the splenic red pulp implies that there would be less haemoglobin available to transport oxygen. This, coupled with the reduction in ventilation frequency reinforces the reduction in tissue oxygen rendering muscles and organs dependent on oxygen hypofunctional. This does not agree with the findings from liver histology which reflected an initial hyperfunction. Thus, the initial stimulation of liver energy consumption (hyperfunction) may reflect an increase in the detoxification process in this organ (Heath 1995). This is in agreement with Felts and Heath (1984) who reported a decrease in oxygen consumption, elevated liver oxygen consumption and no significant alteration in oxygen consumption of gill and brain measured *in vitro*. Copper seems to reduce oxygen consumption at a higher level of integration than at individual tissue level.

The method used for establishing the ventilation frequency in this study could not establish the stroke volume and ventilation volume. Thus, further studies on the effect of Cu on respiration taking into account stroke and ventilation volume and oxygen partial pressures are recommended as these may provide better interpretations of ventilation frequencies.

In conclusion, these findings suggest that fish exposed to Cu may have a depressed appetite, reduced oxygen consumption and depressed muscular activity. At low Cu exposure levels, the fish may be able to maintain the body's homeostatic mechanisms. Thus, low Cu concentration may not cause biological changes at cellular level but could have the ability to affect behaviour. At higher Cu concentrations behavioural changes may serve as clinical signs reflecting the biological changes taking place at cellular level. There is a need to compare changes in behaviour with cellular changes to evaluate the effects of a toxicant on fish.

CHAPTER 8

General discussion

The aim of this study was to investigate the effects of elementary Cu on aspects of bioconcentration, histology and behaviour of *O. mossambicus* under experimental conditions in the laboratory. The challenge is to obtain a concentration-duration-response relationship whereby the response (boiconcentration and histopathology) could be used to predict the Cu concentration to which the fish were exposed and how long the fish were exposed. To obtain such a relationship exposure concentration and duration, and environmental conditions must be closely controlled and monitored. Since it is not possible to control environmental conditions in the field this can only be done under experimental conditions (Kendall 1996). Results from experimental studies may be used to explain field results and estimate exposure time and concentration.

One of the objectives of this study was to establish a relationship between Cu accumulation in tissues, concentration of Cu in the water and duration of exposure under controlled conditions. Copper accumulation in the liver and gills of *O. mossambicus* was positively correlated to concentration and exposure duration. The higher bioconcentration factor and a better fitting model for the accumulation-concentration-duration relationship observed in the liver indicated that this organ was a good indicator organ for Cu toxicity. Thus, the model $Log\ L = 3.35 + 0.85W + 0.31T$, where L = Cu accumulation values in the liver (µg/g dry mass), W = Cu concentration in water (mg/l), T = duration of exposure (days), could be used as a predictor of Cu levels in the liver. Results may need to be corrected for fluctuations of Cu concentration in the water. This model may be tested under different sets of environmental conditions. This will determine the potential of the model in monitoring Cu toxicity in the environment.

The second objective was to establish sequential histological transformation in the gills, liver and spleen in relation to sub-lethal Cu exposure. Histological changes in the gills, liver and spleen were related to the concentration of copper in the water and

duration of exposure showing a gradual increase in damage with larger duration of exposure time and increasing Cu concentration. The sequential histological changes in the liver and gills indicated that the fish were initially able to homeostatically regulate and detoxify Cu and an increase in exposure time produced a higher degree of alteration. However, the lesions observed during this study were non-specific, thus it would be difficult to derive a confirmatory diagnosis for Cu toxicity using liver, gill and spleen histological procedures. Under field conditions, a definitive diagnosis can only be derived if histological findings are correlated with evidence of Cu accumulation in the tissues and history of Cu exposure. At a Cu level of 0.11 ± 0.02 mg/l the histological changes were minor and fish behaviour was not significantly different from that of the control. Because of the less severe gill damage the fish were able to maintain the body homeostatic mechanisms but at the expense of energy thereby experiencing some physiological stress as was manifested by the hyperventilation (figure 8.1). Thus, low Cu concentration may not cause histological effects but could have the ability to cause abnormal behaviour whereas at higher Cu concentrations behavioural changes may serve as clinical signs reflecting the physiological changes. Therefore, a comparison of histological changes with changes in behaviour may assist in evaluating the potential effects of a toxicant on fish.

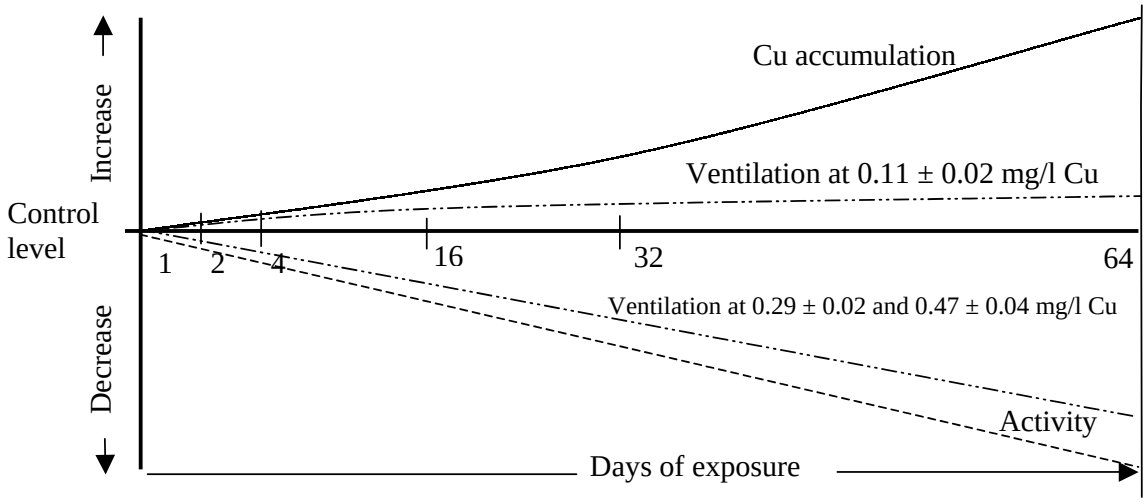


Figure 8.1 Illustration of the changes that occurred in bioaccumulation of Cu in organs and behaviour of *O. mossambicus* during Cu exposure for up to 64 days. The horizontal bold line represents the control treatment. The activity of the Cu exposed fish decreased with increase in Cu exposure. There was hyperventilation at 0.11± 0.02 mg/l Cu and hypoventilation at 0.29 ± 0.02 and 0.47± 0.02 mg/l Cu.

The study produced guidelines for the monitoring of Cu toxicity:

1. The liver had the highest Cu accumulation capacity and Cu accumulation and the sequential appearance of lesions were related to the concentration of Cu in the water and duration of exposure. There was a sequential appearance of lesions in the order of, hepatic vacuolar degeneration, fatty degeneration and necrosis (table 8.1).

2. Sequential histopathological changes indicated an initial liver hyperfunction followed by hypofunction and consequently cell death. The hyperfunction may be related to the homeostatic control and detoxification of Cu by the liver (Heath 1995, Olsson 1998) and explained the slow increase in Cu accumulation after 1 and 32 days of exposure to 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu.

3 The initial lesions in the gills and the slow increase in Cu accumulation after 1 and 32 days of exposure to 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu indicated an initial defence mechanism of the fish against Cu toxicity. Histologically this was manifested as hypertrophy and hyperplasia of the gill epithelium causing increase in the thickness of the secondary lamellae, mucous cell hypertrophy and proliferation, mucous hypersecretion, proliferation of eosinophilic granule cells and hyperplasia of interlamellar cells (table 8.1). Epithelial hypertrophy may indicate an increased cellular metabolism directed towards repair of subcellular damages or towards detoxification, such as synthesis of metallothionein-like metal-binding proteins (Daoust 1984).

4. The advanced lesions in the gills were related to the concentration of Cu in the water and duration of exposure which reinforced the concept that these lesions were indicators of direct toxic effects of the chemical to the gills (Metcalfe 1996). These changes included necrosis of the eosinophilic granule cells, lamellar oedema, epithelial desquamation leading to development of non-cellular gaps especially at the base of the secondary lamellae and increase in severity of lamellar hyperplasia as a result of hypertrophy of epithelial cells, and hyperplasia of epithelial and interlamellar cells (table 8.1).

5. Hypertrophy and hyperplasia of epithelial cells could have affected the gaseous exchange at exposure to 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu thereby causing hypoventilation as was reflected by the decrease in number of opercular movements at these exposure levels. Hypertrophy of the lamellar epithelium hinders the secretory, and excretory function of the gills and also increases the diffusion distance for oxygen transfer. This reduces the efficiency of oxygen uptake and carbondioxide excretion (Eller 1975, Evans *et al*. 1988).

6. A decrease of erythrocytes in the splenic red pulp at exposure to 0.29 ± 0.02 and 0.47 ± 0.04 mg/l Cu may lead to anaemia and reduce the quantity of haemoglobin available to transport oxygen for utilization in the tissues and cells. This coupled with the reduction in ventilatory rate reinforced the reduction in tissue oxygen rendering organs and tissues dependent on oxygen such as muscles hypofunctional. This could explain the gradual decline in fish activity with an increase in exposure time at these exposure levels.

Table 8.1 Summary of the lesions observed in organs and their first appearance at each exposure level. Cu treatments 1 = 0.11 ± 0.02 mg/l, 2 = 0.29 ± 0.02 and 3 = 0.47 ± 0.04 mg/l. Lesions with * were not observed at 0.11 ± 0.02 mg/l Cu level.

Organ	Lesion	Day lesion was first observed	Cu treatment
Liver	Vacuolar degeneration	1	All treatments
	Fatty degeneration	4	3
		16	2
		32	1
	Necrosis *	4	3
		16	2

Table 8.1 continued

Organ	Lesion	Day lesion was first observed	Cu treatment
Gills	Mucous cell hypertrophy	1	All treatments
	Mucous exudate	1	All treatments
	Hyperplasia of eosinophilic granule cells	1	All treatments
	Epithelial cell hypertrophy	1	2 and 3
		2	1
	Lamellar hyperplasia	1	3
		2	2
		4	1
	Mucous cell hyperplasia	2	3
		4	1 and 2
	Lamellar oedema	4	3
		16	2
		32	1
	Epithelial cell desquamation	16	2 and 3
		32	1
	Lamellar fusion*	32	3
		64	2
	Telangiectasis *	32	3
		64	2
Spleen	Haemosiderosis	1	All treatments
	Macrophage centres	1	All treatments
	Expansion of the white pulp	2	2 and 3
		4	1
	Reduction in the red pulp *	4	2 and 3
	Necrosis *	16	2 and 3

In conclusion Cu accumulation and effects on histology of the liver and gills are related to the concentration of Cu in the water and duration of exposure showing a gradual increase in incidence and intensity with larger duration of exposure time and increasing Cu concentration. The fish are initially able to homeostatically regulate and detoxify Cu. However, as the exposure continues, the homeostatic mechanism appears to fail to cope with the increasing metal burden causing advanced cellular

degenerative changes that may lead to anaemia, immunosuppression, impaired osmoregulation, tissue metabolism and excretion of waste products leading to their accumulation, and other organ hypofunction (figure 8.2). In high exposure concentrations physiological alterations occur faster causing death within a shorter time.

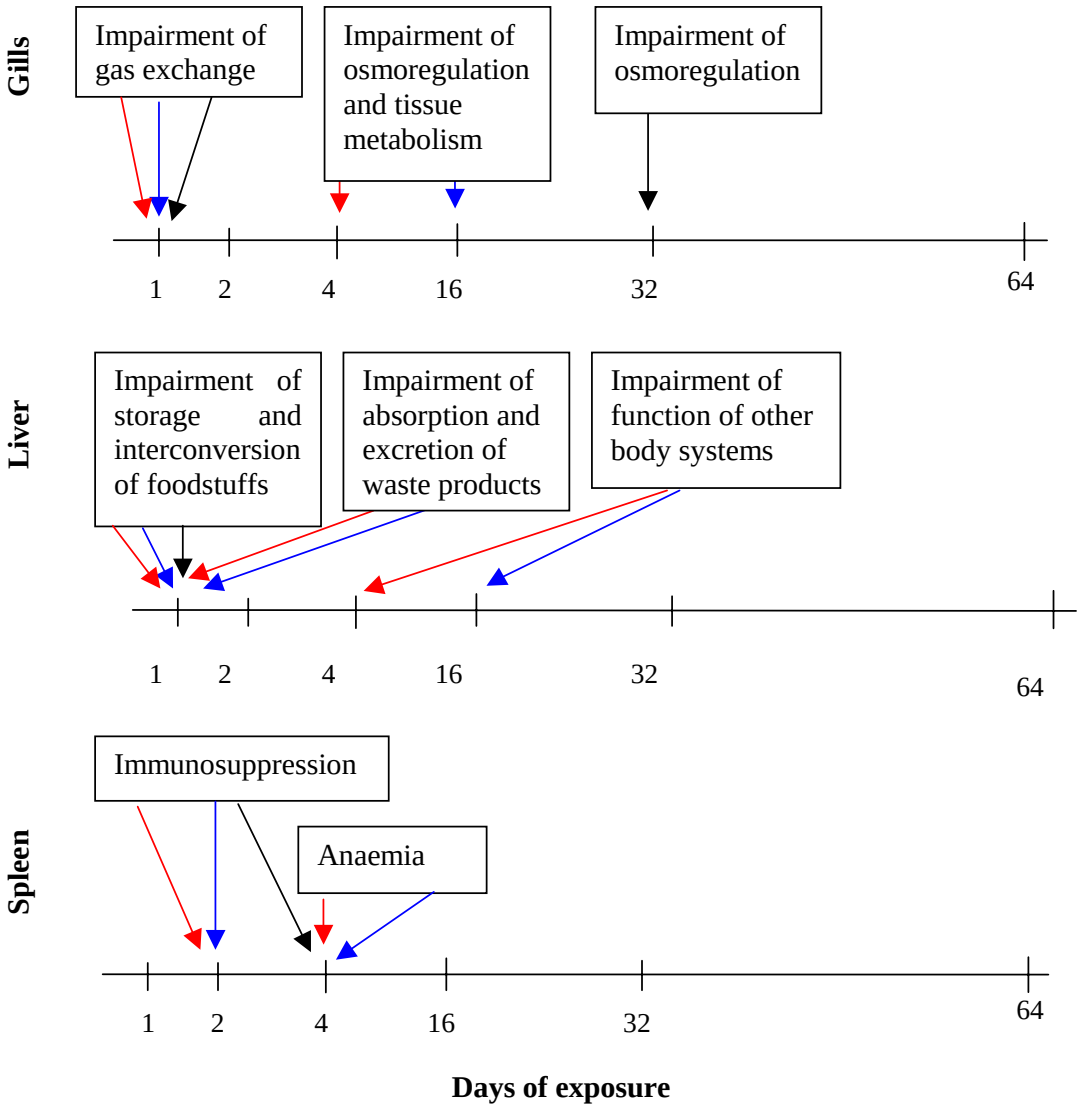


Figure 8.2 An illustration of the physiological changes and the exposure duration at which they are likely to occur following histological alterations due to Cu toxicity to the gills, liver and spleen of *O. mossambicus*. Physiological changes are presented in boxes. Arrows represent treatments where black = 0.11 ± 0.02, blue = 0.29 ± 0.02 and red = 0.47 ± 0.04 mg/l Cu

There are still gaps in Cu toxicology that need to be investigated in order to obtain an integrated picture of the effects of this metal on fish and to be able to predict the dynamics of these effects under different environmental conditions.

A summary of recommendations:

1. Studies on accumulation coupled with histological observations give an insight into the changes in the organs as a result of metal toxicity. For light microscopic observation, Massons' trichrome stain needs to be combined with haematoxylin and eosin in order to appreciate the cellular and fibrotic changes. Specific stains such as Sudan III for neutral fat in cases of fatty degeneration and electron microscopic observations to give the details of cellular organelles' changes due to Cu toxicity are recommended.

2. Variables such as blood cell differentials and the splenic ability to elicit immune responses following Cu exposure need to be investigated given that Cu may cause anaemia and immunosuppression. These factors combined with histopathology may serve as indicators of environmental exposure.

3. The effect of Cu on the physiological performance of the liver should be evaluated as this organ accounts for physiological activities such as vitellogenin synthesis, the reticulo-endothelial and imunoglobulin system, bile secretion, and storage of lipids and carbohydrates. These functions are likely to be affected by liver hypofunction and cell death.

4. More data are needed to model combined effects of exposure duration and concentration on Cu accumulation in fish tissues under different conditions including field conditions. Successful controlling of environmental conditions is unlikely in many field settings. Thus results may need to be corrected for fluctuations of Cu concentration in the water. This will determine the potential of the tissue accumulation approach in monitoring Cu toxicity in the environment.

REFERENCES

Adler, H.E. 1975. Fish behaviour: why fishes do what they do. T.F.H. Publications, Neptune City, USA. 271 pp.

Anderson, D.P., Dixon, O.W., Bodammer, J.E. and Lissio, E.F. 1989. Suppression of antibody-producing cells in rainbow trout spleen sections exposed to copper *in vitro*. Journal of Aquatic Animal Health **1**: 57 – 61.

Ashley, L.M. 1975. Comparative fish physiology. pp 3 – 32. *In*: W.E. Ribelin and G. Migaki (eds.) The pathology of fishes. The University of Wisconsin press, London.

Atchison, G.J., Henry, M.G. and Sandheinrich, M.B. 1987. Effects of metals on fish behaviour: a review 1. Environmental Biology of Fishes **18** (1): 11 – 25.

ATSDR (Agency for Toxic Substances and Disease Registry). 1990. Toxicological profile for copper. U.S. Public Health Service, Atlanta, Georgia. TP-90-08. 142 pp.

Avenant-Oldewage, A. and Marx, H.M. 2000. Bioaccumulation of chromium, copper and iron in the organs and tissues of *Clarias gariepinus* in the Olifants River, Kruger National Park. Water SA **26** (4): 569 – 582.

Banerjee, S. and Homechaudhuri, S. 1990. Haematological monitoring of a bioindicator fish, *Heteropneustes fossilis*, on exposure to copper toxicity. Israeli Journal of Aquaculture Bamigdeh **42**: 46 –51.

Benedetti, I. Albano, A.G. and Mola, L. 1989. Histomorphological changes in some organs of the brown bullhead, *Ictalurus nebulosus* LeSueur, following short- and long-term exposure to copper. Journal of Fish Biology **34**: 276 – 280.

Bezuidenhout, L.M., Schoonbee, H.J. and De Wet, L.P.D. 1990. Heavy metal content in organs of the African sharptooth catfish, *Clarias gariepinus* (Burchell), from a

Transvaal lake affected by mine and industrial effluents. Part 1. Zinc and copper. Water SA **16** (2): 125 – 129.

Bieniarz, K., Epler, P., Sokolowska-Mikolajczyk, M. and Popek, W. 1997. Reproduction of fish in conditions disadvantageously altered with the salts of zinc and copper. Archives of Polish Fisheries **5** (1): 21 – 30.

Bilinski, E. and Jonas, R.E.E. 1973. Effects of cadmium and copper on the oxidation of lactate by rainbow trout (*Oncorhynchus mykiss*) gills. Journal of the Fisheries Research Board of Canada **30** (10): 1553 – 1558.

Birge, W.J. and Black, J.A. 1997. Effects of copper on embryonic and juvenile stages of aquatic animals. pp 383 - 399. *In*: Copper in the environment. Part II. J.O. Nriagu (ed.) Wiley, New York.

Bleckmann, H. 1986. Role of the lateral line in fish behaviour, pp 177 – 202. *In*: T. J. Pitcher (ed.) The behaviour of teleost fishes. Croom Helm Ltd., London.

Carbonell, G. and Tarazona, J.V. 1994. Toxicokinetics of copper in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology **29**: 213 - 221.

Cardeilhac, P.T. and Whitaker, B.R. 1988. Copper treatments: uses and precautions. Veterinary Clinics of North America **18**: 435- 448.

Clesceri, L.S., Greenberg, A.E. and Trussel, R.R. 1989. Standard methods for the examination of water and wastewater 17th edition. American Public Health Association, Washington D.C. 1469 pp.

Conte, F.P. and Lin, D.H.L. 1967. Kinetics of cellular morphogenesis in gill epithelium during seawater adaptation of Coho, *Oncorhynchus kisutch*. Comparative Biochemistry and Physiology **23**: 945 – 957.

Crompton, T.R. 1997. Toxicants in the aqueous ecosystem. John Wiley & Sons, New York. 382 pp.

Daoust, P.Y., Wobester, G. and Newstead, J.D. 1984. Acute pathological effects of inorganic mercury and copper in gills of rainbow trout. Veterinary Pathology **21**: 93 – 101.

Donaldson, E.M. and Dye, H.M. 1975. Corticosteroid concentrations in sockeye salmon (*Oncorhynchus nerka*) exposed to low concentrations of copper. Journal of the Fisheries Research Board of Canada **32** (4): 533 – 539.

Drummond, R.A., Spoor, W.A. and Olson, G.F. 1973. Some short-term indicators of sublethal effects of Cu on brook trout, *Salvelinus fontinalis*. Journal of the Fisheries Research Board of Canada **30**: 698 – 701.

Droy, B.F. and Hinton, D.E. 1988. Allyl formate-induced hepatotoxicity in rainbow trout. Marine Environmental Research **24**: 259 – 264.

Du Preez, H.H. and Steyn, G.J. 1992. A preliminary investigation of the concentration of selected metals in the tissues and organs of the tigerfish (*Hydrocynus vittatus*) from the Olifant River, Kruger National Park, South Africa. Water SA **18** (2): 131 – 136.

Du Preez, H.H., Smit, L., Steyn, G.J. and Buermann, Y. 1996. The effect of sublethal concentrations of natural silt on routine oxygen consumption by Mozambique tilapia (*Oreochromis mossambicus*). Southern African Journal of Aquatic Sciences **22** (1/2): 81 – 89.

Eaton, D.L. and Klaassen, C.D. 1996. Principles of toxicology. pp 13 – 34. *In*: C.D. Klaassen (ed.) Casarett and Doull's toxicology: The basic science of poisons. 5th edition. McGraw-Hill Health Progressions Division, New York.

Eisler R. 1998. Copper hazards to fish, wildlife and invertebrates: A synoptic review. Biological Science Report 33. 100 pp.

Eller, L.L. 1975. Gill lesions in freshwater teleosts. pp 305 – 330. *In*: W.E. Ribelin and G. Migaki (eds.) The pathology of fishes. University of Wisconsin Press, Madison, Wisconsin.

Ellgaard, E.G. and Guillot, J.L. 1988. Kinetic analysis of the swimming behaviour of bluegill sunfish, *Lepomis macrochirus* Rafinesque, exposed to copper: hypoactivity induced by sublethal concentrations. Journal of Fish Biology **33**: 601 – 608.

Ellis, A.E. 1989. The immunology of teleosts. pp 135 –152. *In*: R.J. Roberts (ed.) Fish pathology. 2nd edition. Baillière Tindall, London.

Ellis, A.E., Roberts, R.J. and Tytler, P. 1989. The anatomy and physiology of teleosts. pp 13 – 55. *In*: R.J. Roberts (ed.) Fish pathology. 2nd edition. Baillière Tindall, London.

Evans, R.E., Brown, S.B. and Hara, T.J. 1988. The effects of aluminium and acid on the gill morphology in rainbow trout, *Oncorhynchus mykiss*. Environmental Biology of Fishes **22** (4): 299 – 311.

Fänge, R. 1992. Fish blood cells. pp 2 – 46. *In*: W.S. Hoar, D.J. Randall and A.P. Farrell (eds.) Fish physiology. **XII** (B) The cardiovascular system. Academic Press, Inc. San Diego, Califonia.

Fänge, R. and Nilsson, S. 1985. The fish spleen: structure and function. Experientia **41**: 152 – 158.

Felts, P.A. and Heath, A.G. 1984. Interactions of temperature and sub-lethal environmental copper exposure on the energy metabolism of bluegill, *Lepomis macrochirus* Rafinesque. Journal of Fish Biology **25**: 445 – 453.

Fournie, J.W., Summers, J.K., Courtney, L.E. and Engle, V.D. 2001. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. Journal of Aquatic Animal Health **13**: 105 – 116.

Gardner, R.G and LaRoche, G. 1973. Copper induced lesions in estuarine teleosts. Journal of the Fisheries Research Board of Canada **30** (3): 363 – 368.

Gbem, T.T., Balogum, J.K., Lawal, F.A. and Annune, P.A. 2001. Trace metal accumulation in *Clarias gariepinus* (Teugels) exposed to sublethal levels of tannery effluent. The Science of the Total Environment **271**: 1- 9.

Gogal Jr., R.M., Smith B.J., Robertson, J.L., Smith, S.A. and Holladay, S.D. 1999. Tilapia (*Oreochromis niloticus*) dosed with azathioprine display immune effects similar to those seen in mammals, including apoptosis. Veterinary Immunology and Immunopathology **68**: 209 – 227.

Goyer R.A. 1996. Toxic effects of metals. pp 691 – 736. *In*: C.D. Klaassen (ed.) Casarett and Doull's toxicology: The basic science of poisons. 5th edition. McGraw-Hill Health Progressions Division, New York.

Gregus, Z. and Klaassen, C.D. 1996. Mechanisms of toxicology. pp 35 – 74. *In*: C.D. Klaassen (ed.) Casarett and Doull's toxicology: The basic science of poisons. 5th edition. McGraw-Hill Health Progressions Division, New York.

Griffin, B.R., Hobbs, M.S., Gollon, J.L., Schlenk, D., Kadlubar, F.F. and Brand, C.D. 1997. Effect of waterborne copper sulphate exposure on copper content in liver and axial muscle of channel catfish. Journal of Aquatic Animal Health **9**: 144 – 150.

Gupta, A.K. and Rajbanshi, V.K. 1981. Measurement of acute toxicity of copper to the freshwater teleost, *Mystus bleekeri* (DAY) using bioassay, statistical and histopathological methods. Archives of Hydrobiology **91** (4): 427 – 434.

Hara, T.J. 1986. Role of olfaction in fish behaviour. pp 152 – 176. *In*: T. J. Pitcher (ed.) The behaviour of teleost fishes. Croom Helm Ltd. London.

Heath, A.G. 1995. Water pollution and fish physiology. 2nd edition. Lewis publishers, Boca Raton, Florida. 359 pp.

Hibiya, T., Yokote. M., Oguli, M., Sato, H., Takashima, F., Aida, K. 1982. Introduction, Digestive System – Liver. pp 1 – 7, 82 – 93. *In*: T. Hibiya (ed.) An atlas of fish histology: normal and pathological features. Kodansha Ltd Tokyo.

Hinton, D.E. and Laurén, D.J. 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. American Fisheries Society Symposium **8**: 145 – 166.

Hoar, W.S. and Randall, D. J. (eds.). 1969 – 1985. Fish physiology. Volumes 1 – 10.

Hodson, P.V., Borgmann, U. and Shear, H. 1979. Toxicity of copper to aquatic biota. pp 307 – 372. *In*: J.O. Nriagu (ed.) Copper in the environment. Part 2: Health effects. John Wiley and Sons, New York.

Holladay, S.D., Smith, S.A., El-Habback, H. and Caceci, T. 1996. The influence of Chlorpyrifos, an organophosphate insecticide, on the immune system of tilapia (*Oreochromis niloticus*). Journal of Aquatic Animal Health 8: 104 – 110.

ICA (International Copper Association). 1995. The biological importance of copper: a literature review. ICA Project 223. 106 pp.

Iwama, G.K. and Farrell, A.P. 1998. Disorders of the cardiovascular and respiratory systems. pp 245 – 278. *In*: J.F. Leatherland and P.T.K. Woo (eds.) Fish diseases and disorders. Volume 2: Non-infectious disorders. CABI Publishing, Wallingford, UK.

James, R., Sampath, K. and Selvamani, P. 1998. Effect of EDTA on reduction of copper toxicity in *Oreochromis mossambicus* (Peters). Bulletin of Environmental Contamination and Toxicology **60**: 487 – 493.

Jezierska, B. and Slomińska, I. 1997. The effect of copper on common carp L (*Cyprinus carpio*) during embryonic and post embryonic development. Polish Archives of Hydrobiology **44** (1-2): 261 – 272.

Jones, J.R.E. 1964. Fish and river pollution. Butterworth and Co. Ltd. London. 203 pp.

Kallanagoudar, Y.P. and Patil, H.S. 1997. Influence of water hardness on copper, zinc and nickel toxicity to *Gambusia affinis* (B and G). Journal of Environmental Biology **18** (4): 409 – 413.

Kägi, J.H.R. and Schäffer, A. 1988. Biochemistry of metallothionein. Biochemistry **27**: 8509 – 8511.

Kendall, R.J., Bens, C.M., Cobb III, G.P., Dickerson, R.L., Dixon, K.R., Klaine, S.J., Lacher Jr., T.E., La Point, T.W., McMurry, S.T., Noblet, R. and Smith, E.E. 1996. Aquatic and terrestrial toxicology. pp 883 – 906. *In*: C.D. Klaassen (ed.) Casarett and Doull's toxicology: The basic science of poisons. 5th edition. McGraw-Hill Health Progressions Division, New York.

Kleerekoper, H., Waxman, J.B. and Matis, J.H. 1973. Interaction of temperature and copper ions as orienting stimuli in the locomoter behaviour of the goldfish (*Carassius auratus*). Journal of the Fisheries Research Board of Canada **30** (6) 725 – 728.

Kleerekoper, H., Westlake, G.F., Matis, J.H. and Gensler, P.J. 1972. Orientation of goldfish (*Carassius auratus*) in response to a shallow gradient of a sublethal concentration of copper in an open field. Journal of the Fisheries Research Board of Canada **29**: 45 - 54.

Kim, S.D., Gu, M.B, Allen, H.E. and Cha, D.K. 2001. Physiochemical factors affecting the sensitivity of *Ceriodaphnia dubia* to copper. Environmental Monitoring and Assessment **70** (1-2): 105 – 116.

Kotze, P., Du Preez, H.H. and van Vuren, J.H.J. 1999. Bioaccumulation of copper and zinc in *Oreochomis mossambicus* and *Clarias gariepinus* from the Olifants River, Mpumalanga, South Africa. Water SA **25** (1): 99 – 110.

Kroes, R. 1988. Risk evaluation: the merging of science and art. pp 9 – 17. *In*: H.A.M. de Kruijf, D. de Zwart, P.N. Viswanathan and P.K. de Ray (eds.) Manual on aquatic ecotoxicology. Allied Publishers Private Ltd, New Delhi, India.

Kumar, S. and Pant, S.C. 1981. Histopathological effects of acutely toxic levels of copper and zinc on gills, liver and kidney of *Puntius conchonius* (Ham). Indian Journal of Experimental Biology **19**: 191 – 194.

Leatherland, J.F. and Woo, P.T.K. 1998. Glossary. pp 367 – 377. *In*: J.F. Leatherland and P.T.K. Woo (eds.) Fish diseases and disorders. Volume 2: Non-infectious disorders. CABI Publishing, Wallingford, UK.

Lett, P.F., Farmer, G.J. and Beamish, F.W.H. 1976. Effect of copper on some aspects of the bioenergetics of rainbow trout (*Oncorhynchus mykiss*). Journal of the Fisheries Research Board of Canada **33**: 1335 – 1342.

Lin, H.C. and Dunson, W.A. 1993. The effect of salinity on the acute toxicity of cadmium to the tropical estuarine hermaphroditic fish, *Rivulus marmoratus*: a comparison of Cd, Cu, and Zn tolerance with *Fundulus heteroclitus*. Archives of Environmental Contamination and Toxicology **25**: 41 – 47.

Lloyd, R. 1992. Pollution and freshwater fish. Fishing New Books, Oxford. 176 pp.

Luna, L.G. 1968. Manual of histologic staining methods of the armed forces institute of pathology. 3rd edition. McGraw-Hill, New York. 258 pp.

Mason, C.F. 1981. Biology of freshwater pollution. Longman. London. 250 pp.

Mallatt, J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Canadian Journal of Fisheries and Aquatic Sciences **42**: 630 – 648.

McKim, J.M. and Benoit, D.A. 1971. Effect of long-term exposure to copper on survival, growth, and reproduction of brook trout (*Salvelinus fontinalis*). Journal of the Fisheries Research Board of Canada **28**: 655 – 662.

McKim, J.M., Christensen, G.M. and Hunt, E.P. 1970. Changes in the blood of brook trout (*Salvelinus fontinalis*) after short-term and long-term exposure to copper. Journal of the Fisheries Research Board of Canada **27** (10): 1883 - 1889.

Merck, E. 1974. The testing of water. E. Merck, Darmstadt. 231 pp.

Merron, G.S., Weldrick, S.K., Kaiser, H. and Bruton, M. N. 1994. The third fisheries survey of the Phongolo flood plain, South Africa. JLB Smith Institute of Ichthyology. Investigational report No. 47, 40 pp.

Metcalfe, C.D. 1998. Toxicopathic responses to organic compounds. pp 133 – 162. *In*: J.F. Leatherland and P.T.K. Woo (eds.) Fish diseases and disorders. Volume 2: Non-infectious disorders. CABI Publishing, Wallingford, UK.

Miller P.A., Munkittrick K.R., and Dixon D.G. 1992 Relationship between concentrations of copper and zinc in water, sediment, benthic invertebrates, and tissues of white sucker (*Catostomus commersoni*) at metal-contaminated sites. Canadian Journal of Fisheries and Aquatic Sciences **49**: 978 – 984.

Mis, J., Bieniarz, K., Epler, P., Sokolowska-Mikolajczyk, M. and Chyb, J. 1995. Incubation of fertilized common carp L (*Cyprinus carpio*) eggs in different concentrations of copper. Polish Archives of Hydrobiology **42** (3): 269 – 276.

Moriarty, F. 1988. Ecotoxicology: The study of pollutants in the ecosystems. 2nd edition. Academic Press Ltd., London. 289 pp.

Moslen, M.T. 1996. Toxic responses of the liver. pp 403 – 416. *In*: C.D. Klaassen (ed.) Casarett and Doull's toxicology: The basic science of poisons. 5th edition. McGraw-Hill Health Progressions Division, New York.

Mount, D.I. 1968. Chronic toxicity of copper to the fathead minnows (*Pimephales promelas*, Rafinesque). Water Research **2**: 215 – 223.

Nelson, K., Jones, J., Jacobson, S. and Reimschuessel, R. 1999. Elevated blood urea nitrogen levels in Goldfish as an indicator of gill dysfunction. Journal of Aquatic Animal Health **11** (1): 52 - 60.

Nussey, G., van Vuren, J.H.J. and Du Preez, H.H. 1999 Bioaccumulation of aluminium, copper, iron and zinc in the tissues of the moggel from Witbank Dam, Upper Olifants River Catchment (Mpumalanga). South African Journal of Wildlife Research **29** (4): 130 – 144.

O'Hara, J. 1971. Alterations in oxygen consumption by bluegills exposed to sublethal treatment with copper. Water Research **5**: 321 – 327.

Olsson, P.E. 1998. Disorders associated with heavy metal pollution. pp. 105 – 132. *In*: J.F. Leatherland and P.T.K. Woo (eds.) Fish diseases and disorders. Vol. **2:** Non-infectious disorders. CABI Publishing, Wallingford, UK.

Onwumere, B.G. and Oladimeji, A.A. 1990. Accumulation of metals and histopathology in *Oreochromis niloticus* exposed to treated NNPC Kaduna (Nigeria). Ecotoxicology and Environmental Safety **19**: 123 – 134.

Perkins, E.J., Griffin, B., Hobbs, M., Gollon, J., Wolford, L. and Schlenk, D. 1997. Sexual differences in mortality and sublethal stress in channel catfish following a 10-week exposure to copper sulphate. Aquatic Toxicology **37**: 327 – 339.

Playle, R.C. and Dixon, D.G. 1993. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Canadian Journal of Fisheries and Aquatic Sciences **50**: 2667 – 2677.

Prasada, R.D.G.V. and Khan, M.A.Q. 2000. Zebra mussels: enhancement of copper toxicity by high temperature and its relationship with respiration and metabolism. Water Environment Research **72** (2): 175 – 178.

Pynnönen. 1995. Effects of pH, hardness and maternal pre-exposure on the toxicity of Cd, Cu and Zn to the glochidial larvae of a freshwater clam *Anodonta cygnea*. Water Research **29** (1): 247 – 254.

Randall, D.J. 1970. Gas exchange in fish. pp 253 – 292. *In*: W.S. Hoar and D.J. Randall (eds.) Fish physiology, **IV**, Academic Press, New York.

Reimschuessel, R., Bennett, R.O. and Lipsky, M.M. 1992. A classification system for histological lesions. Journal of Aquatic Animal Health **4**: 135 – 143.

Robinson, J. and Avenant-Oldewage, A. 1997. Cr, Cu, Fe and Mn bioaccumulation in some organs and tissues of *Oreochromis mossambicus* from the lower Olifants River, inside the Kruger National Park. Water SA **23** (4): 387 – 403.

Roberts, R.J. 1975. Melanin-containing cells of teleost fish and their relation to disease. pp 399 – 428. *In*: W.E. Ribelin and G. Migaki. (eds.) The pathology of fishes. The University of Wisconsin Press, London.

Roberts, R.J. 1989. The pathophysiology and systematic pathology of teleosts, pp 56 – 134. *In*: R.J. Roberts (ed.) Fish pathology. 2nd edition. Baillière Tindall, London.

Saucier, D., Astic, L., Rioux, P. and Godinot, F. 1990. Histopathological changes in the olfactory organ of rainbow trout (*Oncorhynchus mykiss*) induced by early chronic exposure to a sublethal copper concentration. Canadian Journal of Zoology **69**: 2239 – 2245.

Schlenk, D., Davis, K.B. and Griffin, B.R. 1999. Relationship between expression of hepatic metallothionein and sublethal stress in channel catfish following acute exposure to copper sulphate. Aquaculture **177**: 367 – 379.

Schroeder, H.A., Nason, A.P., Tipton, I.H and Balassa, J.J., 1996. Essential trace metals in man: copper. Journal of Chronic Diseases **19**: 1007 – 1034.

Sellers, C.C.M., Heath, A.G., and Bass, M.L**.** 1975. The effect of sub-lethal concentrations of copper and zinc on ventilatory activity, blood oxygen, and pH in rainbow trout (*Oncorhynchus mykiss*). Water Research **9**: 401 – 408.

Skjelkvåle, B.L., Andersen, T., Fjeld, E., Mannio, J., Wilander, A., Johansson, K., Jensen, J.P. and Moiseenko, T. 2001. Heavy metal surveys in Nordic lakes; concentrations, geographic patterns and relation to critical limits. Ambio **30** (1): 2 – 10.

Stagg, R.M. and Shuttleworth, T.J. 1982. The accumulation of copper in *Platichthys flesus* L. and its effects on plasma electrolyte concentrations. Journal of Fish Biology **20**: 491 – 500.

Steele, C.W. 1983. Effects of exposure to sublethal copper on the locomotor behaviour of the sea catfish *Arius felis*. Aquatic Toxicology **4**: 83 – 93.

Sultan, S. and Khan, S.M. 1983. Histopathological studies on the liver and gills in *Carassius auratus* exposed to copper sulphate. Indian Journal of Fisheries 30 (1): 96 – 98.

Takashima, F. 1982. Vascular system: spleen. pp 62 – 64. *In*: T. Hibiya (ed.) An atlas of fish histology: normal and pathological features. Kodasha Ltd. Tokyo.

Tao, S., Wen, Y., Long, A., Dawson, R., Cao, J. and Xu, F. 2001. Simulation of acid-base condition and copper speciation in the fish gill microenvironment. Computers and Chemistry **25**: 215 – 222.

Urani, C., Melchioretto, P., Morazzoni, F., Canevali, C. and Camatini, M. 2001. Copper and zinc uptake and hsp70 expression in HepG2 cells. Toxicology in Vitro **15**: 497 – 502.

USEPA (United States Environmental Protection Agency). 1980. Ambient water quality for copper. U.S. Environmental Protection Agency Report 440/5-80-036. pp B 1-67.

Usha Rani, A. and Ramamurthi, R. 1989. Histopathological alterations in the liver of freshwater teleost *Tilapia mossambica* in response to cadmium toxicity. Ecotoxicology and Environmental Safety **17**: 221 – 226.

van den Heever, D.J. and Frey, B.J. 1994. Human health aspects of the metals zinc and copper in tissue of the African sharptooth catfish, *Clarias gariepinus*, kept in treated sewage effluent and in the Krugersdrift dam. Water SA **20** (3): 205 – 212.

Veith, G.D., DeFoe, D.L. and Bergstedt, B.V. 1979. Measuring and estimating the bioconcentration factor of chemicals in fish. Journal of the Fisheries Research Board of Canada **36**: 1040 – 1048.

Waiwood, K.G. and Beamish, F.W.H. 1978. The effect of copper, hardness and pH on the growth of rainbow trout, *Oncorhynchus mykiss*. Journal of Fish Biology **13**: 591 – 598.

Warren, C.E. 1971. Biology and water pollution control. W.B. Saunders Company. Philadelphia, Toronto. pp 11 - 14.

Weeks, B.A., Warinner, J.E., Mason, P.L. and Mcginnis, D.S. 1986. Influence of toxic chemicals on the chemotactic response of fish macrophages. Journal of Fish Biology **28**: 653 – 658.

Westlake, G.F., Kleerekoper, H., and Matis, J.H. 1974. The locomotor response of goldfish to a steep gradient of copper ions. Water Resources Research **10**: 103 – 105.