Fish health, condition and biomarkers: a mechanistic and environmental perspective on copper pollution

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Thesis submitted in fulfilment of the requirements of the degree Philosophiae Doctor in Zoology of the North-West University, Potchefstroom Campus

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The world turns softly
Not to spill its lakes and rivers,
The water is held in its arms
And the sky is held in the water.
What is water,
That pours silver,
And can hold the sky?

Hilda Conkling

Preface

The experimental work conducted and discussed in this thesis was carried out in the School for Environmental Sciences and Development, Zoology, North-West University, Potchefstroom, South Africa and the Laboratory of Animal Physiology, University of Turku, Finland. The study was conducted during the period of August 2001 to November 2004 under the supervision of Dr. André Vosloo, North-West University and co-supervision of Prof. Mikko Nikinmaa, University of Turku.

This thesis is presented as a compilation of published papers and unpublished manuscripts, where each paper is an individual entity and some repetitions between the papers have been unavoidable. The research conducted represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. Appropriate acknowledgements in the text have been made where the use of work conducted by other researchers have been included. Permission of the co-author(s) of the papers/manuscripts used in the study has been included.

The references in this thesis have been listed according to the specifications given by the Council of Biology Editors (CBE) Scientific Style (http://writing.colostate.edu/referen-

ces/sources/cbe/index.cfm), using the name-year system. The references for the respective manuscripts were written according to the guide to authors of the journals where the manuscripts will be submitted to. The guides to authors for each manuscript have been included.

This thesis is based on the following publications, which will be referred to in the text by Roman numerals:

I. Van Heerden D, Vosloo A, Nikinmaa M. (2004) Effects of short-term copper exposure on gill structure, metallothionein and hypoxia inducible factor-1α (HIF-1α) in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 69 : 271-280.

II. Van Heerden D, Tiedt, L and Vosloo A. (2004) Gill damage in *Oreochromis mossambicus* and *Tilapia sparrmanii* after short term copper exposure. In: A Vosloo and S Morris, editors. Animals and Environment. International Congress Series 1275C:195-200., Amsterdam: Elsevier.

III. Van Heerden D, Vosloo A, Nikinmaa M. *Tilapia sparrmanii* and *Oreochromis mossambicus* metallothionein expression in response to short term copper exposure (to be submitted to *Aquatic Toxicology*).

IV. Van Heerden D, Jansen van Rensburg P, Vosloo A and Nikinmaa M. Gill damage, metallothionein gene expression and metal accumulation in *Tilapia sparrmanii* from selected field sites in Rustenburg and Potchefstroom, South Africa (to be submitted to *African Journal of Aquatic Science*).

The papers represent original research conducted by the author of the thesis. The papers have been written in whole by the student, with co-authors being responsible for assistance in final editing, except for ICP-MS analysis, performed by Peet Jansen van Rensburg (Paper IV).

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Manuscripts of papers III and IV are preceded by *Instructions to Authors* of the relevant Journals.

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TO WHOM IT MAY CONCERN

12 May 2006

Dear Sir / Madam

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned, as co-authors of the research articles listed below, hereby give permission to Ms. Daléne van Heerden to submit the papers as part of the degree Ph.D. in Zoology at the North-West University, Potchefstroom campus.

I. Van Heerden D, Vosloo A, Nikinmaa M. (2004) Effects of short-term copper exposure on gill structure, metallothionein and hypoxia inducible factor-1 α (HIF-1α) in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 69:271-280.

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IV. Van Heerden D, Janse van Rensburg P, Vosloo A and Nikinmaa M. Gill damage, metallothionein gene expression and metal accumulation in *Tilapia sparrmanii* from selected field sites in Rustenburg and Potchefstroom, South Africa.

Yours sincerely,

Dr. A. Vosloo

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In accordance with section 23(1) of the Higher Education Act, 1997 (Act No. 101 of 1997), as amended, the Potchefstroom University for Christian Higher Education and the University of North-West merged to form the North-West University on 1 January 2004. In accordance with section 24(1) of the Higher Education Act, 1997 (Act No. 101 of 1997) as amended, the staff and students of the Sebokeng Campus of the Vista University were incorporated into the North-West University on 2 January 2004.

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Finally, yet importantly, I would like to thank my Creator for the enormous opportunity to study and explore His creation. *"Nature is the glass reflecting God, as by the sea reflected is the sun, too glorious to be gazed on in his sphere" – Brigham Young*

Opsomming

Suid-Afrika het beperkte waterhulpbronne, wat tot gevolg het dat die beskikbare water van baie hoë kwaliteit moet wees. Ongelukkig word die kwaliteit van ons hulpbronne deur antropologiese impakte bedreig.

Tot baie onlangs het roetine monitering van natuurlike akwatiese stelsels slegs die fisies-chemiese eienskappe in ag geneem. Biologiese monitering is egter baie voordelig omdat dit die algemene integriteit van akwatiese sisteme reflekteer. Dit kan soms 'n baie goeie aanduiding gee van die langtermyn-effek van korttermyn-impakte.

Die doel van hierdie studie was om die effek van koper op sekere aspekte van visfisiologie gedurende laboratoriumblootstellings te ondersoek. Dieselfde parameters is ook in vis wat in riviere in die Noordwes Provinsie voorkom, gemeet om te bepaal of daardie parameters as moontlike biomerkers van hoë omgewingskopervlakke gebruik kan word.

Gedurende die laboratoriumblootstellings (4 en 24 uur blootstelling, gevolg deur 'n 48 uur herstelperiode) op reenboogforel (*Oncorhynchus mykiss*), bloukurper (*Oreochromis mossambicus*) en vleikurper (*Tilapia sparrmanii*) is die volgende resultate verkry:

(1) Kieuskade, soos aangedui deur 'n verhoging in die gemiddelde epiteeldikte in sekondêre kieulamellae (H_{ar}) het na kort blootstelling aan hoë kopervlakke in al drie visspesies voorgekom. Die reaksie van *O. mossambicus* and *T. sparrmanii* was minder intens en effe stadiger as dié van *O. mykiss*. Die verhoging in epiteeldikte in die kieue het tot gevolg dat die vis hipoksiese toestande ondervind, soos aangedui deur die opeenhoping van Hipoksie-induseerbare faktor-1α (HIF-1α) in die kieue van *O. mykiss* vroeg in die blootstellingstydperk. Die epiteeldikte het egter na die 48 uur herstelperiode weer verminder; (2) Induksie van metallotionien geenuitdrukking het tydens die blootstellingstydperk in die kieue van al drie visspesies voorgekom en weer na die 48 uur herstelperiode vlakke naby aan die van kontrolevisse bereik. Die induksie in die kieuweefsel van was na 4 uur blootstelling in *O. mykiss* en *O. mossambicus* reeds statisties beduidend hoër as in kontrolevisse. Geen induksie is in die lewerweefsel van enige van die visspesies aangetref nie; (3) Alhoewel koper by die water van blootstellingstenks gevoeg is, was daar geen beduidende koperakkumulasie in die kieu- en lewerweefsel van enige van die visspesies nie.

Visse wat in Boskopdam, Potchefstroom gevang is het 'n hoër graad van kieuskade gehad as in Klerkskraaldam, wat verklaar word deur die kopervlakke in die viskieue. Daar was egter geen beduidende verskil in die metallotionieninduksie in die lewer en kieue van die visse van enige van die opnamepersele nie. Volgens 'n meervoudige regressie, kan die variasie in beide kieu- en lewerweefsel deur die teenwoordigheid van kadmium by die persele verklaar word.

Daar kan uit bogenoemde resultate afgelei word dat die graad van verdikking van kieuepiteel 'n baie sensitiewe respons gedurende koperblootstelling is. Die tegniek is ook baie goedkoop en maklik om te gebruik. Metallotionieninduksie is 'n duurder tegniek en minder sensitief waneer dit in die omgewing gebruik word. Dit is egter baie sensitief gedurende laboratoriumblootstellings aan koper. Hipoksie-induseerbare faktor-1α proteinakkumulering kan moontlik 'n baie sensitiewe indikator van hipoksie, veroorsaak deur die verhoging in die diffusie-afstand tussen bloed en water, in *O. mykiss* wees. Die antiliggaam teen Hipoksie-induseerbare faktor-1α (HIF-1α) in die Tilapia-agtige visse moet nog geoptimaliseer word.

Sleutelwoorde: Biomerkers; Metallotionien; Kieuskade; Hipoksie-induseerbare faktor-1α (HIF-1α); Koper; Reenboogforel; *Oncorhynchus mykiss*; Bloukurper; *Oreochromis mossambicus*; Vleikurper; *Tilapia sparrmanii*

During the laboratory exposures (4 and 24 h exposure with 48 h recovery after the exposure) on rainbow trout (*Oncorhynchus mykiss*), Mozambique tilapia (*Oreochromis mossambicus*) and banded tilapia (*Tilapia sparrmanii*) the following observations were made: (1) Gill damage, as indicated by an increase in the arithmetic epithelial thickness of secondary gill lamellae (H_{ar}), occurred after short term exposure to high levels of copper in all three fish species. *O. mossambicus* and *T. sparrmanii* had slower and/ less intense responses to higher levels of copper than *O. mykiss*. The increase in epithelial thickness in gills causes the fish to experience hypoxia, as indicated by the accumulation of Hypoxia inducible factor-1α (HIF-1α) in the gills of *O. mykiss*; (2) Induction in metallothionein gene expression occurred in all three fish species, with levels in *O. mykiss* and *Oreochromis* mossambicus gills being significantly higher than control values after only 4 h of exposure. Again *T. sparrmanii* reacted slower. No significant induction in metallothionein gene expression was detected in the liver of any of the species; (3) Although copper was added to the water of experimental tanks, there were no significantly higher levels of copper in gills and liver from any of the exposed animals at any time during the experiments.

During the field based studies it was detected that fish from Boskop Dam, Potchefstroom had a higher degree of gill damage than fish from Klerkskraal Dam, Potchefstroom, which was explained by the levels of copper in the gills. There were no significant trends in either gill or liver metallothionein levels between any of the field sites, although according to a multiple regression cadmium was indicated to have a influence on the metallothionein levels measured in fish from the selected sites.

It could be concluded that determining the degree of thickening of gill epithelium is a sensitive indicator of the overall response of fish to especially copper exposure during this study. The technique is easy and inexpensive to use. Metallothionein gene expression is more expensive, and probably not sensitive enough when lower levels of metals are found in rivers. It is, however, very sensitive in experimental situations, under controlled circumstances. Although the accumulation of HIF-1α promises to be a sensitive indicator of hypoxia, caused by the increased water-to-blood barrier due to increased gill epithelial

thickness in *O. mykiss*, properly working antibody against HIF-1α in the Tilapiines still need to be investigated.

Keywords: Biomarkers; Metallothionein; Gill damage; Hypoxia-inducible factor-1α (HIF-1α); Copper; Rainbow trout; *Oncorhynchus mykiss*; Mozambique tilapia; *Oreochromis mossambicus*; Banded tilapia; *Tilapia sparrmanii*

Abstract

South Africa is a country with limited water resources, where many of the areas with high water demand receive little rainfall. This makes it imperative for our water to be of high quality. Unfortunately our water resources are impacted by anthropological practices including agriculture and mining.

Until recently, routine water monitoring programmes only included monitoring of physical and chemical properties of water bodies, while biological monitoring has the advantage of reflecting the overall integrity of aquatic ecosystems. Some biomarkers can give a clear indication of long term effects of short term impacts.

The aim of this study was to determine the effects of copper on selected aspects of fish physiology during laboratory exposures and to measure the same parameters in fish caught from rivers in the North-West Province to determine if those parameters could be considered as possible biomarkers of environmental copper insult.

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Chapter 1

Introduction

1.1 The water dilemma in South Africa

South Africa is classified as an arid to semi-arid region where the average rainfall is less than 500 mm per annum, with areas with high water demand receiving little rainfall. To add to the dilemma, almost 40 % of the total length of South Africa's rivers is subjected to seasonal flows and land-use and vegetation changes in catchments is causing constant flowing rivers to become seasonal. This and the high evaporation rate cause water to be a very scarce resource in the country (O'Keeffe *et al.* 1994). Although South Africa has rather large quantities of exploitable ground water, their distribution is limited. This causes rivers to be our most vital water resource (Rabie and Day 1994).

According to Davies and Day (1998) South Africa's water demand will exceed the supply between 2003 and 2040. With the slowest estimated population growth, water demand will exceed supply between 2020 (when all surface water will be used) and 2040 (when

both surface and ground water will be used). With the highest population growth, surface water will be fully committed by 2003 and both surface and ground water by 2015. Estimates of when water demand will exceed water supply range between 2005 and 2040 (Davies and Day 1998, Figure 1.1). However, it is not only the quantity, but also the quality of our water which is important. Unfortunately, the availability and quality of our water is being impacted by the following anthropological practices (Rabie and Day 1994):

Agriculture: By 1994, 67% of direct water use in SA could be attributed to agricultural practices. Excessive abstraction of water as well as impounding of water for irrigation leads to a decline in downstream availability of water. In arid areas irrigation leads to salinisation of ground water and, ultimately, of rivers. The destruction of riparian vegetation leads to soil erosion, which causes an increase in the silt load in rivers. Fertilizers and biocides used in agriculture eventually reach rivers and illegal dumping of toxicants in rivers, to control waterborne diseases, has been recorded.

Urbanisation: Sixteen percent of South Africa's water is used for municipal and domestic consumption. Water is impounded at water-storage facilities and natural water resources are impacted by sewage effluent, seepage from urban refuse-disposal sites and by storm water drainage.

Human population concentrated in rural areas: In rural areas people usually concentrate around water while water abstraction, use and return to the source are often not controlled. Sewage and effluent are usually left untreated.

Industrial use of water: Sixteen percent of the direct water use is accounted for by industrial use. Effluents however have a high impact on water resources because they contain highly toxic substances. Industrial water use includes use by mines, manufacturing industries and power generation companies.

Aquaculture: Water loss occurs by means of evaporation and seepage and the quality of the water returned to streams is usually degraded.

Road-building: The construction of roads along or across rivers may impair stream flow.

Recreation: Although recreation may stimulate water conservation because of the need for clean water, recreational activities may cause destabilisation of river banks, destruction of riparian vegetation, littering and water pollution when practicing power boating.

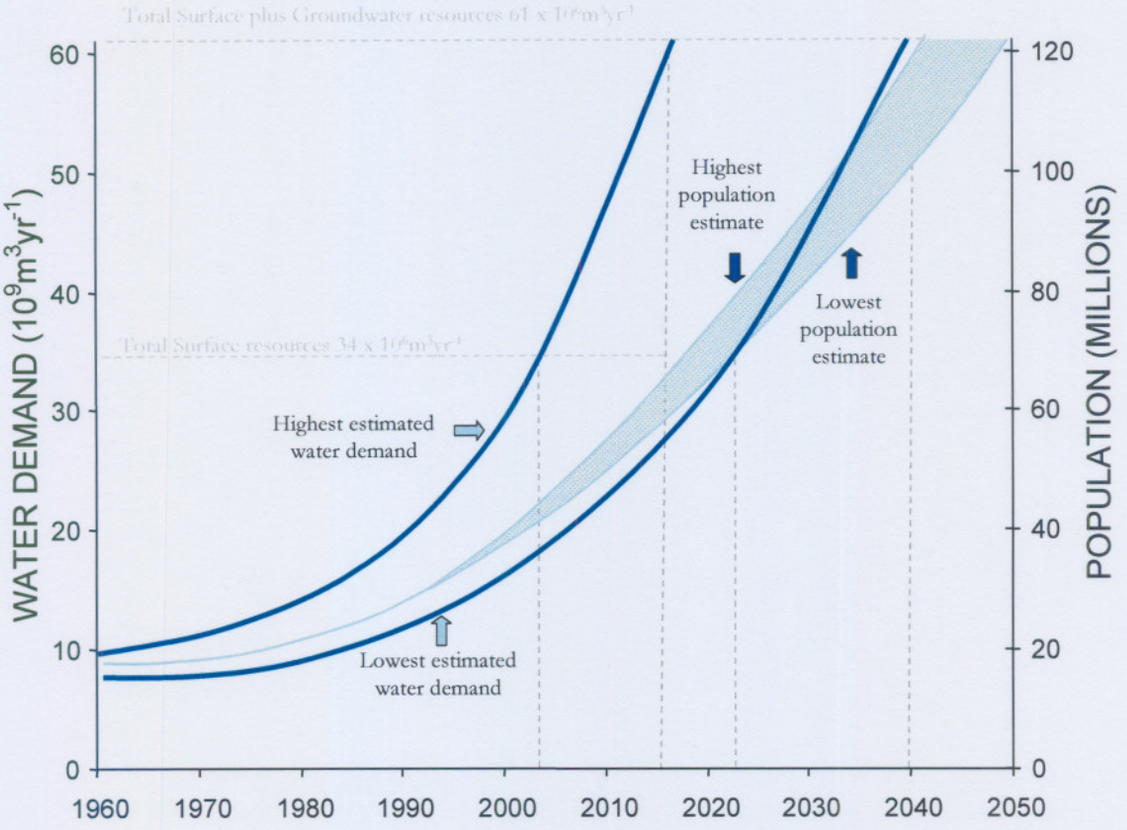

Figure 1.1. The relationship between demand for water and size of the human population of South Africa. The two dotted curves represent the fastest and slowest estimated rates of population growth. The two solid curves are the highest and lowest estimates of the amount of water needed to satisfy human requirements. The amount of surface water available is fairly accurately estimated, but the amount of usable groundwater is based on guesswork (Adapted from Davies and Day 1998).

1.2 Monitoring of water quality

The aquatic environment is often the ultimate reservoir for contaminants, either by direct effluent discharges or by natural hydrologic and/or atmospheric processes (Stegeman and Hahn 1994). As a result of the anthropogenic practices discussed in 1.1, the quality of South Africa's water is becoming an increasing problem. It is therefore important to continuously monitor the state of water sources.

Because of an increased awareness of, and concern for, environmental pollution, since the early sixties, and the subsequent changes in legislation to control unwanted emissions, the demand for water quality monitoring and surveillance schemes is on the increase (Howells 1976; Stegeman and Hahn 1994). Biological monitoring is also starting to play an increasingly important role in the monitoring of aquatic environments.

1.2.1 Chemical *vs.* biological monitoring

Until recently, routine monitoring programmes did not include biological monitoring techniques. They have been introduced due to the following shortcomings in standard chemical and physical measurements: (1) it is difficult and expensive to analyse every possible pollutant in a water sample, whereas biological monitoring is cost effective and usually obtained easily and rapidly; (2) by only performing physical and chemical analysis of the medium one does not get an idea of past and present history of water quality, while biological monitoring reflects both because the exposure of aquatic organisms to pollutants is continuous. Biological monitoring, therefore, allows detection of disturbances in the ecosystem that might have been missed by performing chemical analysis alone; (3) chemical and physical analysis do not reflect the overall integrity of ecosystems. By integrating all possible stressors, monitoring of biological communities allows integrated measure of the integrity and health of rivers (Eekhout *et al.* 1996; Chutter 1998; De La Rey *et al.* 2004).

It is difficult to determine dose/response relationships once toxicants have been identified in a chemical monitoring program. Figure 1.2 gives a schematic view of the steps in pollution investigation and assessment. Dose/response studies requires intensive, detailed studies on all the different life stages of organisms as well as the effects of other parameters on the effect of toxicants on these organisms. Another factor to take into account when studying the effects of toxicant on organisms is that the organisms are seldom (if ever) exposed to constant concentrations of toxicants. This calls for investigations into (1) the effect of different gradients of exposure concentration, taking into account that toxicant levels in the environment often fluctuates, (2) incidents where large amounts of toxicants is released at once into the environment, where animals are

exposed to high toxicant concentrations and have time between incidents to recover and (3) the biological status of organisms together with water quality, where the level of exposure is below the level of acute responses (Howells 1976).

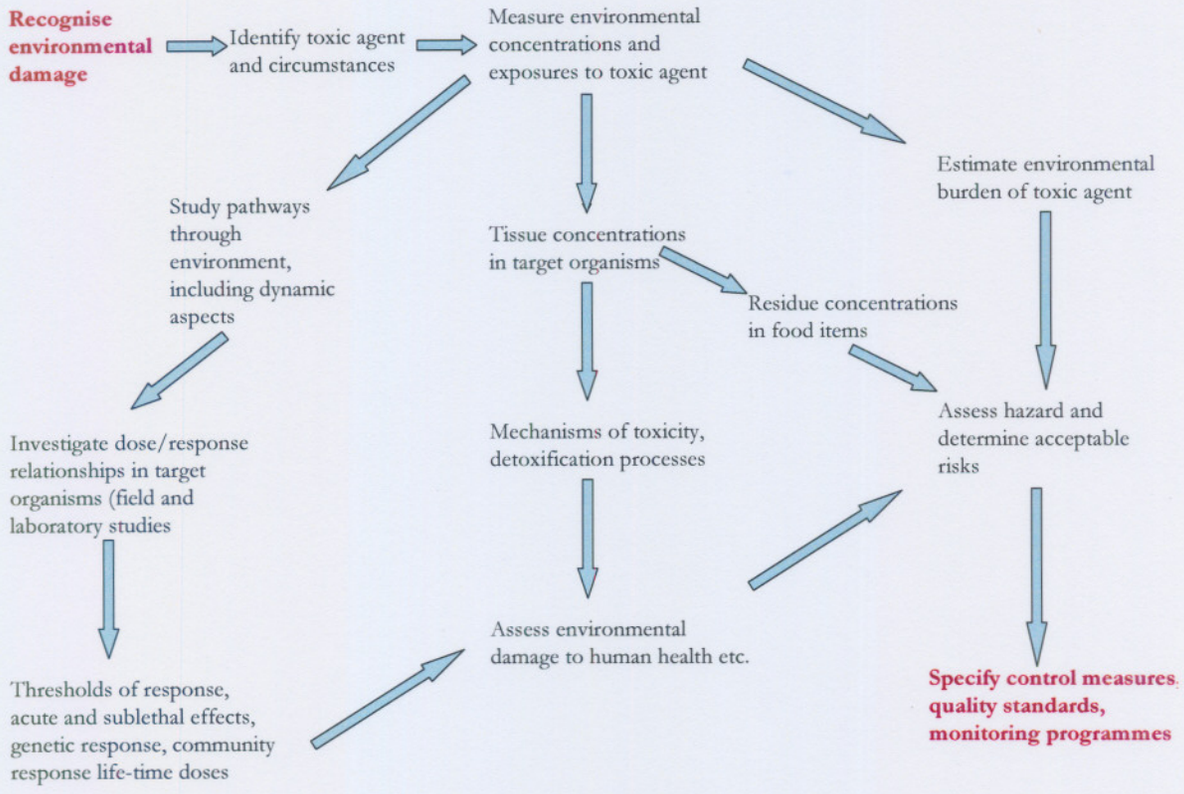

Figure 1.2. Schematic view of the steps in pollution investigation and assessment (Adapted from Howells 1976).

1.2.2 The use of biomarkers in aquatic pollution monitoring

While physical and chemical analysis of water and/or sediment samples from polluted water bodies give the pollutant concentration present in the water, by measuring chemical concentrations in animal tissue one can determine the fraction of the pollutant which is bioavailable and therefore can have an effect on organisms. Because only measuring the pollutant concentration in animal tissue does not give information on the effect toxicants have on the organism, biomarkers should be used in biomonitoring programmes to assess the effects of pollutants on animals. These effects measured are the function of the

toxicity of single chemicals or a mixture of chemicals, as well as the duration of exposure to these chemicals (Lam and Wu 2003).

Aquatic organisms are targets of contaminated water from, amongst other, mining, industrial and agricultural activities. These waters usually contain heavy metals such as copper (Cu), zinc (Zn), cadmium (Cd) and mercury (Hg) (Zafarullah *et al.* 1988). The heavy metal concentrations in natural waters are usually low, with slightly higher levels occurring in rivers and estuaries. When heavy metals from industrial activities are released into rivers, aquatic organisms can easily be exposed to metals at concentrations in excess of background levels normally encountered. Some of these organisms could be more tolerant to metal exposure than others (Bryan 1976). The tolerance of some organisms to high metal concentrations without obviously being affected could be detrimental for human health if these organisms are used as a food source. It is therefore important to develop biomarkers sensitive enough to determine whether an organism is affected by exposure to metals, without showing obvious signs of deterioration in health. These biomarkers can be short-term indicators of long-term toxicant effects, allowing human intervention in aquatic systems before the effects of toxicants become irreversibly detrimental (McCarthy and Shugart 1990). The term *biomarker* refers to any measurement that indicates interactions between biological systems and environmental agents, which can be chemical, physical or biological (WHO 1993). A biomarker can more specifically be defined as "*a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence or exposure to and/or effects of, one or more chemical pollutants (and/or radiations)*" (Depledge 1993). Biomarkers can be divided into three classes namely:

(1) Biomarkers of exposure, where an exogenous substance or metabolite thereof is detected and measured;

(2) Biomarkers of effect, where the effect of the toxicant on biochemical of physiological changes, associated with exposure, can be measured; and

(3) Biomarkers of susceptibility, where the ability of an organism to respond or adapt to toxicant exposure is indicated (WHO 1993).

Although it is difficult to determine the relationship between the level of exposure to xenobiotics and the magnitude by which biomarkers respond, testing for biomarkers can still give important information on whether or not aquatic organisms are affected by the presence of metals in water (Olsvik *et al.* 2001). Biomarker response occurs on different levels of organisation ranging from subcellular level to whole-organisms with molecular level biomarkers responding first, followed by biochemical, physiological and organismal responses. The higher the level of response the less reversible and more damaging it is (Lam and Wu 2003, Figure 1.3).

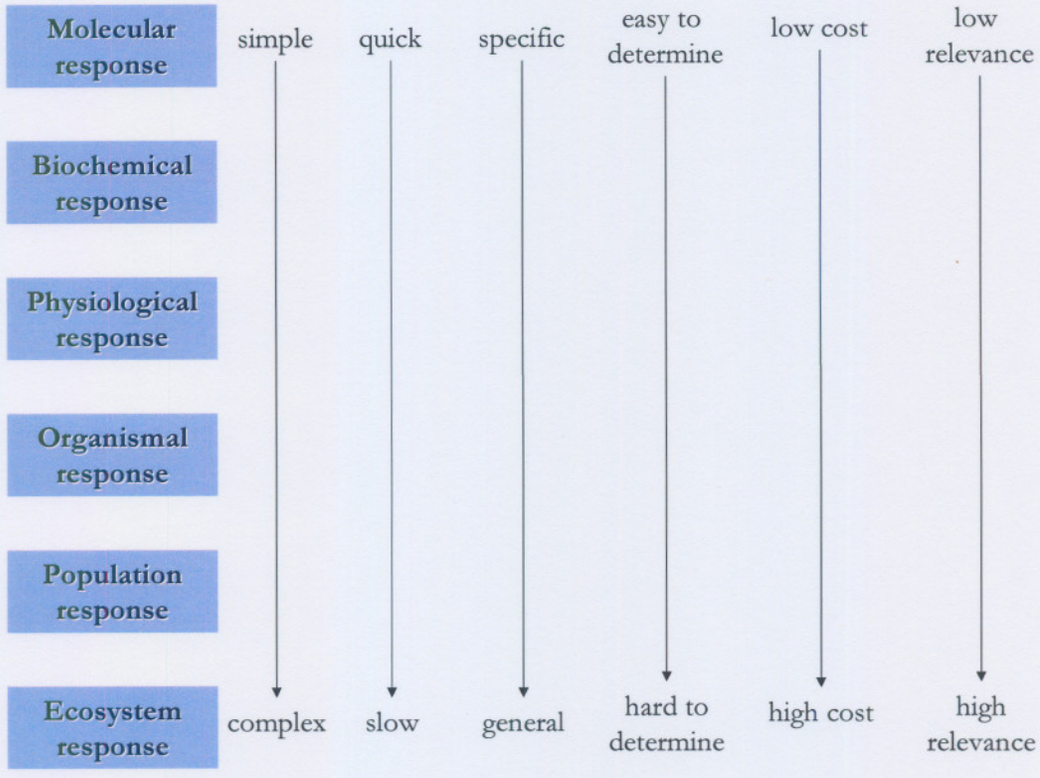

Figure 1.3. The pros and cons of different levels of responses to pollutant exposure (Adapted from Lam and Wu 2003).

Above a certain threshold in either exposure level (dose and time), the response of biomarkers differs from normal range where the animal is not stressed. The intensity of the response can increase until detrimental effects such as impaired reproduction and reduced life span can be observed (Van der Oost *et al.* 2003, Figure 1.4). While the levels of biomarker responses can provide valuable information on the effects of pollutants on

fauna, it is also important (and usually difficult) to determine the relationship between biomarker responses at the different levels of organisation as well as the relevance and time scale of these responses. The biomarker responses at each level of organisation provide valuable information to understand the relationship between exposure and effect and to determine the ecological relevance of biomarkers (Adams *et al.* 1989, Figure 1.5).

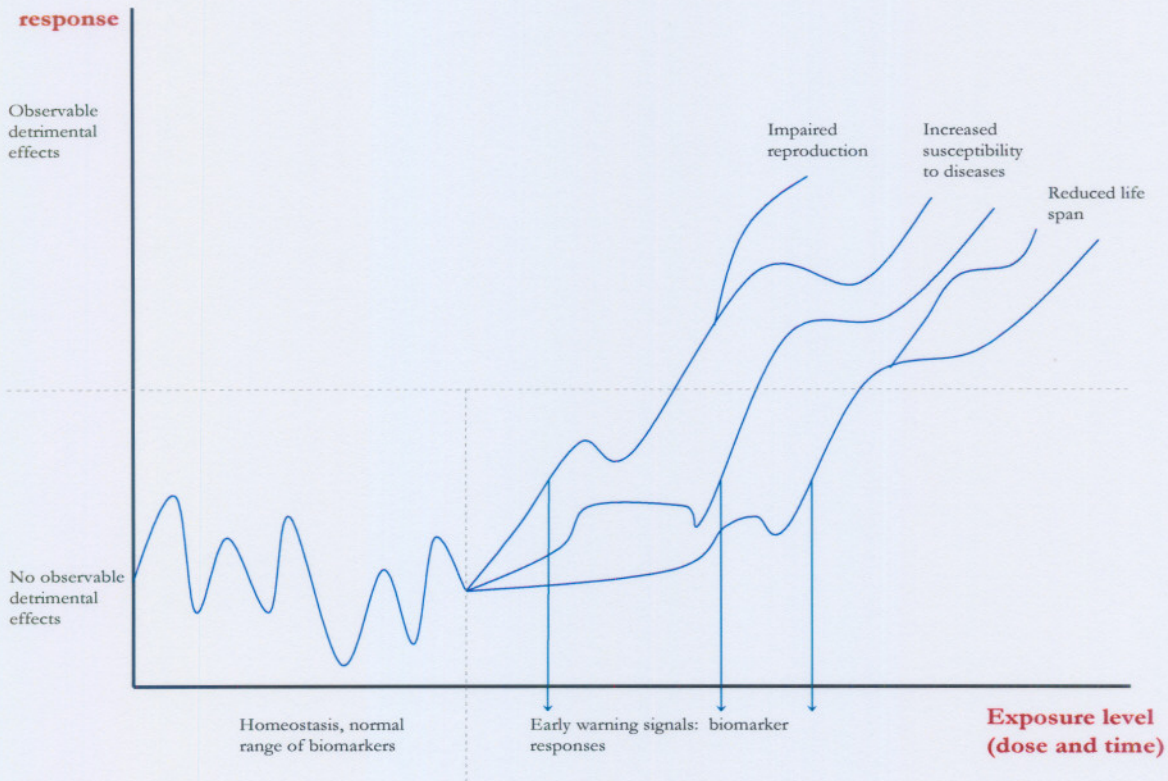

Figure 1.4. Responses to pollutant exposure in organisms' ranges from exposure levels where no effects could be observed to levels where detrimental effects could be seen (Adapted from Van der Oost et al. *2003).*

The toxicity of copper has been studied extensively because of its use in agriculture and water treatment (Schlenck *et al.* 1999). Although Cu is an essential nutrient in numerous enzymatic systems, it becomes toxic when in high concentrations in water (Heath 1995; Cerqueira and Fernandes 2002). High Cu levels overwhelm typical copper homeostasis mechanisms of fish (Schlenk *et al.* 1999). Amongst others, it can cause rapid generation of reactive oxygen species (Harris and Gitlin 1996) and binds histidine-, cystein- and methionine-containing proteins, resulting in dysfunction (Grosell and Wood 2002).

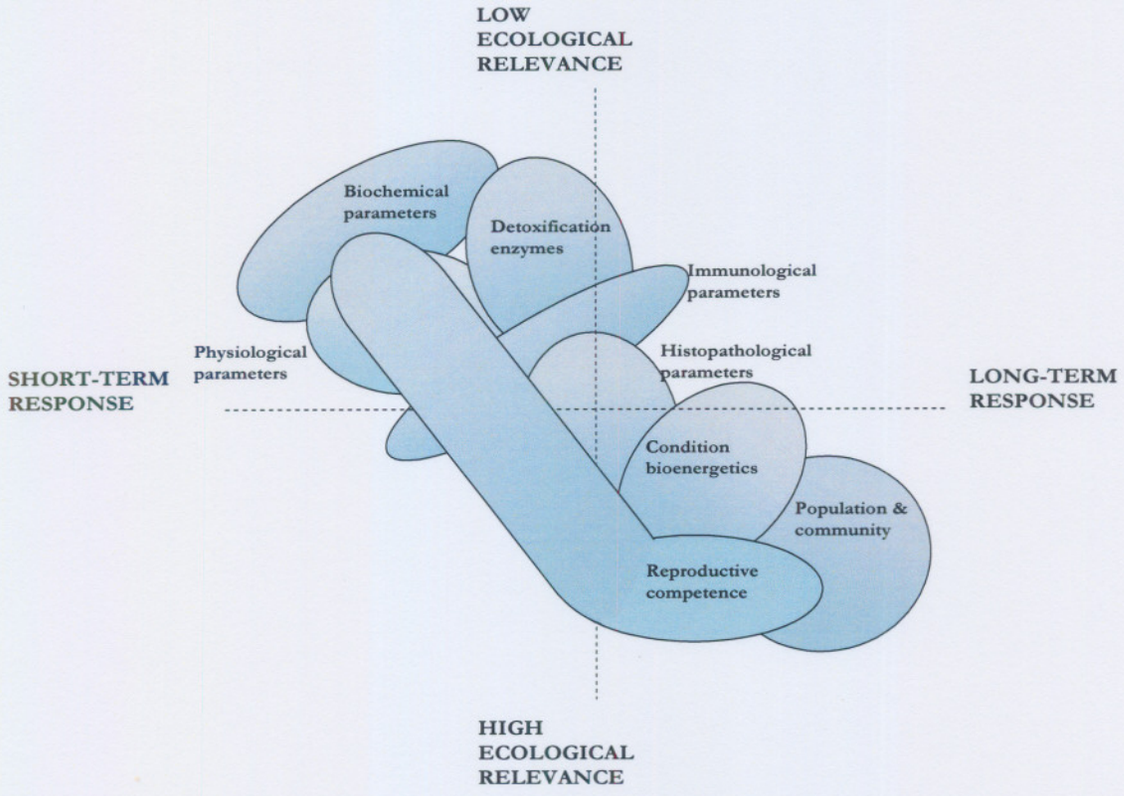

Figure 1.5. Diagram showing the relationship between biomarker responses, time-scale of response and ecological relevance proposed by Adams et al. *(1989).*

The present study focussed on the following effects of Cu on fish physiology:

- Gill damage
- Metal accumulation and metallothionein (MT) induction
- Induction of hypoxia inducible factor (HIF 1α) proteins.

Each of these potential biomarkers will now be discussed in more detail.

1.2.2.1 Gill damage

Fish gills are, due to their large surface area, the prime target for copper in water. In low-sodium water (typically ion-poor soft water) copper will be taken up through the apical sodium pathway in gills, inhibiting sodium (Na) uptake via gills. This is associated with the inhibition of Na^+/K^+ ATP-ase (for review see Wood 2001; Grosell and Wood 2002).

The disruption of brancial ionregulation, due to high copper levels in water, can cause mortality in fish (Laurén and McDonald 1987).

Copper is reported to cause severe gill damage in *Prochilodus scrofa*. The damage included epithelial lifting, cell swelling and chloride and mucus cell proliferation, but showed recovery only after 7 days of recovery from copper exposure for 96 h (Cerqueira and Fernandez 2002). An increase in epithelial thickness, caused by hypertrophy of epithelial cells, was found to be most characteristic of heavy metal exposure (Lappivaara *et al.* 1995, for review see: Mallat 1985). Gill damage can be linked to impaired physiological function (Woodward *et al.* 1983). Histopathology biomarkers are in generally assumed to be useful as an indicator of the general health of fish. It could be used as an early-warning tool for monitoring the effects of an array of anthropogenic pollutants, where the alteration caused by exposure to toxicants can persist even after the exposure has ceased, making it ideal in monitoring long-term effects of short-term exposure (Hinton *et al.* 1992; Hinton 1994; Lease *et al.* 2003).

1.2.2.2 Metal accumulation and metallothionein induction

Certain animals are known to live in water contaminated by toxicants, without showing obvious distress. These animals however, accumulate the contaminants they are exposed to in their bodies. Animals exposed to toxicants acts as natural accumulators, thereby reducing the detection limits required by analytical methods. The levels accumulated also represent long term average contaminant levels and not only the current levels on the day of water/sediment sampling (STAP 2003). By analysing the accumulation of contaminants in aquatic animals both the amount of toxicants taken from the environment and the accumulation of the toxicants could be studied. Because accumulation of toxicants in aquatic animals causes enrichment in body toxicant levels of these animals, species at higher trophic levels could be negatively affected by water pollution (Lam and Wu, 2003).

The effects of heavy metals are organ specific (Pelgrom1994). Freshwater teleosts are known to accumulate Cu in both liver and gills (Sorensen 1991). Pelgrom (1994), however, found no significant increases in liver copper content in rainbow trout within the first 11 days of exposure, where significant copper load was found in both kidneys and gills. Apart from Cu accumulating in gills, liver and kidney, a 59% increase of plasma Cu levels were observed during the first 3 h of copper exposure, whereafter normal levels were detected (Grosell *et al.* 1997).

Metal accumulation is often associated with an increase in both gill and liver MT levels. The induction of MT in response to metals could therefore be an important biomarker used in assessing the magnitude of metal contamination in areas with high mining and industrial activities. MTs are small (6 to 7 kDa), cysteine-rich, nonenzyme proteins. They have been essential for the homeostasis of essential metals (*e.g.*Cu and Zn) while they detoxify non-essential metals like Cd and Hg in biological systems (Cousins 1985; Suzuki and Koizumi 2000). They also act as scavengers of free radicals and reactive oxygen species (Kagi and Shaffer 1988).

Transcription of MT mRNA is upregulated by a number of factors of which metals, such as Cu, Cd and Zn, are the most common inducers (Figure 1.6). Transcriptional induction of MT by metals is mediated by metal-responsive elements (MRE's), which are situated in the promotor region of the MT gene. Differential responses of MT induction to metals are caused by MRE's reacting differently for different metals. MRE's consist of a conserved 7-base pair core sequence (TGC(G/A)CNC) surrounded by semiconservative flanking sequences. It is also possible that mutations in MRE sequences occur, causing either the inactivation of MRE function or flexibility of the MRE consensus nucleotides (Samson and Gedamu 1998).

MT proteins act as chelators through the formation of metal-thiolate bonds (Samson and Gedamu 1995, 1998) after which the metal-MT complexes are transported to the liver and excreted through bile (Cousins 1985). Rainbow trout MT protein concentration is known to increase with exposure to Cu (Kling and Olson 1995). De Boeck *et al.* (2002)

found hepatic MT protein levels to be indicative of long-term Cu exposures in rainbow trout.

The effect of metals on both accumulation of MT proteins and upregulation of MT gene expression have been studied extensively in different fish species, which include rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and zebrafish (*Danio rerio*) (Kling and Olsson 1995; Oslvik *et al.* 2001; De Boeck *et al.* 2002; Riggio *et al.* 2003; Paper I).

Evaluating gene expression at mRNA level instead of protein level poses the advantage that interference from post transcriptional processes, which may influence protein levels or activity, need not be taken into consideration (Kaplan *et al.* 1995). Upregulation of gene expression does not necessarily result in changes in the activity of gene products such as proteins. In experimental treatment for instance, the treatment may lead to changes in gene transcription without changing the concentration of the expressed protein if the treatment influences the stability of the protein or mRNA. This scenario could mean that the level of gene transcription is increased to simply sustain the levels of protein needed to maintain important cellular processes that must be closely regulated for preserving cellular function. This type of gene regulation is just as, if not more, important as regulation where changes in protein concentrations in cells can be observed as result of the upregulation in gene expression (Probansky and Somero 2004). The increase in the level of gene transcription nevertheless indicates that, in the case of metal insult, MT protein levels should at least be maintained at normal levels because metals are bound to the protein and transported to the liver for detoxification, which will decrease the normal protein levels in cells when animals experience metal insult.

Assessing MT gene induction requires that at least the MT gene coding sequence be known to construct species-specific primers. The MT gene coding sequence is known for rainbow trout (*O. mykiss,* GenBank accession numbers X59395 and X59394) and Mossambique tilapia (*Oreochromis mossambicus* GenBank accession number AY257202) and thus it was imperative that the MT gene coding sequence for banded tilapia (*Tilapia sparrmanii*) be investigated in order to use *T. sparrmanii* as experimental animal.

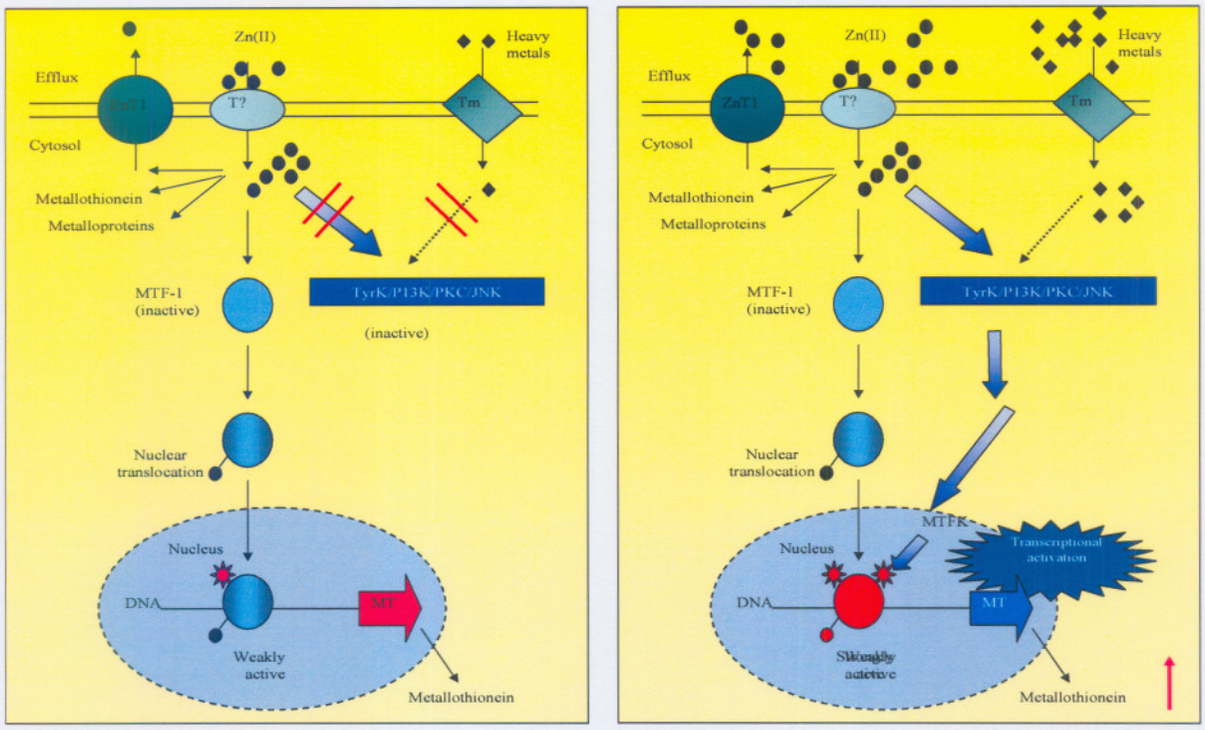

Figure 1.6. Regulation of metallothionein transcription factor (MTF-1) activity and metallothionein gene expression under basal and induced conditions in mice. Under basal conditions Zn enters the cytosol of the cell by means of a transporter protein and is metabolised by metallothionein and other metalloproteins. It also weakly activates an inactive (non DNA-binding) MTF-1 to be transported into the nucleus of the cell and bind to DNA to facilitate the transcription of metallothionein mRNA. In Zn induced cells MTF-1 activation and transcriptional activity is enhanced. A kinase transduction cascade, which involves tyrosine-specific protein kinase (TyrK), P13K, PKC and JNK, controls the activity of MTF kinase (MTFK). Cadmium and other metals stimulate one of several kinases in the MTFK pathway to activate and induce metallothionein gene expression (Adapted from La Rochelle et al. *2001).*

1.2.2.3 Induction of Hypoxia-inducible-factor (HIF-1) proteins

Hypoxia inducible factors (HIF's) are regulatory proteins of oxygen homeostasis in animals. In mammals, genes regulated by HIF-1 include the 11 glycolytic enzymes aldolase A, aldolase C, enolase 1, glyceraldehyde-3-phosphate dehydrogenase, hexokinase 1, hexokinase 2, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1, pyruvate kinase M, and triosephosphate isomerase. It also targets, amongst others, genes for erytrhopoietin, ceruloplasmin, tranferrin and vascular endothelial growth

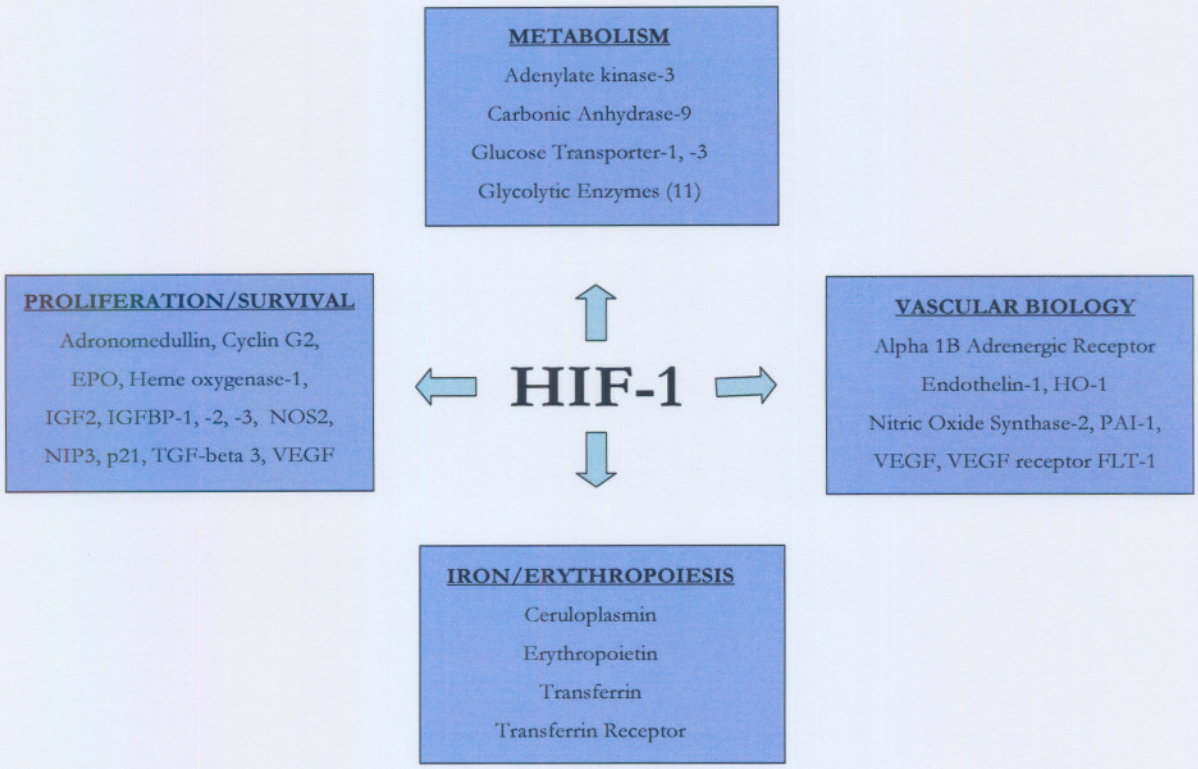

Figure 1.7. The different target genes of HIF-1. Abbreviations: EPO, erythropoietin; HO, heme oxygenase;IGF, insulin-like growth factor; IGFBP, IGF binding protein; NOS, nitric oxide synthase; PAI, plasminogen activator inhibitor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor, NIP3, Nineteen kilodalton interacting protein 3; FLT-1, Fms-like tyrosine kinase 1; (Semenza 2001).

factor (Semenza 2001, Figure 1.7). HIF-1 consists of two subunits namely HIF-1α and HIF-1ß. HIF-1ß protein expression can be detected in most normoxic cells, but HIF-1α is degraded by means of the ubiquitin-proteasome system (Semenza 2001, Figure 1.8). HIF-1α protein rapidly accumulates in hypoxic conditions and could, therefore, indicate respiratory problems, caused by metal exposure. For example, in the experiments of Jewell *et al.* (2000), HIF-1α concentration of human HeLaS3 cells peaked after only one hour of hypoxia. After the initial peak, HIF-1α protein level decreased towards the baseline level even in continuous hypoxia: *e.g.*, in brain tissue the levels reached maximum after 5 h hypoxia, but returned to baseline levels within 12 h (Stroka *et al.* 2001). Various authors have observed that HIF-1α increases even in normoxic cells, if they are treated with metalloids and metals like arsenite (As), chromium (VI) and both soluble and

insoluble nickel (Ni) (Duyndam *et al.* 2001; Gao *et al.* 2002; Costa *et al.* 2003 and Salnikow *et al.* 2003), suggesting that, in addition to hypoxic conditions, metal exposure can affect the stability of the protein. One possibility accounting for the metal responses of HIF-1α is that its stability is affected by the redox-state of the cells, which is influenced by metals. Recently, Nikinmaa *et al.* (2004) have demonstrated that the stability of HIF-1α in rainbow trout is redox-sensitive.

Assessment of HIF-1α protein levels in rainbow trout after different exposures has recently become possible, since the protein has been identified in this species, and antibodies towards it have been produced (Soitamo *et al.* 2001).

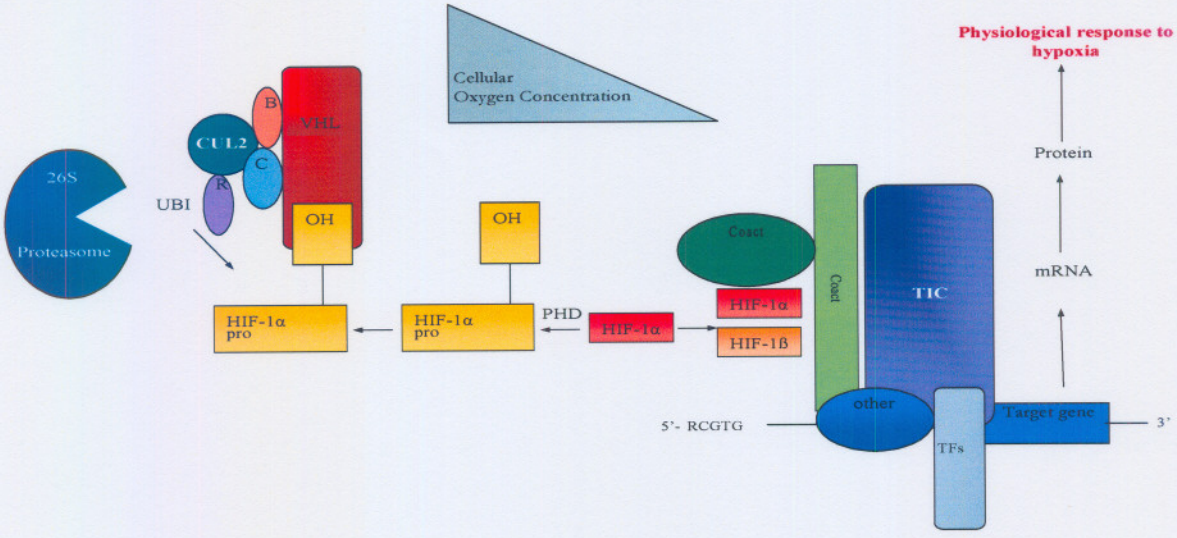

Figure 1.8. During normoxia, HIF-1 a is hydoxylised by prolyl hydroxilation domains 1-3 (PHDs 1-3), a prerequisite for HIF-1a to bind the Von Huppel-Lindau tumor-suppression protein (VHL) together with elongins B and C, Cullin 2 (CUL2) and RBX1 (R). This assosiation is called a functional E3 ubiquitin-protein ligase complex, which is then degraded by the 26S proteasome. During hypoxia HIF-1a dimerizes with HIF-1ß, which escapes prolyl hydroxylation, ubiquitination and degradation. The HIF-1 heterodimer binds to hypoxia response elements (HRE's), containing the core recognition sequence 5'-RCGTG-3', and coactivator (Coact) molecules resulting in the formation of an increased transcription initiation complex (TIC) and mRNA synthesis. This ultimately causes the production of proteins associated with physiologic responses to hypoxia. (Adapted from Semenza 2001).

1.3 Aims of the present study

Because South Africa has an active mining industry, with an overall contribution of 12% to the GDP (South African Chamber of Mines 2004), it would be useful to find biomarkers in selected fish species to assess the impact of metal levels in rivers on aquatic fauna. Cichlid fish were selected as model fish because they are widespread across Southern Africa and because they are omnivorous, and represent a trophic level high up in the food chain. Physiological and biochemical responses to environmental stress have been studied extensively in rainbow trout (*O. mykiss*). For this reason, rainbow trout has been selected as a model to identify possible biomarkers of Cu exposure, before it was tested on Southern African fish species. *T. sparrmanii* and *O. mossambicus* were used in this study to represent Southern African species. These species were selected because both are economically important species: both species are common components of subsistence fisheries while *O. mossambicus* is used in commercial fisheries, aquaculture and in biological, physiological and behavioural research (Skelton 1993).

In this study we investigated if short-term Cu exposure was associated with quantitative damage on gill structure, thus reducing the capacity for oxygen uptake in gills, with the consequent result that cellular oxygen levels are reduced. In addition, we investigated whether MTs and hypoxia-inducible factor could be used to indicate the metal-induced effects in fish. Furthermore, the reversibility of the metal-induced responses was evaluated during a 48 h recovery period.

The specific aims of the present study were:

1. to determine, using rainbow trout (*O. mykiss*), if short term Cu exposure caused thickening of gill epithelium, induction in the production of HIF-1α proteins and induction in MT gene expression,

2. to clone and sequence the MT coding sequence for banded tilapia (*T. sparrmanii*),

3. to determine if there are changes in gill epithelial thickness, HIF-1α protein production and MT gene expression after Cu exposure in banded tilapia (*T. sparmanii*) and Mossambique tilapia (*O. mossambicus*)

4. to test the use of MT gene expression and gill epithelial thickness at selected field sites and correlate this with metal concentrations in water and sediment samples from these locations.

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Chapter 2

Materials and Methods

2.1 Experimental animals

2.1.1 Rainbow trout (*Oncorhynchus mykiss*)

Rainbow trout are found in dams and mountain streams of south-western, southern, eastern, and north eastern Cape, Lesotho, Natal, Mpumalanga, Swaziland and the eastern parts of Zimbabwe (Figure 2.1). It preys opportunistically on a variety of insects, crabs, frogs and fish. It is mostly used in South Africa in aquaculture and angling (Skelton 1994).

2.1.2 Banded tilapia (*Tilapia sparrmanii*)

Banded tilapia are found northwards from the Orange River and Natal southcoast to southern Zaire, Lake Malawi and the Zambezi system. It has also been translocated into

the Cape, south of the Orange River (Figure 2.2). It is omnivorous, feeding on algae, plants, invertebrates and small fish. It is important in South Africa as a component of subsistence fisheries (Skelton 1994).

2.1.3 Mozambique tilapia (*Oreochromis mossambicus*)

Mozambique tilapia is mostly found in rivers along the East Coast from the Zambezi system south towards the Eastern Cape Province. It occurs in estuaries and coastal reaches south of the Pongola system. It is also dispersed inland and the south-west and west coastal rivers (Figure 2.3). It feeds on diatoms and detritus but could also feed on insects. In Southern Africa is important for aquaculture as well as commercial and subsistence fisheries and is widely used in biological research (Skelton 1994).

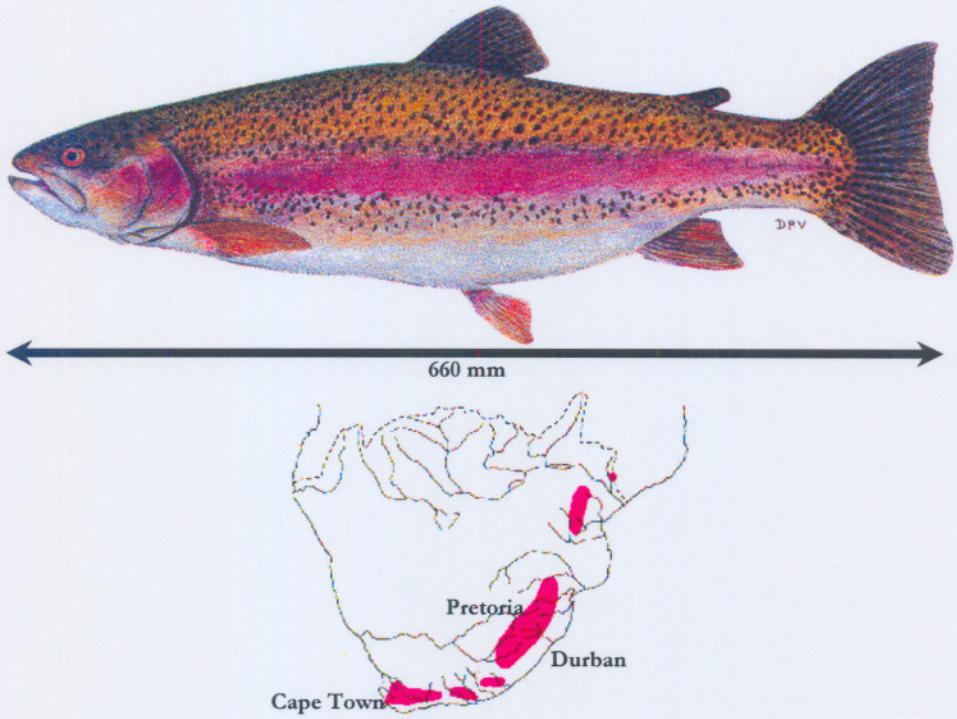

*Figure 2.1. A map of Southern Africa showing the distribution of rainbow trout (*O. mykiss*) (Adapted from Skelton 1994).*

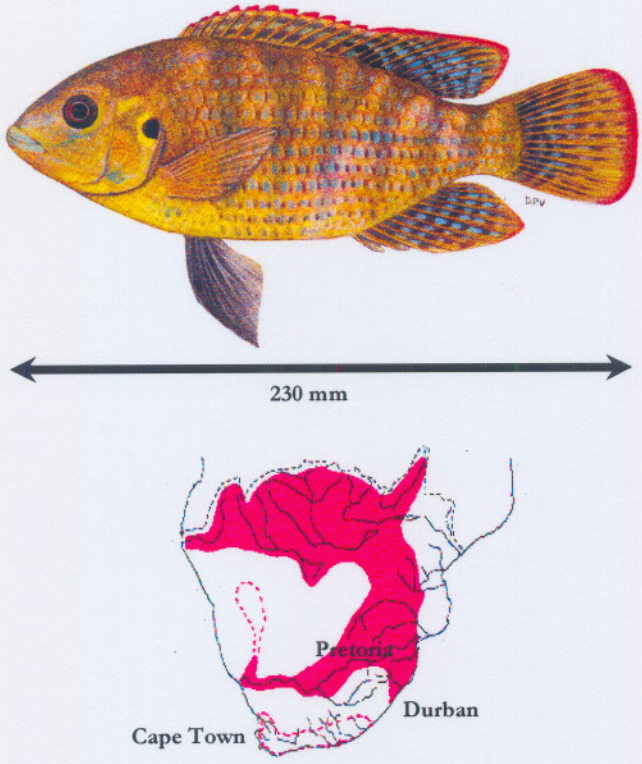

*Figure 2.2. A map of Southern Africa showing the distribution of banded tilapia (*T. sparrmanii*) (Adapted from Skelton 1994).*

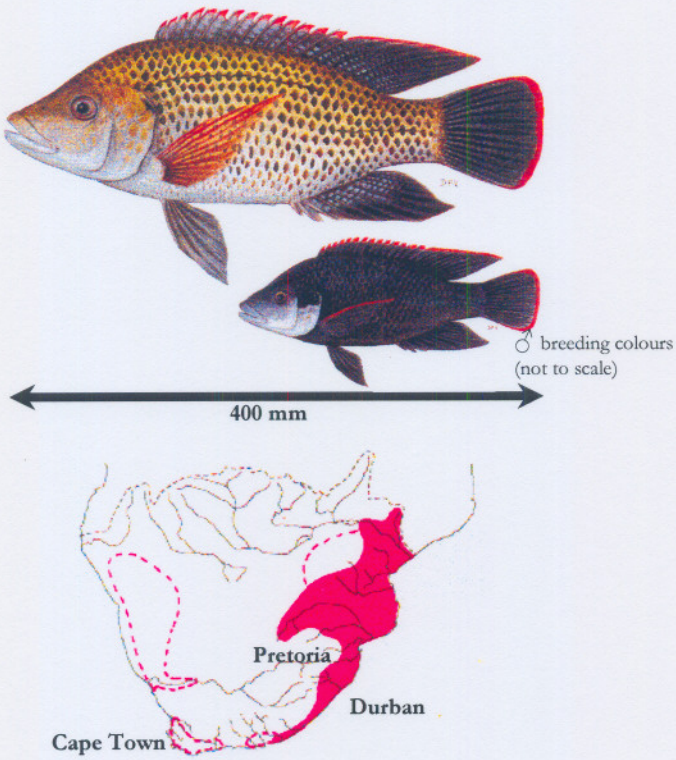

*Figure 2.3. A map of Southern Africa showing the distribution of Mozambique tilapia (*O. mossambicus*) (Adapted from Skelton 1994).*

2.2 *Experimental setup for short-term copper exposures*

Rainbow trout (*O. mykiss*) with a body mass of 107-220 g were obtained from the Finnish Institute for Fisheries and Environment, Parainen Finland and the experiments were conducted at the Laboratory for Animal Physiology, University of Turku, Finland. A second experiment was set up at the North-West University, Potcheftroom Campus, South Africa, with fish provided by Lunsklip Fisheries, Lydenburg, South Africa. Fish were allowed to acclimate to laboratory conditions for 1 week prior to experiments at 14°C (12 h : 12 h light : dark cycle). The fish were fed every second day and feeding stopped 2 days before experiments. Fish were randomly divided into four groups: 2 exposure groups and 2 control groups of 9 fish each and transferred to 4 aerated tanks with a volume of 110 L each (water temperature of 14°C). After the transfer, fish were allowed to recover overnight. Copper, in the form of copper sulphate ($CuSO_4 \cdot 5H_20$), was added to the water of tanks of exposure groups for a final calculated Cu concentration of 1.65 μmol L^{-1} (105 μg L^{-1}). No variations in tank conditions were noted. Tap water, purified and deionized by reverse osmosis, was used during acclimation and the experiment. After the reverse osmosis, $CaCl_2$, $MgSO_4$ and NaCl were added in the water to reach final ion concentrations of 0.64 mmol L^{-1} Ca^{2+}, 2.8 mmol L^{-1} Cl^-, 0.31 mmol L^{-1} Mg^{2+}, 0.31 mmol L^{-1} SO_4^{2-} and 1.5 mmol L^{-1} Na^+.

Mozambique tilapia (*O. mossambicus*) were obtained from the Aquaculture study group of the Stellenbosch University, Stellenbosch South Africa, where these experiments were conducted. Fish were divided into four groups: 2 exposure and 2 experimental groups of 9 fish each and transferred to four aerated tanks, filled with water form the holding systems (dechlorinated Stellenbosch tap water), at least 12 h before the onset of experiments. Cu, in the form of copper sulphate ($CuSO_4 \cdot 5H_2O$) was added to the exposure tanks for a final calculated Cu concentration of 600 μg L^{-1}.

Banded tilapia (*T. sparrmanii*) were caught in Boskop dam, Potchefstroom South Africa. The same feeding regime and experimental setup as for *O. mossambicus* were followed. The water from the holding systems was dechlorinated Potchefstroom tap water. Cu, in the

form of copper sulphate ($CuSO_4 \cdot 5H_2O$) was added to the exposure tanks for a final calculated Cu concentration 4.4 mg L^{-1}.

After 4 and 24 h respectively 6 exposed fish (three from each exposure tank) and 6 control fish (three from each control tank) were stunned by a blow to the head, weighed and measured. The second left gill arch was clamped both dorsally and ventrally with forceps. After dissecting the gill arch from the gill chamber it was immersed in ice cold fixative (5% glutaraldehyde in 0.16 mol L^{-1} s-collidine buffer, pH 7.4 for trout one set of trout samples and Todd's fixative (Todd 1986) for the second set of trout samples and samples for both Tilapias. After forceps were removed, the samples were stored in the fixative at 4°C proir to preparation for light microscopy. The rest of the gill arches were dissected from the gill chamber and the gill tissue scraped from the cartilage before being snap-frozen in liquid nitrogen. Liver tissue was also collected, without puncturing the gall bladder, and snap-frozen.

2.3 Sampling at selected field sites

Fish were collected from three sites in the Rustenburg area and two in the Potchefstroom area (Figure 2.4). In the Rustenburg area, one site was chosen close to the origin of the Hex River, one in the Hex River, downstream from the town and one site downstream from where the Hex River and Leragane River flows into the Elands River. In the Potchefstroom area one site was chosen close to the origin of the Mooi River and one site downstream from mining, agricultural and industrial development. Fish were collected with nets, stunned by a blow on the head and the total length measured. The second left gill arch as well as liver and gill tissue were collected as described in 2.2.

At each of the sites pH, dissolved oxygen concentration (DO) and temperature were measured (YSI 556 MPS, YSI Incorporated, USA). Water samples were collected in labelled plastic bottles, acidified to 0.1 mol L^{-1} with HNO_3 and stored until metal analysis. Water hardness (mg $CaCO_3$) was determined by means of EDTA titration (Clesceri et al.

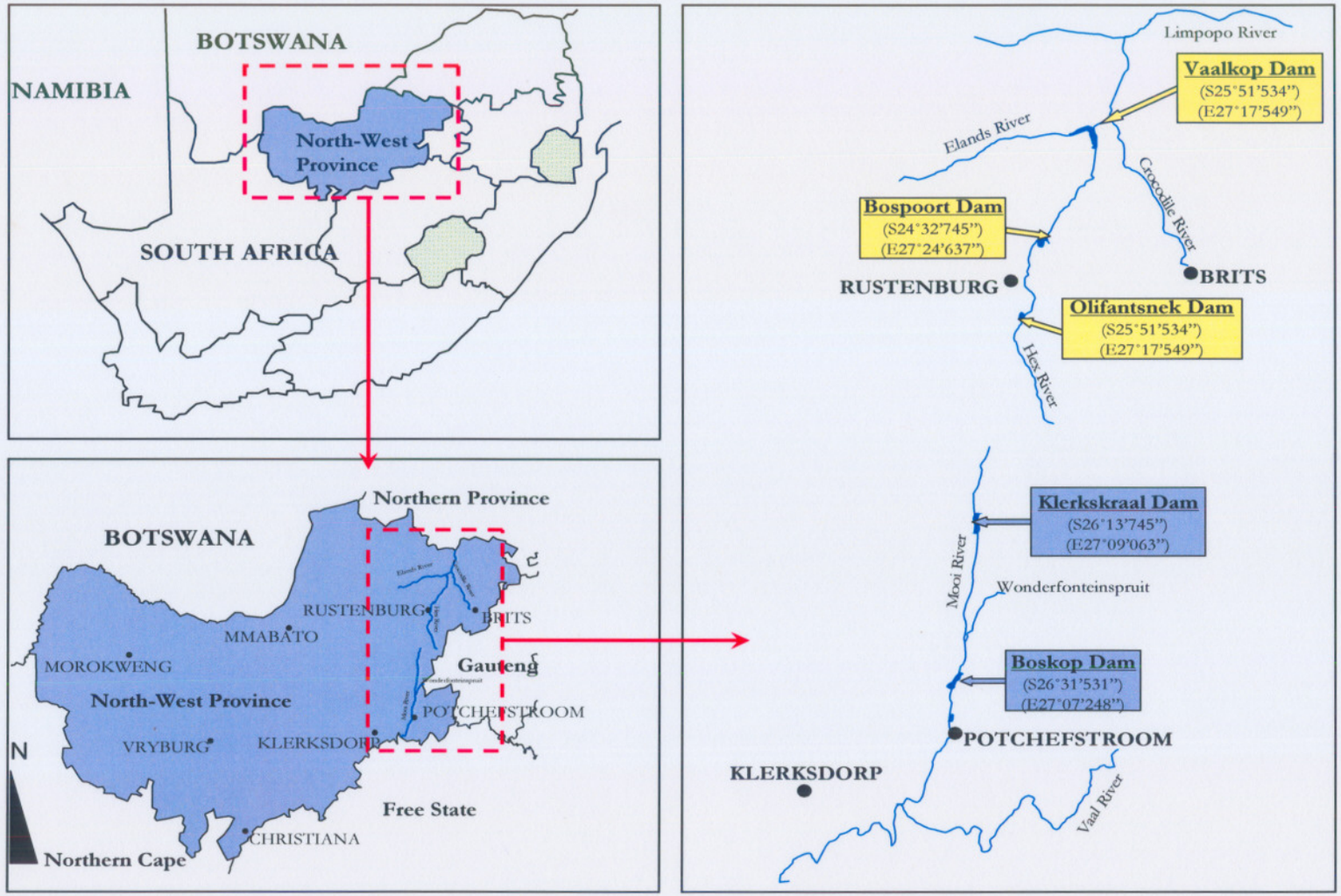

Figure 2.4. Maps of South Africa and the North-West Province indicating the field sites (Flags with arrows) selected for testing biomarkers on wild caugh Tilapia sparrmanii. *Samples were collected in both the Limpopo and Vaal River catchments. No other rivers and streams than those used for gathering samples were shown on maps.*

1998). Sediment samples were collected with a PVC corer from below the water surface. The sediment samples were stored in labelled plastic bags for transport to the lab.

2.4 Gill morphometric studies

Samples from one set of rainbow trout gills were prepared by the Laboratory of Laboratory of Electron Microscopy, University of Turku, Finland. After initial fixing, gill filaments were cut from the gill arch at the same region for every arch, post fixed in 2% osmium tetroxide and 3% K-ferrocyanide (Karnovsky 1971), dehydrated in ethanol and embedded in Epon. Gill samples of the second rainbow trout experiment as well as those of the Mossambique and banded tilapia were treated at the Laboratory of Electron Microscopy, North-West University, South Africa. In short, after fixing with Todd's fixative, gill filaments were cut from gill arch, post-fixed in 2% osmium tetroxide, stained with 2% uranile asetate and dehydrated in acetone. Filaments were then embedded in Spurr resin (Spurr 1969). Semithin (1 μm) saggital sections were cut from gill filaments and stained with 0.5% toluidine blue. Digital images were taken at 200 times magnification for the first rainbow trout experiment and 100 times magnification for the other experiments.

Samples were analysed using a cycloidal grid (Howard *et al.* 1998, grid C1, see Figure 2.5). The points counted were: (1) the points on the epithelial cells (EPT); (2) points on non-tissue (lymphatic) space of the epithelium (EPN) and (3) intersections between grid lines and the outer surface of the epithelium (I_o). From these points the arithmetic mean thickness of the epithelium was calculated as $H_{ar} = [(EPT + EPN) \times 20\ \mu m]/(2 \times I_o)$, where 20 μm is the grid constant in the 200 times magnification pictures of rainbow trout and 16.5μm for the 100 times magnification pictures (Weibel and Knight, 1964, Tuurala 1983).

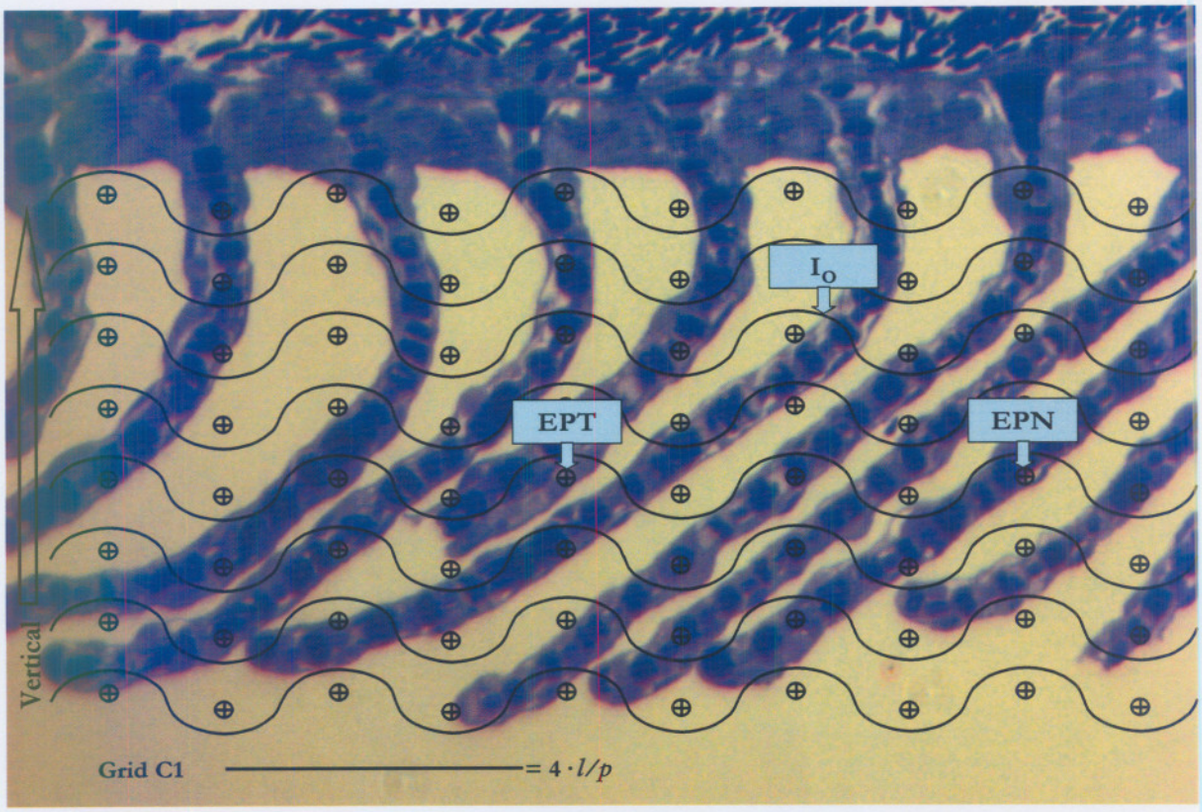

Figure 2.5. A cycloidal grid superimposed onto a micrograph of secondary lamellae of fish gills showing the points counted for calculating the arithmetic mean epithelial thickness (H_{ar}) after exposure to copper. The points counted were: (1) the points on the epithelial cells (EPT); (2) points on non-tissue (lymphatic) space of the epithelium (EPN) and (3) intersections between grid lines and the outer surface of the epithelium (I_o).

2.5 *Cloning of* Tilapia sparrmanii *metallothionein gene*

Total RNA was isolated from liver tissue form *Tilapia sparrmani* that were exposed to copper for 4 h, using a Nucleo Spin® RNA II kit (Macherey-Nagel, Germany) according to the supplier's instructions. PCR was performed using primers designed from the MT coding sequence of *O. mossambicus* (forward 5'ccaaga-ctggaacctgcaac3', reverse 5'tggtgtcgcat-gtctttcct3'). The PCR product was separated on a 1.2 % agarose gel in modified TAE (Millipore, Billerica, Massachusetts, USA). PCR product was cut from the gel and isolated (Montage gel extraction kit, Millipore) and sequenced by the Sequencing Centre, Department of Biology, University of Turku, Finland by means of automated fluorescent sequencing. Gene specific primers for rapid amplification of cDNA ends

PCR (RACE-PCR) were designed to create overlapping sequences, forward primer for 3'-RACE-PCR and reverse primer for 5'-RACE-PCR (forward 3'atgcaaaggaaagacatgcgacacc5'and reverse 3'ggtgtcgcatgtctttcctttgcat5'). 5'- and 3'-RACE-ready-cDNA were synthesised respectively and 3'RACE- and 5'RACE-PCR reactions performed (BD SMART™ RACE cDNA Amplification Kit BD Biosciences Clontech). PCR product was separated by electrophoresis and extracted from the gel as previously described. All isolated fragments were cloned into pGEM®-T Easy Vector (pGEM®-T Easy Vector SystemII, Promega, Madison, WI, USA). After ligation of PCR products into the vectors, vectors were transformed into JM109 high-efficiency competent cells (Promega, Madison, WI, USA), plated on LB/ampicillin/IPTG/x-gal plates and incubated for 24 h at 37°C. Four white colonies were selected from each of the transformation samples and incubated in LB solution (containing 100μg mL^{-1} Ampicillin) for 24 h at 37°C. PCR were performed with 1μL aliquots of each liquid culture, using primers for the T7 and SP6 promotor regions of the plasmid vector, for identifying cultures containing inserts. Plasmid DNA from 1 mL of each bacterial culture containing inserts was purified (Wizard® *Plus* Minipreps DNA purification system, Promega, Madison, WI, USA). Purified DNA samples from both 3'- and 5'-RACE PCR reactions were sent for sequencing.

2.6 Semi-quantitative analysis of metallothionein gene expression with RT-PCR

2.6.1 Rainbow trout

Liver and gill samples were homogenized on ice (ULTRA-TURRAX, Ika Labortechnik, Germany) in Tri Reagent (Sigma-Aldrich Inc, USA). Total RNA was isolated using the Tri reagent method (Sigma Technical Bulletin MB-205). RNA purity was determined by measuring the absorbance ratio at wavelengths of 260 and 280 nm. cDNA synthesis was performed with 2 μg RNA using general oligo$(dT)_{15}$-primer (Promega, Madison, WI, USA), together with avian myeoloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). MT DNA was amplified using gene-specific primers designed for rainbow trout MT A (GenBank accession no. X59395) and B (Genbank accession no. X59394)

coding sequences (forward primer: 5'-ggatccttgtgaatgctc-3', reverse primer: 5'-tcactgacaacagctgg-3'). The forward primer was designed to anneal to base 3-20 on both MT A and B. The reverse primer was designed to anneal to base 170–186 on the MT A and 167–183 on MT B coding sequences. The optimal annealing temperature (49°C) was determined using the gradient program of the HYBAID thermal cycler. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was used to normalize the signals for MT and was amplified using sequences in GenBank (GenBank accession no. AF027130, forward primer: 5'-ggaatcaaagtcgttgccat-3', reverse primer: 5'-ggatctcatggggcttcata-3'). An annealing temperature of 60°C was used and a product of 178 base pairs was obtained. PCR-products were run on 1.2% agarose gel at 85 V for 30 minutes. Gels were analyzed and photographed under UV light with Chemi-Imager (Alpha Innotech Corp., San Leandro, CA, USA). The MT/GAPDH ratio was determined for each fish. While the present studies give an accurate picture of changes in the MT/GAPDH ratio, it should be noted that if metal exposure affects GAPDH levels, this response will remain undetected.

2.6.2 Mozambique and banded tilapia

Liver and gill tissue were homogenized on ice (ULTRA-TURRAX, Ika Labortechnik, Germany) in Tri Reagent (Sigma-Aldrich Inc, USA). Total RNA was isolated using the Tri reagent method (Sigma Technical Bulletin MB-205). RNA integrity was determined by separation with 1.2 % agarose gel electrophoresis and both the yield and purity of RNA was determined by measuring absorbance at 260 and 280 nm. cDNA were synthesized from 2 μg of RNA using standard Oligo(dT)$_{15}$primer (Promega, Madison, WI, USA), together with avian myeoloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland). DNA was amplified using polymerase chain reaction (PCR) with gene specific primers from the coding sequence for *O. mossambicus* MT (GenBank accession no. AY257202; forward 5'ccaagactggaacctgca- ac3', reverse 5'tggtgtcgcatgtctttcct3') for a product size of 150 base pairs. An annealing temperature of 60°C was used for amplification. PCR product was separated with 1.2 % agarose gel electrophoresis in TBE buffer. Image analysis was performed under UV light with Chemi-Imager (Alpha Innotech Corp., San Leandro, CA, USA). Signals were normalized with Glyceraldehyde

3-phosphate dehydrogenase (GAPDH) expression (GenBank accession no. AY140649; forward primer 5'gggtgtcaaccaa-aagt3', reverse primer 5'cgtcaaccgtcttctgtg3'). To confirm that the amplified sequence was indeed MT, PCR product was separated on 1.2% agarose gel in modified TAE gel extraction buffer (Millipore, Billerica, Massachusetts, USA) and isolated from the gel with Montage gel extraction kit (Millipore, Billerica, Massachusetts, USA). The extracted PCR product was sequenced by the Sequencing Center, Department of Biology, University of Turku, Finland by means of automated fluorescent sequencing to confirm that the PCR product truly was MT.

2.7 *Analysis of HIF-1α protein accumulation with western blot immunodetection*

Whole cell extraction was performed with frozen gill and liver samples. Briefly, tissues were cut into pieces, washed with ice cold PBS and homogenized in a buffer used by Mosser *et al.* (1988) (25% glycerol (v/v), 420 mmol L^{-1} NaCl, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} EDTA, 20 mmol L^{-1} Hepes, 0.5 mmol L^{-1} phenylmethylsulphonyl fluoride and 0.5 mmol L^{-1} dithiothreitol) mixed with an inhibition cocktail containing 2 μg ml^{-1} LAP (leupeptin, antipan, pepstatin), 1 mmol L^{-1} Na_3VO_4 and 2 μg ml^{-1} aprotinin. Samples were centrifuged for 30 min (4°C, 13 500 G) and supernatant collected. Protein quantification was done using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules California, USA), according to the manufacturer's instructions. Equal amounts of protein samples (20 μg) were resolved by denaturing electrophoresis on discontinuous 8% SDS-polyacrylamide slab gels and transferred to PROTRAN nitrocellulose filter (PerkinElmer, Boston MA, USA) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 3% non-fat milk powder in PBS and 0.3% Tween 20 for one and a half hours. Immunodetection was done according to the ECL Western blotting manual (Amersham Pharmacia Biotech, Buckinghamshire, England) using 1:20 000 C-terminal rabbit-anti-trout HIF-1α in 1% BSA-PBS and 0.02% NaN_3 as primary antibody (Soitamo *et al.* 2001) and 1:15 000 horseradish peroxidase (HRP)-conjugated anti-rabbit in blocking solution as secondary antibody. Membranes were incubated overnight in the primary antibody. Detection was done using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, England). Alpha-tubulin was used to

normalize signal and detected using a mouse anti-α-tubulin antibody diluted 1:10 000 as primary antibody and HRP-conjugated anti-mouse immunoglobulin as secondary antibody. The signal was captured on X-ray film and the photographed with Chemi-imager (Alpha Innotech Corporation). The results were analyzed with AlphaEase™ software (Alpha Innotech Corporation).

2.8 Metal analysis for tissue metal accumulation and metal contents of sediment and water samples

For the determination of tissue Cu concentration frozen tissue samples were thawed, weighed and digested in a 5:1 mixture of 65% nitric acid and 70% perchloric acid. Samples were digested for 2 h (80°C in round bottom flasks with distillation condensers) after which digest were made up to a final volume of 20 mL with deionized water.

Sediment and water samples were collected from the field sites in the Rustenburg and Potchefstroom areas. Samples were dried for at least 24 h at 70°C, ground with a mortar and pestle, passed through a 500 micron sieve and the larger particles discarded. The <500 micron fraction were stored in dry plastic containers until digestion and analysis.

One gram fractions of sediment were digested in 12 mL of a 5:1 mixture of 65% nitric acid and 70% perchloric acid. Samples were digested for 2 h (80°C in round bottom flasks with distillation condensers) after which digest was filtered and made up to a final volume of 20 mL with deionized water.

Cu concentration of the digested tissue, sediment and acidified water was determined by means of ICP-MS.

Statistical analyses are described in detail in each paper.

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Chapter 3

Original papers

Paper I

*Effects of short-term copper exposure on gill structure,metallothionein and hypoxia-inducible factor-1a (HIF-1a) levels in rainbow trout (*Oncorhynchus mykiss*)*

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Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1α (HIF-1α) levels in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) were exposed to 1.65 μM of waterborne copper for 24 h. Fish were then transferred to metal-free water. Metallothionein mRNA induction in rainbow trout liver and gill tissue, hypoxia-inducible factor-1 (HIF-1α) accumulation in gill tissue and arithmetic mean thickness of gill epithelium (H_{ar}) were determined at 4 and 24 h of exposure as well as 48 h after transfer to metal-free water. The arithmetic mean distance from water to blood was significantly elevated after both 4 and 24 h of exposure (H_{ar} was 4.67 and 4.66 μm, respectively in exposed fish, compared to 3.81 and 3.62 μm for the corresponding control fish). During the 48 h recovery H_{ar} returned towards the control values; the recovery value of 4.21 μm was significantly lower than values during exposures. There was also a significant increase in gill metallothionein mRNA levels after the 4 h exposure with MT/GAPDH ratio of 1.288 versus the control value of 0.988. In liver, metallothionein induction was not observed. HIF-1α protein showed an increased accumulation in gills after 4 h, with the HIF-1α/α-tubulin ratio of 0.562 being significantly higher than the 24 h exposure value of 0.232. These results suggest that exposure to copper for four hours causes hypoxia in the gill epithelium, which is adequate for the activation of HIF-1α.
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Keywords: Copper; Rainbow trout; *Oncorhynchus mykiss*; Metallothionein; Hypoxia-inducible factor (HIF); Gill damage; Diffusion distance

1. Introduction

Although copper is an essential nutrient in numerous enzymatic systems, it becomes toxic when in high concentrations in water (Heath, 1995; Cerqueira and Fernandes, 2002). Because gills are directly exposed to metals in water and because they play an important role in metal uptake, gill damage can be indicative of metal exposure. In soft water copper will be taken up through apical sodium pathway in gills, inhibiting sodium uptake via gills (see e.g. Wood, 2001). This is associated with the inhibition of Na^+/K^+ ATPase (Wood, 2001; Grosell and Wood, 2002). Cerqueira and Fernandes (2002) found gill damage in fish exposed to

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sublethal concentrations of copper for 96 h. Recovery of gill damage occurred after 7–15 days. Disruption of gill function in fish by copper exposure was found on several occasions (Dang et al., 2000a; Daglish and Nowak, 2002). Lappivaara et al. (1995) used the arithmetic mean thickness of the epithelium (H_{ar}) as indicator of gill damage in *Oncorhynchus mykiss* after acute exposure to zinc and during recovery. Changes in H_{ar} indicate changes in the diffusion distance across gill epithelium (Weibel and Knight, 1964), which might impede gas exchange, leading to tissue hypoxia.

Freshwater teleosts accumulate copper in both liver and gills (Sorensen, 1991). Metal accumulation is often associated with an increase in both gill and liver metallothionein levels. Metallothioneins are small (6–7 kDa), cysteine-rich, nonenzyme proteins. They have been implicated in metal homeostasis and detoxification of heavy metals in biological systems (Cousins, 1985). Their expression is upregulated in response to Zn, Cd and Cu and the proteins act as chelators through the formation of metal-thiolate bonds (Samson and Gedamu, 1998). Rainbow trout metallothionein protein concentration is known to increase with exposure to copper (Kling and Olsson, 1995) and De Boeck et al. (2002) found hepatic metallothionein levels to be indicative of long-term copper exposures in rainbow trout.

Hypoxia-inducible factors (HIF's) are regulatory proteins of oxygen homeostasis in animals (Semenza, 2001). HIF-1 consists of two subunits namely HIF-1α and HIF-1β. HIF-1β protein expression can be detected in most normoxic cells, but HIF-1α is degraded by means of the ubiquitin-proteasome system (Semenza, 2001). HIF-1α protein rapidly accumulates in hypoxic conditions and could, therefore, indicate respiratory problems, caused by metal exposure. For example, in the experiments of Jewell et al. (2000), HIF-1α concentration of human HeLaS3 cells peaked already after one hour of hypoxia. After the initial peak, HIF-1α protein level decreases towards the baseline level even in continuous hypoxia: e.g. in brain tissue the levels reached maximum after 5 h hypoxia, but returned to baseline levels within 12 h (Stroka et al., 2001). Various authors have observed that HIF-1α increases, if normoxic cells are treated with metalloids and metals like arsenite, chromium (VI) and both soluble and insoluble nickel (Duyndam et al., 2001; Salnikow et al., 2003; Gao et al., 2002; Costa et al., 2003), suggesting that, in addition to hypoxic conditions, metal exposure can affect the stability of the protein. One possibility accounting for the metal responses of HIF-1α is that its stability is affected by the redox-state of the cells, which is influenced by metals. Recently, Nikinmaa et al. (2004) have observed that the stability of HIF-1α in rainbow trout is redox-sensitive.

Assessment of HIF-1α protein levels in rainbow trout after different exposures has recently become possible, since the protein has been identified in this species, and antibodies towards it have been produced (Soitamo et al., 2001).

In this study we investigated if copper exposure was associated with quantitative damage on gill structure, thus reducing the capacity for oxygen uptake in gills, with the consequent result that cellular oxygen levels are reduced. In addition, we investigated, if metallothioneins and hypoxia-inducible factor could be used to indicate the metal-induced damage in rainbow trout. Furthermore, the reversibility of the metal-induced responses was evaluated during a 48 h recovery period.

2. Material and methods

Rainbow trout (*O. mykiss*) with a body mass of 107–220 g were obtained from the Finnish Institute for Fisheries and Environment, Parainen Finland. Fish were allowed to acclimate to laboratory conditions for 1 week prior to experiments at 14 °C (12 h:12 h light:dark cycle). The fish were fed every second day and feeding stopped 2 days before experiments. Fish were randomly divided into four groups: two exposure groups and two control groups of nine fish each and transferred to four aerated tanks with a volume of 110 L each (water temperature of 14 °C). After the transfer, fish were allowed to recover overnight. Copper, in the form of copper sulfate ($CuSO_4 \cdot 5H_2O$), was added to the water of tanks of exposure groups to give a final calculated copper concentration of 1.65 μM (105 $\mu g\,L^{-1}$). No variations in tank conditions were noted.

Tap water, purified and deionized by reverse osmosis, was used during acclimation and the experiment. After the reverse osmosis, $CaCl_2$, $MgSO_4$ and NaCl were added in the water to reach final ion concentrations of 0.64 $mmol\,L^{-1}$ Ca^{2+}, 2.8 $mmol\,L^{-1}$ Cl^-,

0.31 mmol L^{-1} Mg^{2+}, 0.31 mmol L^{-1} SO_4^{2-} and 1.5 mmol L^{-1} Na^+.

After 4 and 24 h, respectively, six fish exposed to copper (three fish from each exposure tank) and six control fish (three fish from each control tank) were stunned with a blow to the head. For gill morphometric analysis the second left gill arch was clamped both dorsally and ventrally with tongue forceps to stop blood flow, cut outside the forceps and immersed in ice-cold fixative (5% glutaraldehyde in 0.16 mol L^{-1} s-collidine buffer, pH 7.4) with forceps for at least 20 min. Forceps were then removed and fixing continued for at least 24 h at +20 °C (Tuurala, 1983).

For RT-PCR and Western blot immunodetection gills and liver were dissected on ice. Samples were immediately snapfrozen in liquid nitrogen and then stored at −85 °C until analysis. Water in exposure tanks was replaced with metal-free water after 24 h exposure and exposed fish were allowed to recover for 48 h before another set of samples were collected. Similar water changes and sampling regime was also applied to the control group.

2.1. Gill morphometric analysis

Gill samples for morphometry were treated by the Laboratory of Electron Microscopy, University of Turku, Finland. After initial fixing, gill filaments were cut from the gill arch at the same region for every arch, postfixed in 2% osmium tetroxide and 3% K-ferrocyanide (Karnovsky, 1971), dehydrated in ethanol and embedded in Epon. Semithin (1 μm) saggital sections were cut from gill filaments and stained with 0.5% toluidine blue. Digital images of the sections were taken at 200 times magnification with a Leica DM RXA microscope and a Hamamatsu ORCA UV digital camera (Hamamatsu Corporation, Bridgewater, NJ, USA). Samples were analysed using a cycloidal grid (Howard and Reed, 1998, grid C1). The points counted were: (1) the points on the epithelial cells (EPT); (2) points on non-tissue (lymphatic) space of the epithelium (EPN); (3) intersections between grid lines and the outer surface of the epithelium (I_0). From these points the arithmetic mean thickness of the epithelium was calculated as $H_{ar} = [(EPT + EPN) \times 20\,\mu m]/(2 \times I_0)$, where 20 μm is the grid constant (Weibel and Knight, 1964).

2.2. Reverse transcripase polymerase chain reaction (RT-PCR) for metallothionein mRNA detection

Liver and gill samples were homogenized on ice (ULTRA-TURRAX, Ika Labortechnik, Germany) in Tri Reagent (Sigma–Aldrich Inc., USA). Total RNA was isolated using the Tri reagent method (Sigma Technical Bulletin MB-205). RNA purity was evaluated by determining the absorbance ratio at wavelengths of 260 and 280 nm. cDNA synthesis was performed with 2 μg RNA using general oligo$(dT)_{15}$-primer (Promega, Madison, WI, USA), together with avian myeoloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified using gene-specific primers designed for rainbow trout metallothionein A (GenBank accession no. X59395) and B (Genbank accession no. X59394) coding sequences (forward primer: 5′-ggatccttgtgaatgctc-3′, reverse primer: 5′-tcactgacaacagctgg-3′). The forward primer was designed to anneal to base 3–20 on both Metallothionein A and B. The reverse primer was designed to anneal to base 170–186 on the Metallothionein A and 167–183 on Metallothionein B coding sequences. The optimal annealing temperature (49 °C) was determined using the gradient program of the HYBAID thermal cycler. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the signals for MT and cDNA was amplified using sequences in GenBank (GenBank accession no. AF027130, forward primer: 5′-ggaatcaaagtcgttgccat-3′, reverse primer: 5′-ggatctcatggggcttcata-3′). An annealing temperature of 60 °C was used and a product of 178 base pairs was obtained. PCR-products were run on 1.2% agarose gel at 85 V for 30 min. Gels were analyzed and photographed under UV light with Chemi-Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The MT/GAPDH ratio was determined for each fish. While the present studies give an accurate picture of changes in the MT/GAPDH ratio, it should be noted that if metal exposure affects GAPDH levels, this response will remain undetected.

2.3. Western blot immunodetection for hypoxia-inducible factor

Whole cell extraction was performed with frozen gill and liver samples. Briefly, tissues were cut into

pieces, washed with ice cold PBS and homogenized in a buffer used by Mosser et al. (1988) (25% glycerol (v/v), 420 mmol L^{-1} NaCl, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} EDTA, 20 mmol L^{-1} Hepes, 0.5 mmol L^{-1} phenylmethylsulphonyl fluoride and 0.5 mmol L^{-1} dithiothreitol) mixed with an inhibition cocktail containing 2 μg mL^{-1} LAP (leupeptin, antipan, pepstatin), 1 mmol L^{-1} Na_3VO_4 and 2 μg mL^{-1} aprotinin. Samples were centrifuged for 30 min (4 °C, 13 500 × g) and supernatant collected. Protein quantification was done using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Equal amounts of protein samples (20 μg) were resolved by denaturing electrophoresis on discontinuous 8% SDS-polyacrylamide slab gels and transferred to PROTRAN nitrocellulose filter (Perkin-Elmer, Boston, MA, USA) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 3% non-fat milk powder in PBS and 0.3% Tween 20 for one and a half hours. Immunodetection was done according to the ECL Western blotting manual (Amersham Pharmacia Biotech, Buckinghamshire, England) using 1:20 000 C-terminal rabbit-anti-trout HIF-1α in 1% BSA-PBS and 0.02% NaN_3 as primary antibody (Soitamo et al., 2001) and 1:15 000 horseradish peroxidase (HRP)-conjugated anti-rabbit in blocking solution as secondary antibody. Membranes were incubated overnight in the primary antibody. Detection was done using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, England). Alpha-tubulin was used to normalize signal and detected using a mouse anti-α-tubulin antibody diluted 1:10 000 as primary antibody and HRP-conjugated anti-mouse immunoglobulin as secondary antibody. The signal was captured on X-ray film and the photographed with Chemi-Imager (Alpha Innotech Corporation). The results were analyzed with AlphaEase™ software (Alpha Innotech Corporation).

2.4. Statistical analysis

A modified Kolmogorov–Smirnov (KS) test was used for determining normal distribution (Dallal and Wilkinson, 1986). Results were analysed with two-way analysis of variance (ANOVA), using Tukey HSD post-hoc test (Statistica Version 6.1, STATSOFT Inc.). All results were given as mean ± standard error of the mean (S.E.M.). Significant differences between data of exposed animals at different exposure times is indicated on graphs with ∗ and significant differences between data of exposed and control animals at the same sampling time is indicated with # (# or ∗ indicates $P < 0.05$, ## or ∗∗ indicates $P < 0.01$ and ### or ∗∗∗ indicates $P < 0.001$).

3. Results

The 1.65 μM (105 μg L^{-1}) copper, also used by Dalglish and Nowak (2002) used for exposure

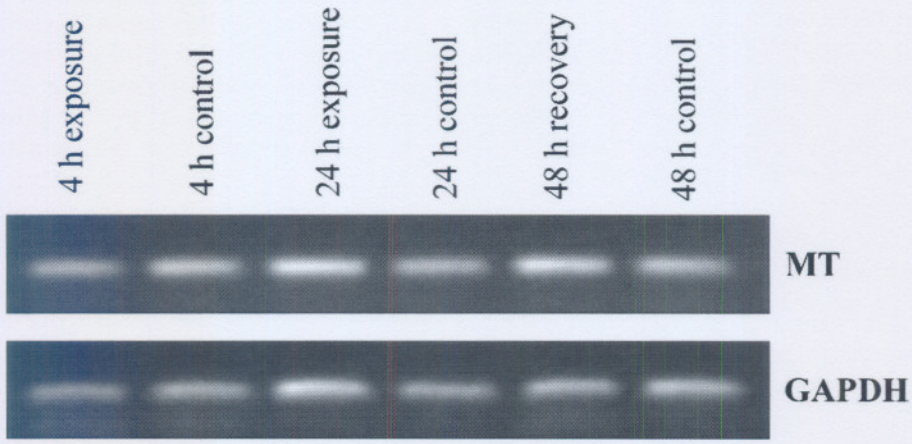

Fig. 1. An example of metallothionein and GAPDH expression during 4 and 24 h exposure to 1.65 μM copper and 48 h after recovery. Corresponding control samples are also given.

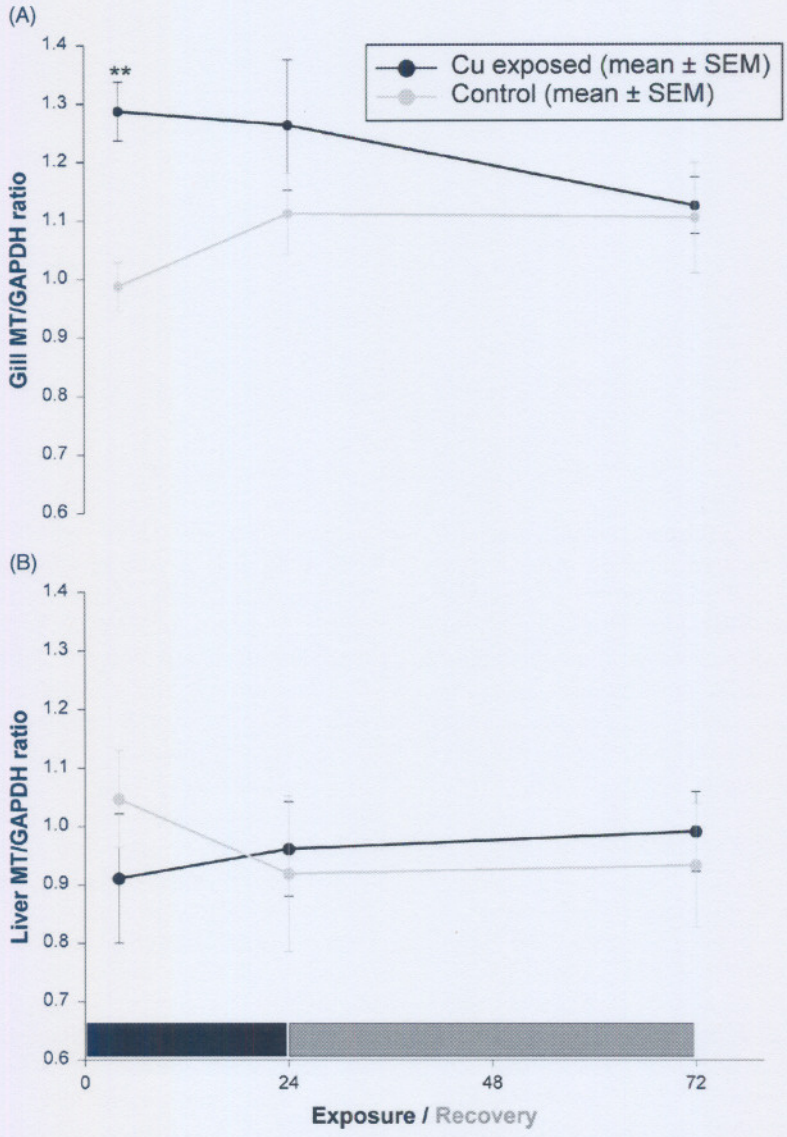

Fig. 2. (A) Metallothionein gene expression in gill tissue, given as metallothionein/GAPDH ratio, of fish exposed to 1.65 μM copper sulphate for 4 or 24 h and during a 48 h recovery, and in the gills of control fish sampled at the same time points as exposed fish. Means ± S.E.M. is given. ** indicates a statistically significant ($P < 0.01$) difference between copper-exposed and control fish at the same time point. Two-way ANOVA followed by Tukey's post-hoc test or *t*-test was used for comparisons ($N = 6$). (B) Metallothionein gene expression in liver tissue, given as metallothionein/GAPDH ratio, in fish exposed to 1.65 μM copper sulphate for 4 or 24 h and during a 48 h recovery, and in the liver tissue of control fish sampled at the same time points as exposed fish. Means ± S.E.M. is given. There is no significant upregulation of metallothionein gene expression in liver tissue ($N = 6$).

in this experiment is below the 96 h LC_{50} value of 210 μg L^{-1} reported earlier for rainbow trout (Anderson and Spear, 1980). Since water chemistry undoubtedly affects the toxicity of metals, we ascertained that all the fish survived the 24 h exposure to copper.

The exposure of rainbow trout to a nominal concentration of 1.65 μM of Cu was adequate to cause a

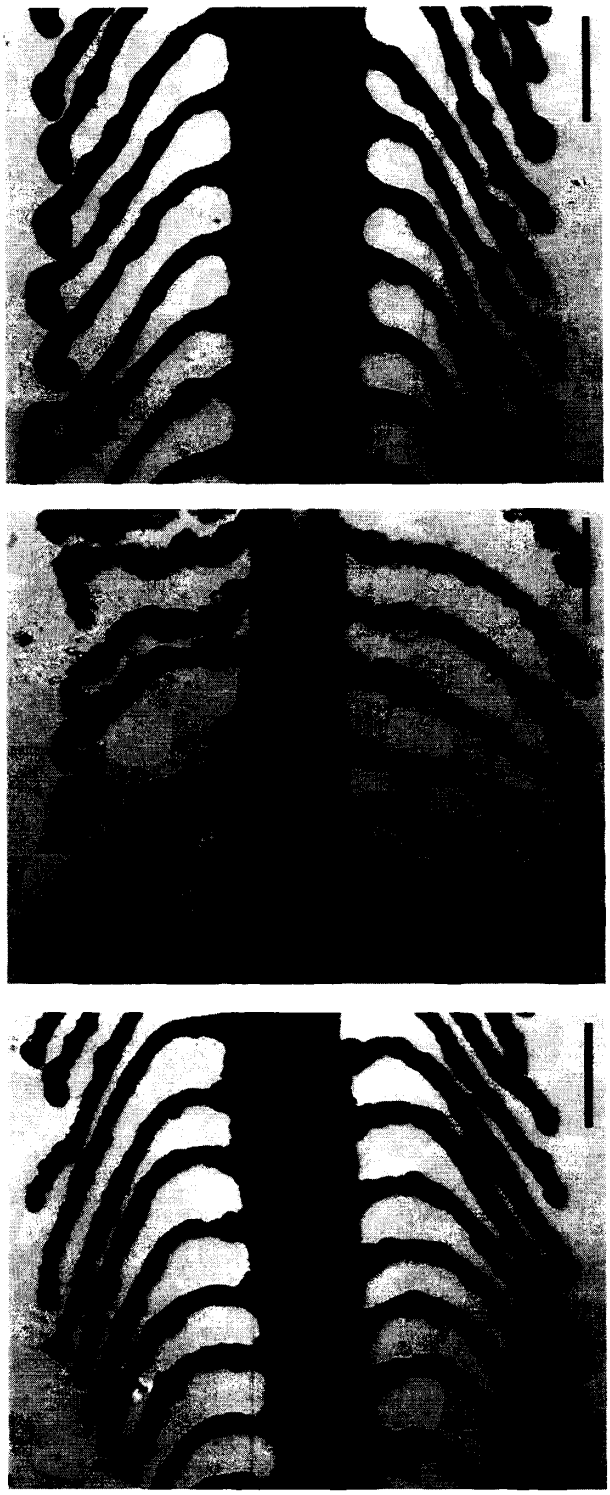

Fig. 3. Light micrographs of saggital sections through secondary lamellae of rainbow trout gills. Micrograph A shows gills of control fish while micrograph B shows gills of fish exposed to 1.65 μM copper for 24 h. Thickening of the epithelium because of hypertrophy of pavement cells (▶) and chloride cells (→) as well as lamellar telangiectasis (★) can clearly be seen in the gills of exposed fish. After 48 h recovery some epithelial thickening and telangiectasis is still visible although to much lesser extent than in exposed fish (micrograph C).

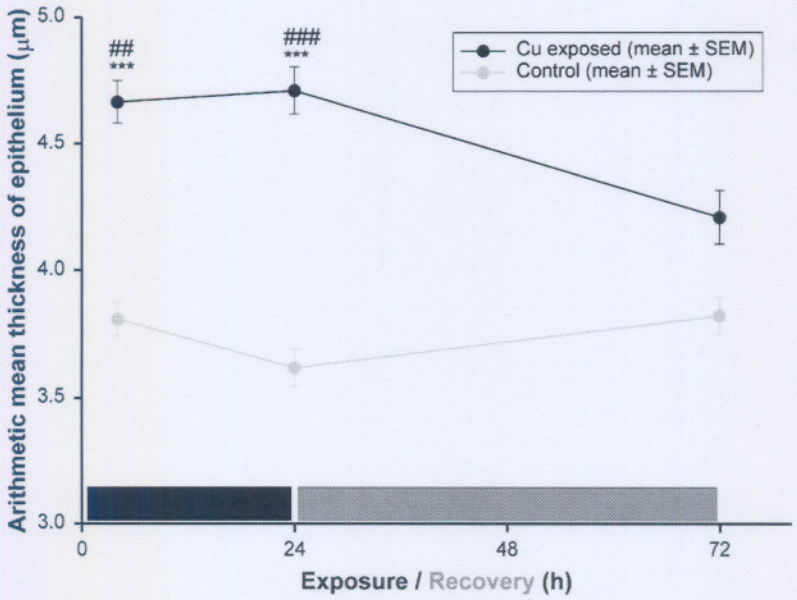

Fig. 4. The arithmetic mean thickness of gill epithelium (H_{ar}) of fish exposed to 1.65 μM copper sulphate for 4 or 24 h and during a 48 h recovery, and in the gills of control fish sampled at the same time points as exposed fish. Means ± S.E.M. is given. Asterisks indicate statistically significant ($^{***}P < 0.001$) difference between copper-exposed and control fish at the same time point, #'s indicate significant difference between exposed fish and fish after 48 h recovery ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$). Two-way ANOVA followed by Tukey's post-hoc test was used for comparisons ($N = 25$).

significant Cu load to the animals, as indicated by the induction of gill metallothionein (MT) after 4 h of exposure to copper. The MT/GAPDH ratio of 1.288 ± 0.050 after the 4 h exposure was significantly higher than the corresponding value (0.988 ± 0.041) in control fish. In control animals, gill MT/GAPDH ratios did not change during the 72 h experiment, and in experimental animals decreased towards control values after 24 h exposure and 48 h recovery (Figs. 1 and 2). In the liver, MT/GAPDH ratios did not differ significantly between control and exposed groups at any time point.

The environmental copper load was adequate to cause significant structural changes in the gills, as indicated in Fig. 3. A 4 h exposure to copper caused epithelial thickening, which was largely reversed by the end of the 48 h recovery. When the disturbances of gill structure were quantified by measuring the arithmetic mean thickness of epithelium (H_{ar}), a significant increase was observed in exposed fish as compared to control fish at both 4 and 24 h (Fig. 4). After the 48 h recovery, H_{ar} was significantly lower than at 4 and 24 h of exposure, but did not differ significantly from the corresponding control value. The epithelial thickening was associated with an increased level of hypoxia-inducible factor HIF-1α in the early stages of exposure. The HIF-1α/α-tubulin ratio of 0.562 ± 0.115 of fish exposed to copper for 4 h was significantly higher than that (0.232 ± 0.042) after 24 h exposure, whereas no significant changes were observed in control fish (Fig. 5).

4. Discussion

The present results indicate that, although sublethal, 1.65 μM copper concentration is already enough to result in gill damage and induction of MT in gills. Furthermore, there is a clear difference in HIF-1α levels in exposed fish between 4 and 24 h exposure, whereas similar difference is not observed in control animals.

4.1. Gill damage

Gill damage occurred in fish exposed to copper already at 4 h (Fig. 3B). Cerqueira and Fernandes

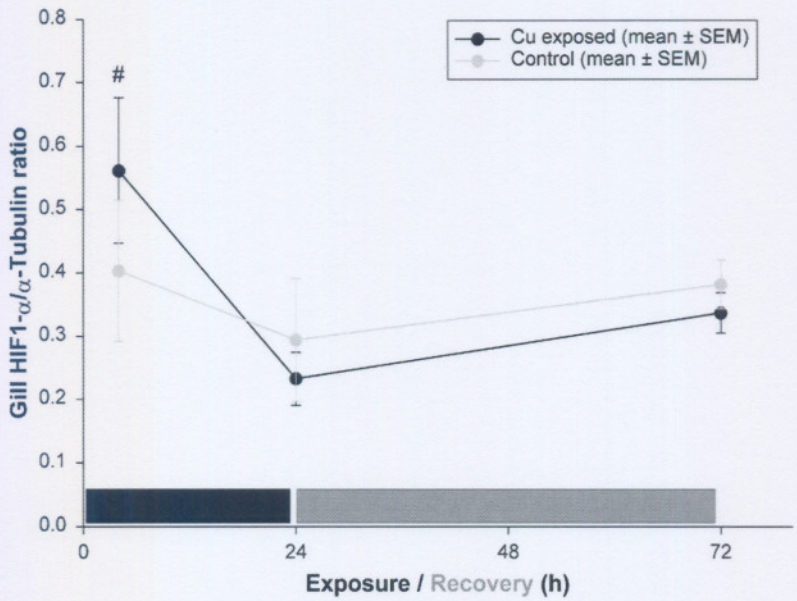

Fig. 5. Levels of HIF-1α, given as HIF-1α/α-tubulin ratio, in gill epithelium of fish exposed to 1.65 μM copper sulphate for 4 or 24 h and during a 48 h recovery, and in the gills of control fish sampled at the same time points as exposed fish. Means ± S.E.M. is given. # indicates a significant difference ($P < 0.05$) between exposed fish at 4 and 24 h of exposure. Two-way ANOVA followed by Tukey's post-hoc test was used for comparisons ($N = 6$) (except for 4 and 24 h control, where $N = 5$).

(2002) found thickening in gill epithelium as result of hypertrophy in both pavement and chloride cells as well as lamellar telangiectasis (both as seen in Fig. 3B) in *Prochilodus scrofa* as a result of copper exposure. After 48 h in copper-free water, recovery took place to a certain extent (Fig. 3C). Changes in gill morphology due to exposure to metals are found to be a compensatory response to keep metal from entering through gill cells. These changes include cell proliferation, mucus secretion and hypertrophy (Mallat, 1985; Dang et al., 2000b). Although hypertrophy of chloride cells (Fig. 3B) is considered a compensatory response, Dang et al. (2000b) found that increased chloride cell turnover, resulting from hypertrophy, can result in increased numbers of necrotic and apoptotic cells. Since changes in the mean arithmetic distance between water and blood occurred early during copper exposure, gill morphometric measurements could be used to indicate toxic exposure. The samples are quite easily taken and although time consuming, the analysis is easily carried out.

4.2. HIF-1α protein accumulation

Lappivaara et al. (1995) found the relative diffusion capacity and arterial oxygen tension of *O. mykiss* to decrease when exposed to zinc. We did not measure arterial oxygen tension and relative diffusion capacity. However, an increase in mean arithmetic distance between water and blood indicates that relative diffusion capacity is decreased. In accordance with this, we found evidence of hypoxia in gill tissue: HIF-1α protein levels in gill cells of exposed fish were higher (although not statistically significant) than those of control fish after only 4 h of exposure. HIF-1α protein levels returned to normal within the next 20 h—a reduction of HIF-1α protein level appears to occur after the initial, early peak in rainbow trout cells exposed to hypoxia (E. Rissanen and M. Nikinmaa, unpublished data). Mice liver, kidney and brain cells (Stroka et al., 2001) and human lung epithelium (Uchida et al., 2004) also have an initial accumulation of HIF-1α protein, with a decrease in HIF-1α during prolonged hypoxia. This could be attributed

to the destabilization of the HIF-1α during prolonged times of hypoxia (Uchida et al., 2004). The physiological consequences of the initial accumulation and consequent destabilization of HIF-1α are unknown at present time.

4.3. Metallothionein gene expression

We observed upregulation of the metallothionein gene in reaction to copper exposure within 4 h after the onset of exposure. This upregulation was significantly higher than in control fish but was not significantly different from fish exposed to copper for 24 h. Within 48 h of transfer of fish to copper-free water, metallothionein gene expression was downregulated to levels close to those of control fish as seen from the MT/GAPDH ratio. Since no metallothionein gene upregulation was observed in the liver tissue, most of the changes in copper level in this tissue must have been below the induction threshold for liver metallothionein responsive to copper.

5. Conclusion

During short-term exposure to a nominal concentration of copper, sublethal effects such as gill damage, tissue hypoxia and induction of metallothionein gene expression can be observed. It can thus be concluded that gill morphometric analyses, HIF-1α protein accumulation and upregulation of metallothionein gene expression in gills are more sensitive as biomarkers of short-term copper or other metal exposures than the mere accumulation of the metal in tissue or general accumulation of metallothionein proteins.

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Paper II

Gill damage in Oreochromis mossambicus *and* Tilapia sparrmanii *after short-term copper exposure*

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RE: Publication credits from peer-reviewed conference volume

Dear Ms Prinsloo

The aim of this letter is to:

(1) provide background to the ICCPB in Africa Conferences,

(2) explain the process of publication of the 2005 volume and,

(3) to inform our colleagues that the *International Congress Series* is a peer-reviewed publication and thus the collected papers in International Congress Series 1275 (2005: ISBN 0 444 51763 4) should qualify for publication rewards / credits in the South African academic arena (VERKA, DNE).

Accordingly, we attach a copy of the abstract book and the final proceedings volume of the Third International Conference of Comparative Biochemistry and Physiology in Africa that was held in August 2004 in the Ithala Nature Reserve, KwaZulu-Natal, South Africa.

Background to ICCPB in Africa

The ICCPB in Africa conferences have several aims. Among these are our efforts to showcase South African animal physiology, to foster international linkages by bringing specialists together in a unique nature setting and to facilitate the attendance and interactions of indigenous African delegates at these conferences. (Please see the ICS editorial for more information).

The conferences were held at the following venues:

1997 Skukuza, Kruger National Park, South Africa
2001 Kasane, Chobe National Park, Botswana
2004 Ithala Nature Reserve, KwaZulu-Natal, South Africa
2008 Maasai Mara National Reserve, Kenya (*in planning*)

Publication of the ICCPB in Africa

The organizers work hard to elevate the profile of the ICCPB in Africa conferences (and thereby research in southern Africa), in part through high level publication of the scientific contributions presented at the conferences. Selected papers from the first Conference was published in the (then) South African Journal of Zoology (Vol 33: 53 - 140, 1998). For the second conference we selected Comparative Biochemistry and Physiology (Vol 133A: 419 - 899). The outputs of the third and most recent conference appear/will appear in 2 formats: the first is via Elsevier's *International Congress Series* (ICS) (Vol 1275: 1-379, 2005) and the second is via *Physiological and Biochemical Zoology* (PBZ *in prep*).

We elected to publish collected, peer reviewed research papers through the ICS route, since it is a respected international publication and because the editors of the relevant regularly published scientific journals were unable to dedicate sufficient space to publish papers on widely differing topics. A brief perusal of the contents pages of the ICS volume will indicate phylogenetic groupings from bacteria to mammals and organizational levels from genetics to whole animal physiology. The regular journals are very constraining and specific about the research topics they wish to publish, whereas Elsevier offered the ICS format which allowed us the freedom to properly encapsulate the full scope and spirit of the meeting - which is what we aimed for. Selected review papers will be published in addition in *Physiological & Biochemical Zoology* but we were anxious to ensure that ALL delegates had the opportunity to published peer-reviewed papers.

As will be apparent from the accompanying material, not all of the 113 oral and 30 poster contributions at the conference made it into the ICS volume (45 collected papers). Papers were invited before the start of the conference and were peer-reviewed during and after the conference. Scientific and technical revisions to each paper were required before acceptance into the volume. Authors had to sign off on page proofs and completed the appropriate copyright paperwork. The rigorous review process required that several papers required multiple revisions before the reviewers and editors were satisfied with the scientific and technical quality of the MS. The ICS series is indexed through ISI Proceedings, ISI Current Contents and well as Science Direct.

This review process was regulated through the Elsevier electronic submissions process and, if required, examples of reviewers comments can be provided.

We, the editors agree with Elsevier publishing that the ICS series is an important quality publication and we thus feel confident that the series, and our volume, meet the requirements of national evaluation panels and give credit to those South African authors who contributed to the huge success of this publication volume.

Best wishes

André Vosloo

Dr André Vosloo

North-West University, South Africa

Dr Steve Morris

University of Bristol, United Kingdom

International Congress Series 1275 (2004) 195–200

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Gill damage in *Oreochromis mossambicus* and *Tilapia sparrmanii* after short-term copper exposure

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Abstract. *Oreochromis mossambicus* and *Tilapia sparrmanii* were exposed to 600 μg L^{-1} and 4.4 mg L^{-1}, respectively, of waterborne copper for 24 h. The arithmetic mean thickness of gill epithelium (H_{ar}), using a morphometric technique, was measured after 4 and 24 h of exposure for both fish species. Copper exposure caused the arithmetic thickness of gill epithelium in *O. mossambicus* to increase to 4.92±0.210 μm, after 24 h of exposure, which was significantly higher than the corresponding control value of 2.97±0.131 μm and that of the 4 h exposure value (3.08±0.142 μm), while the 4 h exposure value did not differ from the control values. The gill epithelial thickness of 6.07±0.156 μm for *T. sparrmanni* exposed to copper was already significantly higher than the control value of 4.96±0.205 μm after only 4 h of exposure. The increase in epithelial thickness remained during the entire 24 h of exposure, with the value for the exposed fish after 24 h (5.56±0.190 μm) being significantly higher than that of the control fish (4.99±0.137 μm). © 2004 Published by Elsevier B.V.

Keywords: Tilapia; *Oreochromis mossambicus*; *Tilapia sparrmanii*; Gill damage; Copper; Gas exchange

1. Introduction

Although an essential element for numerous enzymatic systems in fish, copper is extremely toxic in high concentrations [1]. Amongst others, high copper levels can cause fast generation of reactive oxygen species [2]. It also binds histidine-, cystein- and methionine-containing proteins, resulting in dysfunction [3].

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Fish gills are, due to their large surface area, the prime target for copper in water. In low-sodium water (typically ion-poor soft water) copper will be taken up through the apical sodium pathway in gills, inhibiting sodium uptake via gills. This is associated with the inhibition of Na^+/K^+ATP-ase [3,4]. The disruption of branchial ion regulation, due to high copper levels in water, can cause mortality in fish [5].

Copper was reported to also cause severe gill damage in *Prochilodus scrofa*, which included epithelial lifting, cell swelling and chloride and mucus cell proliferation. The damage from a 96-h copper exposure recovered only after 7 days [6]. Copper exposure for 24 h caused a significant increase in epithelial thickness in *Oncorhynchus mykiss* gills. Recovery of the increase epithelial thickness occurred within 48 h after exposure was terminated [7]. This increase in epithelial thickness was largely due to hypertrophy of epithelial cells, which was found to be most characteristic of heavy metal exposure (for review see Ref. [8]). Gill damage can be linked to impaired physiological function in fish [9].

Lease et al. [10] found gill histopathology to be useful as an early-warning tool to monitor fish health in the environment.

The present study compares changes in epithelial thickness in both *Tilapia sparrmanii* and *Oreochromis mossambicus* during a 24-h exposure to copper with control values at the same sampling times taken from fish where no copper was added to holding tanks.

2. Materials and methods

Mozambique tilapia (*O. mossambicus*) were obtained from the Aquaculture study group of the Stellenbosch University, Stellenbosch, South Africa. Banded tilapia (*T. sparrmanii*) were caught in Boskop Dam, Potchefstroom, South Africa. Fish were kept in dechlorinated tap water in the laboratory for at least 1 week prior to the experiments. Feeding was stopped 2 days before the experiments. Fish were divided into four groups (two exposure and two experimental groups of nine fish each) and transferred to four aerated tanks at least 12 h before the onset of experiments. Copper, in the form of copper sulphate ($CuSO_4 \cdot 5H_2O$) was added to the exposure tanks for a final calculated copper concentration of 600 μg L^{-1} for Mozambique Tilapia and 4.4 mg L^{-1} for Banded Tilapia. Abovementioned concentration for exposing *O. mossambicus* was based on concentrations used by Nussey et al. [11] for 96 h copper exposure but, due to the unavailability of data, the exposure concentration for Banded Tilapia was estimated from concentrations where physiological changes were detected (Van Aardt, unpublished results).

After 4 and 24 h of exposure, six copper-exposed fish and six control fish were stunned with a blow to the head and the second left gill arch was exposed and clamped both dorsally and ventrally with tongue forceps to stop blood flow. The arch was cut outside the forceps and immersed in ice-cold Todd's fixative [12]. Forceps were removed and fixing continued for at least 24 h at +20 °C.

Gill samples were treated at the Laboratory of Electron Microscopy, Potchefstroom University, South Africa. After fixing with Todd's fixative, gill filaments were cut from gill arch, post-fixed in 2% osmium tetroxide, stained with 2% uranile acetate and dehydrated in acetone. Filaments were then embedded in Spurr resin [13].

Semithin (1 μm) saggital sections were cut from gill filaments and stained with 0.5% toluidine blue. Digital images were taken at 100 times magnification and pictures were analyzed using a cycloidal grid [14] (Grid C) as described in Refs. [7,15], where the grid constant was 16.5 μm.

Data sets were tested for normal distribution by using a modified Kolmogorov–Smirnov (KS) test [16]. Two-way analysis of variance (ANOVA), using Tukey HSD post-hoc test (Statistica Version 6.1, STATSOFT) was used to determine statistical significance of data. All results were given in mean±standard error of the mean (S.E.M).

3. Results

Since water chemistry affects the bioavailability of copper, we cannot compare the concentrations of copper, as used in the experiments, to available copper LC_{50} values for *O. mossambicus* and *T. sparrmanii*. We ascertained that all fish survived the 24-h exposure period.

The two fish species reacted differently to copper exposure, as indicated by the changes in epithelial thickness (Figs. 1 and 2). Exposure to copper caused an increase in the epithelial thickness in the gills of *O. mossambicus* only after 24 h of exposure, where the epithelial thickness of 4.92±0.210 μm showed significant increase compared to both the control value of 2.97±0.131 μm and the 4 h exposure value of 3.08±0.142 μm. The 4 h exposure value was not significantly higher than the corresponding control value of 3.09±0.085 μm (Fig. 1).

Unlike *O. mossambicus*, significant increase in epithelial thickness in gills of *T. sparrmanii* occurred already after 4 h of copper exposure, where the value of exposed fish was 6.07±0.156 μm compared to the control value of 4.96±0.205 μm. The increased epithelial thickness of copper-exposed fish compared to that of control fish remained

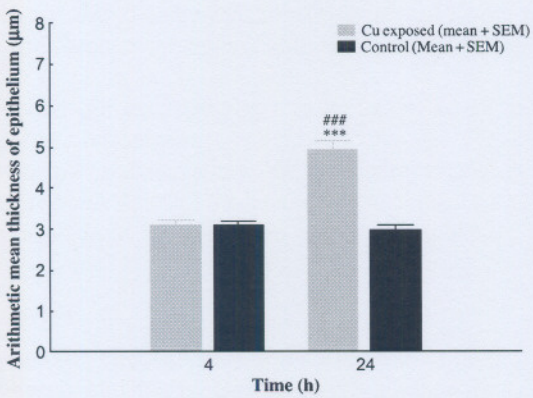

Fig. 1. Arithmetic mean thickness of gill epithelium of *O. mossambicus* exposed to 600 μg L^{-1} copper for 4 and 24 h as well as in gills of control fish sampled at these same time points as exposed fish. Means±S.E.M are given. Asterisks indicate statistically significant differences ($P<0.001$) between copper-exposed and control fish sampled at the same time point. Significant differences ($P<0.001$) between fish exposed for 4 and 24 h, respectively, are indicated with #'s ($N=23$ for 4 h exposed fish and $N=25$ for the others).

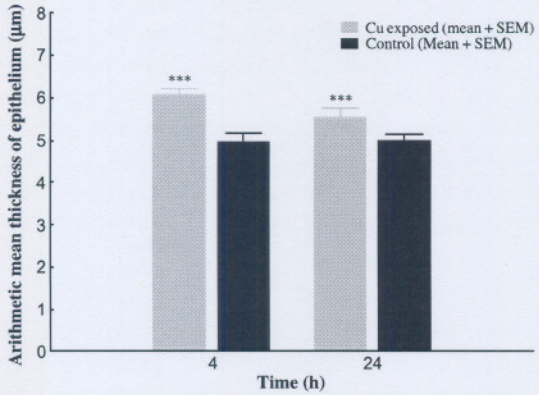

Fig. 2. Arithmetic mean thickness of gill epithelium of *T. sparrmanii* exposed to 4.4 mg L^{-1} copper for 4 and 24 h as well as in gills of control fish sampled at these same time points as exposed fish. Means±S.E.M is given. Asterisks indicate statistically significant differences (P<0.001) between copper-exposed and control fish sampled at the same time point. Significant differences (P< 0.001) between fish exposed for 4 and 24 h, respectively, are indicated with #'s (N=25).

constant for the rest of the experiment, with the value after 24 h exposure being 5.56±0.190 µm and that of the control 4.99±0.137 µm (Figs. 2 and 3).

4. Discussion

There are differences between basal H_{ar} values of different fish species. *O. mossambicus* has a mean basal H_{ar} value of 3.03 µm while *T. sparrmanni* has a mean basal H_{ar} value of 4.98 µm (this study). Studies done in Refs. [7,17] showed basal H_{ar} values for *O. mykiss* of 3.73 and 4.2 µm, respectively. In another study on the effects of copper on gill structure of *O. mykiss*, the mean basal H_{ar} value was found to be 3.9 µm (Van Heerden and Vosloo, unpublished results). Since there was no significant difference between the basal H_{ar} in three separate studies on *O. mykiss*, measuring mean arithmetic epithelial thickness indicates that this technique could be used to compare the changes in

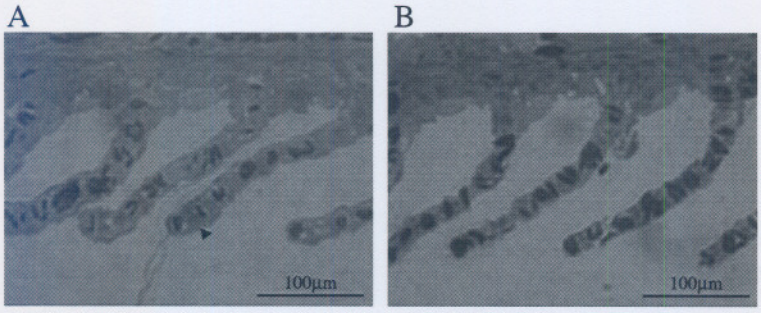

Fig. 3. Light micrographs of saggital sections through secondary lamellae of *T. sparrmanii* gills. Micrograph A shows gills exposed to copper for 4 h, where thickening of epithelium because of hypertrophy (►) can clearly be seen as opposed to gills from control fish (B).

epithelial thickness within species from different experiments or collected from different locations during field based studies.

Although sublethal, the concentrations of copper used during these experiments were enough to cause severe gill damage in both *O. mossambicus* and *T. sparrmanii* as indicated by the highly significant increase in gill epithelial thickness during the 24 h copper exposure. An increase in gill epithelial thickness can lead to a decrease in the relative diffusion capacity of gills epithelium [17], which in turn could cause tissue hypoxia, as indicated in rainbow trout by an accumulation of HIF-1α proteins [7].

The thickening of epithelium in gills of fish exposed to copper was caused by hypertrophy in both pavement cells as well as lamellar telangiectasis as found during previous studies [6,7]. This is believed to be a compensatory response to keep metals from entering gill cells. Although it is indicated that the effects of copper exposure on gills could be reversed to a certain extent [6,7], permanent damage, for instance an increase in the number of necrotic and apoptotic cells, due to hypertrophy could occur [18].

Since we have found downstream physiological effects associated with the thickening of gill epithelium due to copper exposure in rainbow trout, further studies are now undertaken to determine whether or not H_{ar} can successfully be used as a general biomarker of environmental stress from metal impacted field sites. Downstream physiological effects measured in rainbow trout included an accumulation of hypoxia inducible factor-1α (HIF-1α) [7], as well as decrease in both pH_a and P_aO_2 (Van Heerden and Vosloo, unpublished results) after 4 h exposure to copper.

5. Conclusion

The results of this study supports the results from a similar study done on rainbow trout, proving that morphometric measurements on gills of fish could be an easy and effective indicator of toxic exposure. Since the gill damage occurred early during copper exposure, toxic effect could be detected before irreversible damage occurs.

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Paper III

Tilapia sparrmanii *and* Oreochromis mossambicus *metallothionein expression in response to short term copper exposure*

Aquatic Toxicology

Guide for Authors

Types of contribution

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Letters to the Editor
5. Book Reviews.

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles can be divided into three types:
- *Regular reviews* covering subjects falling within the scope of the journal which are of active current interest. These should generally not exceed 12 printed pages (approx. 6000 words).
- *Mini-reviews*. These will be short reviews or overviews (not exceeding 2-3 printed pages, approx. 1000-1500 words) on topics of *above-average* emerging interest.
- *Commentaries*. This label will be given to mini-reviews which clearly contain the personal opinions of the author concerned.

All types of review articles will be solicited by the Reviews Editor, M.N. Moore, Centre for Coastal and Marine Sciences, Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, UK. E-mail: m.moore@pml.ac.uk.

Short Communications will be restricted to papers describing short, complete studies. They should not exceed 3 printed pages, including figures and tables (approx. 1500 words), and should be written in a continuous style, *without* subdivisions of introduction, materials and methods, results, discussion and acknowledgements; they should always begin with a summary. A short communication, although brief, should be a complete and final publication, and figures and tables from the communication should not occur in a later paper.

Letters to the Editor should either offer comment on a paper published in the journal, or comment on any general matter providing that this is relevant to the scope of the journal. In the case of letters commenting on published papers, the author(s) of the latter will be given the opportunity to react to the letter and the two items will subsequently be published together in the journal. All Letters to the Editor should be submitted directly to Dr Nikinmaa or Dr Stegeman.

Book Reviews will be included in the journal on a range of relevant books which are not more than 2 years old. Book reviews will be solicited by the Book Review Editor. Unsolicited reviews will not usually be accepted, but suggestions for appropriate books for review may be sent to the Book Review Editor, Dr. A.S. Kane, Aquatic Pathobiology Center, University of Maryland School of Medicine, Department of Pathology, 10 South Pine Street, Baltimore, MD 21201-1192, USA; Tel: (+1) 410 706 7230, Fax: (+1) 410 706 8414.E-mail: akane@umaryland.edu

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Preparation of manuscripts

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2. Manuscripts should be typewritten, typed on one side of the paper (if possible with numbered lines), with wide margins and double spacing throughout, i.e. also for abstracts, footnotes and references. Every page of the manuscript, including the title page, references, tables, etc. should be numbered in the upper right-hand corner. However, in the text no reference should be made to page numbers; if necessary, one may refer to sections. Underline words that should be in italics,and do not underline any other words. Avoid excessive usage of italics to emphasize part of the text.

3. Manuscripts in general should be organized in the following order:
   Title (should be clear, descriptive and concise)
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   Keywords (normally 3-6 items). Please refer to the cumulative index
   Introduction
   Material studied, area descriptions, methods, techniques
   Results
   Discussion
   Conclusion
   Acknowledgements and any additional information concerning research grants, etc.
   References
   Tables
   Figure captions

4. In typing the manuscript,titles and subtitles should not be run within the text. They should be typed on a separate line, without indentation. Use lower-case lettertype.

5. SI units should be used.

6. If a special instruction to the copy editor or typesetter is written on the copy it should be encircled. The typesetter will then know that the enclosed matter is not to be set in type. When a typewritten character may have more than one meaning (e.g. the lower case letter l may be confused with the numeral 1), a note should be inserted in a circle in the margin to make the meaning clear to the typesetter. If Greek letters or uncommon symbols are used in the manuscript, they should be written very clearly, and if necessary a note such as "Greek lower-case chi" should be put in the margin and encircled.

7. Elsevier reserves the privilege of returning to the author for revision accepted manuscripts and illustrations which are not in the proper form given in this guide.

Abstracts

The abstract should be clear,descriptive and not longer than 400 words. It should provide a very brief introduction to the problem and a statement about the methods used in the study. This should generally be followed by a brief summary of results, including numerical data (means and standard errors, for example). The abstract should end with an indication of the significance of the results.

Tables

1. Authors should take notice of the limitations set by the size and lay-out of the journal. Large tables should be avoided. Reversing columns and rows will often reduce the dimensions of a table.

2. If many data are to be presented, an attempt should be made to divide them over two or more tables.

3. Drawn tables, from which prints need to be made, should not be folded.

4. Tables should be numbered according to their sequence in the text. The text should include references to all tables.

5. Each table should be typewritten on a separate page of the manuscript. Tables should never be included in the text.

6. Each table should have a brief and self-explanatory title.

7. Column headings should be brief, but sufficiently explanatory. Standard abbreviations of units of measurements should be added between parentheses.

8. Vertical lines should not be used to separate columns. Leave some extra space between the columns instead.

9. Any explanation essential to the understanding of the table should be given as a footnote at the bottom of the table.

Illustrations

1. All illustrations (line drawings and photographs) should be submitted separately, unmounted and not folded.

2. Illustrations should be numbered according to their sequence in the text. References should be made in the text to each illustration.

3. Each illustration should be identified on the reverse side (or - in the case of line drawings - on the lower front side) by its number and the name of the author. An indication of the top of the illustrations is required in photographs of profiles, thin sections, and other cases where doubt can arise.

4. Illustrations should be designed with the format of the page of the journal in mind. Illustrations should be of such a size as to allow a reduction of 50%.

5. Lettering should be in Indian ink or by printed labels. Make sure that the size is big enough to allow a reduction of 50% without becoming illegible. The lettering should be in English. Use the same kind of lettering throughout and follow the style of the journal.

6. If a scale should be given, use bar scales on all illustrations instead of numerical scales that must be changed with reduction.

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Tilapia sparrmanii and _Oreochromis mossambicus_ metallothionein expression in response to short term copper exposure

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Abstract

The metallothionein coding sequence cDNA of *Tilapia sparrmanii* was amplified by means of RACE-PCR, cloned into plasmids and sequenced. In order to determine the effect of copper on metallothionein mRNA production in *Tilapia sparrmanii* and *Oreochromis mossambicus*, fish were exposed to copper for 4 and 24 h respectively. Fish were then allowed to recover in water with no added copper for 48 h. At 4 h of exposure, metallothionein gene expression in *T. sparrmanii* gills, as expressed by MT/GAPDH ratio, tended to increase but was not significantly different from the control (1.563 ± 0.154 vs. 1.245 ± 0.073, respectively). After 24 h of exposure, metallothionein gene expression in the gills of *T. sparrmanii* (1.861 ± 0.154) was significantly higher than control levels (1.220 ± 0.078). During the 48 h recovery period, MT/GAPDH ratio of both fish decreased to a level close to the control levels (1.474 ± 0.097 and 1.433 ± 0.119, respectively). MT gene expression in *O. mossambicus* gills (1.558 ± 0.094) was significantly higher than control values (1.142 ± 0.086) after only 4 h of copper exposure.

Keywords: Metallothionein, coding sequence, RT-PCR, *Tilapia sparrmanii*, copper

Introduction

Metallothioneins (MTs) are small, highly conserved, cysteine-rich proteins. Transcription of the MT gene to MT-mRNA is upregulated by a number of factors of which metals, such as copper, cadmium and zinc, are the most common inducers (Samson and Gedamu, 1995). MTs have also been implicated in both metal homeostasis and detoxification (Suzuki and Koizumi, 2000, Cousins, 1985).

Aquatic organisms are targets of contaminated water from, *e.g.*, mining, industrial and agricultural activities. These waters often contain metals such as Cu, Zn, Cd and Hg (Zafarullah et al., 1988). The induction of MT in response to metals is, therefore, often used as a biomarker of metal contamination and the effect of metals on both accumulation of MT proteins and upregulation of MT gene expression have been studied extensively using several fish species including rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), zebrafish (*Danio rerio*) and the tilapiine genus *Oreochromis* (Kling and Olsson, 1995, Van Heerden et al., 2004, De Boeck et al., 2002, Olsvik et al., 2001, Riggio et al., 2003, Chan, 1994, Cheung et al., 2004).

South Africa has an active mining industry, and, consequently, high metal concentrations may be measured in various aquatic systems. Thus, biomarkers to assess the impact of metals on aquatic fauna are particularly useful. Cichlid fish are particularly useful in biomarker research because they are geographically widely distributed across Southern Africa and because they are omnivorous and thus at a high trophic level in the food chain. Although cited as studies on the genus *Tilapia*, all previous studies done on chichlid fish were on the genus *Oreochromis*. The genus *Tilapia* used to include all tillapiine species but is now only restricted to the

substrate spawning tilapiine species (Skelton, 1995). The mouthbrooding species are classified as the genus *Oreochromis* and *Sarotherodon* (Skelton, 1995, Klett and Meyer, 2002). *Tilapia mossambica* (Chan, 1994), *Tilapia nilotica* (Cheung et al., 2004), and *Tilapia aurea* (Cheung et al., 2004) are all grouped under the genus *Oreochromis* and are now known as *O. mossambicus, O. niloticus* and *O. aureus* respectively (Skelton, 1995, www.Fishbase.org). This split in the genus *Tilapia* has been confirmed by means of molecular phylogenetic studies, classifying *Tilapia sparrmanii* as the only true *Tilapia* species (Klett and Meyer, 2002).

The gene for *Oreochromis mossambicus* (previously known as *Tilapia mossambica*, abundant in the eastern and western parts of the Southern Africa) MT has already been sequenced (GenBank accession number AY257202).

The aim of the present study were to (1) identify and sequence the coding sequence for *Tilapia sparrmanii* (occurring in the northern and eastern parts of SA) MT gene in order to design PCR-primers that recognize this gene and (2) determine how *T. sparrmanii* MT gene expression responds to exposure to a high concentration of copper sulphate.

Materials and methods

Banded tilapia (*Tilapia sparrmanii*) were caught in Boskop Dam, Potchefstroom, South Africa one year before the onset of experiments and kept in specially designed recirculating holding systems as described previously (Van Aardt and Booysen, 2004). The holding systems were at ambient temperature during the summer, but additional heating was provided during winter to maintain fish at >20°C. Mossambique tilapia (*Oreochromis mossambicus*)

were supplied by the Aquaculture Study Group, University of Stellenbosch, South Africa. Feeding was stopped 2 days before experiments. Fish were randomly divided into four groups of 2 exposure and 2 experimental groups (9 fish each) and transferred to four aerated tanks at least 12 h before the onset of metal exposure experiments. Copper, in the form of copper sulphate ($CuSO_4 \cdot 5H_2O$) was added to the exposure tanks in a single bolus to give a final calculated copper concentration of 4.4 mg L^{-1} for *T. sparrmanii* and 600 μg L^{-1} for *O. mossambicus*.

After 4 and 24 h of exposure, 6 fish exposed to copper and 6 control fish were stunned with a blow to the head and gill arches were dissected from the gill chamber. Gill tissue was scraped from the gill arches with a blunt object, transferred to 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Liver tissue was also dissected out, collected in microcentrifuge tubes and snap frozen in liquid nitrogen. All dissections were carried out on ice.

Cloning of Tilapia sparrmanii *metallothionein coding sequence*

Total RNA was isolated from liver tissue form *Tilapia sparrmanii* that were exposed to copper for 4 h, using a Nucleo Spin® RNA II kit (Macherey-Nagel, Germany) according to the supplier's instructions. RT-PCR was performed using primers designed from the MT coding sequence of *Oreochromis mossambicus* (forward 5'ccaagactggaacctgcaac3', reverse 5'tggtgtcgcatgtctttcct3'). The PCR product was separated on a 1.2 % agarose gel in modified TAE (Millipore, Billerica, Massachusetts, USA). PCR product was cut from the gel, isolated (Montage gel extraction kit, Millipore), and sequenced by the Sequencing Center, Department of Biology, University of Turku, Finland by means of automated fluorescent sequencing. Gene specific primers for rapid amplification of cDNA ends (RACE) PCR were designed,

forward primer for 3'-RACE-PCR and reverse primer for 5'-RACE-PCR (forward 3'atgcaaaggaaagacatgcgacacc5' and reverse 3'ggtgtcgcatgtctttcctttgcat5'). 5'- and 3'-RACE-ready-cDNA were synthesized respectively and 3'- and 5'RACE-PCR reactions performed (BD SMART™ RACE cDNA Amplification Kit BD Biosciences Clontech). PCR product was separated by electrophoresis and extracted from the gel as described above. All isolated fragments were cloned into pGEM®-T Easy Vector (pGEM®-T Easy Vector SystemII, Promega, Madison, WI, USA). After ligation of PCR products into the vectors, vectors were transformed into JM109 high-efficiency competent cells (Promega, Madison, WI, USA), plated on LB/ampicillin/IPTG/x-gal plates and incubated for 24 h at 37°C. Four white colonies were selected from each of the transformation samples and incubated in LB solution (containing 100 μg mL^{-1} Ampicillin) for 24 h at 37°C (Promega technical manual no 042). PCR was performed with 1 μL aliquots of each liquid culture, using primers for the T7 and SP6 promoter regions of the plasmid vector, for identifying cultures containing inserts. Plasmid DNA from 1 mL of each bacterial culture containing inserts was purified (Wizard® *Plus* Minipreps DNA purification system, Promega, Madison, WI, USA). Purified DNA samples from both 3'- and 5'-RACE PCR reactions were sent for sequencing.

Metallothionein gene expression in response to waterborne copper

Gill tissue from both *T. sparrmanii* and *O. mossambicus* from all treatments (control, copper exposed and recovery) was homogenized on ice (ULTRA-TURRAX, Ika Labortechnik, Germany) in Tri Reagent (Sigma-Aldrich Inc, USA). Total RNA was isolated using the Tri reagent method (Sigma Technical Bulletin MB-205). RNA integrity was determined by separation with 1.2% agarose gel electrophoresis and both the yield and purity of RNA was determined by measuring absorbance at 230 and 260 nm. For each sample cDNA was

synthesized from 2 μg of RNA using standard Oligo(dT)$_{15}$primer (Promega, Madison, WI, USA), together with avian myeloblastosis virus reverse transcriptase (AMV-RT, Finnzymes, Espoo, Finland). DNA was amplified using polymerase chain reaction (PCR) with gene specific primers (forward 5'ccaagactggaacctgcaac3', reverse 5'tggtgtcgcatgtctttcct3'). The annealing temperature for amplification was 60°C. PCR product was separated with 1.2 % agarose gel electrophoresis in TBE buffer. Image analysis was performed under UV light with Chemi-Imager (Alpha Innotech Corp., San Leandro, CA, USA). Signals were normalized with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (forward primer 5'gggtgtcaaccaaaagt3', reverse primer 5'cgtcaaccgtcttctgtg3' designed from GAPDH sequence of *Oreochromis mossambicus*). To confirm that the amplified sequence was indeed MT, PCR product was separated on 1.2% agarose gel in modified TAE gel extraction buffer (Millipore, Billerica, Massachusetts, USA) and isolated from the gel with Montage gel extraction kit (Millipore, Billerica, Massachusetts, USA). The extracted PCR product was sequenced by the Sequencing Center, Department of Biology, University of Turku, Finland by means of automated fluorescent sequencing.

Water analysis

To determine if the measured copper levels in the tanks portray the calculated values, water from the tanks of the *T. sparrmanii* experiments were analysed for copper content. Water samples were taken from experimental tanks immediately after addition of copper and then 4 h and 24 h after addition of copper. A separate tank, without fish, was set up and the same water sampling regime was followed. Water was then acidified to 0.1 mol L^{-1} nitric acid and analysed by means of semi-quantitative ICP-MS. Water hardness (mg $CaCO_3$ L^{-1}) was also determined by means of EDTA titration (Clesceri et al., 1998).

Statistical analysis

Normal distribution was determined by means of a modified Kolmogorov-Smirnov (KS) test (Dallal and Wilkinson, 1986). Two-way analysis of variance (ANOVA), using Tukey HSD post-hoc test (Statistica Version 6.1, STATSOFT Inc.) was used to analyse results. All results were given as mean ± standard error of the mean (S.E.M). Significant differences between data of exposed animals and control animals at the same sampling time were indicated by asterisks, where ***/### indicated $P < 0.001$, **/## indicated $P < 0.01$ and */# indicated $P < 0.05$.

Results

Cloning and sequencing of metallothionein coding sequence

From sequencing the RACE-PCR products the following was identified as the coding sequence of *Tilapia sparrmanii* MT:

1 ATGGACCCCT GCGAGTGCGC CAAGACTGGA ACCTGCAACT GCGGAGGATC
51 CTGCACGTGC ACAAAGTGCT CATGCAAGAG CTGCAAGAAG AGCTGCTGCG
101 ACTGCTGCCC ATCCGGCTGC AGCAAATGCG CCTCTGGCTG CGTATGCAAA
151 GGAAAGACAT GCGACACCAG TGA

The coding sequence of *T. sparrmanii* shows a 98 % correspondence to the MT coding sequence of two other cichlids, *Oreochromis mossambicus* (GenBank accession number: **AY257202**) and *Oreochromis aureus* (GenBank accession number: **AY257201**):

```
T. sparrmanii      1 atggacccctgcgagtgcgccaagactggaacctgcaactgcggaggatcctgcacgtgc 60
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
O. mossambicus     1 atggacccctgcgagtgcgccaagactggaacctgcaactgcggaggatcctgcacgtgc 60

T. sparrmanii     61 acaaagtgctcatgcaagagctgcaagaagagctgctgcgactgctgcccatccggctgc 120
                     |||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
O. mossambicus     61 acaaagtgctcctgcaagagctgcaagaagagctgctgcgactgctgcccatccggctgc 120

T. sparrmanii     121agcaaatgcgcctctggctgcgtatgcaaaggaaagacatgcgacaccagctgctgccag 180
                     ||||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||
O. mossambicus     121agcaaatgcgcctctggctgcgtgtgcaaaggaaagacatgcgacaccagctgctgccag 180

T. sparrmanii     181tga 183
                     |||
O. mossambicus    181tga 183
```

```
T. sparrmanii      1 atggacccctgcgagtgcgccaagactggaacctgcaactgcggaggatcctgcacgtgc 60
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
O. aureus          1 atggacccctgcgagtgcgccaagactggaacctgcaactgcggaggatcctgcacgtgc 60

T. sparrmanii     61 acaaagtgctcatgcaagagctgcaagaagagctgctgcgactgctgcccatccggctgc 120
                     |||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
O. aureus          61 acaaagtgctcctgcaagagctgcaagaagagctgctgcgactgctgcccatccggctgc 120

T. sparrmanii     121 agcaaatgcgcctctggctgcgtatgcaaaggaaagacatgcgacaccagctgctgccag 180
                     ||||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||
O. aureus          121 agcaaatgcgcctctggctgcgtgtgcaaaggaaagacatgcgacaccagctgctgccag 180

T. sparrmanii     181 tga 183
                     |||
O. aureus         181 tga 183
```

Metallothionein gene expression in response to waterborne copper sulphate

After 4 h of exposure to copper, *T. sparrmanii* gill MT/GAPDH ratio of 1.563 ± 0.154 tended to be elevated (the difference to controls was not significant) compared to the control value of 1.245 ± 0.073. After 24 h of exposure the levels were even higher than after 4 h, resulting in the exposed value of 1.861 ± 0.154 being significantly higher than the corresponding control value of 1.220 ± 0.078 ($P < 0.01$). After 48 h in water with no added copper, the MT/GAPDH

ratio of 1.474 ± 0.097 had returned towards the control value of 1.433 ± 0.119 (Figure 1). There were no time dependent changes in the MT/GAPDH ratio of the 3 groups of control fish.

After only 4 h exposure to copper, *O. mossambicus* gill MT expression (expressed as MT/GAPDH ratio) increased to 1.558 ± 0.0941, which was significantly higher than the corresponding control level of 1.142 ± 0.086. After 24 h of exposure the initial rise in MT expression decreased to 1.068 ± 0.042, significantly lower than the 4 h exposure value and not significantly lower than the 24 h control value of 0.95 ± 0.016. After 48 h of recovery in water with no added copper the MT expression for the exposed animals (0.898 ± 0.048) is close to the value for the corresponding control samples (0.974 ± .0361).

Water copper levels during experiment

No significant changes occurred in copper levels between different times in control tanks. The measured copper concentration immediately after addition of copper for the *T. sparrmanii* experiments was 4.026 ± 0.148 mg L^{-1} for exposure tanks with fish, decreasing to 3.731 ± 0.495 mg L^{-1} after 4 h and was determined as 3.851 ± 0.333 mg L^{-1} after 24 h of exposure (Figure 5). The calculated copper concentration was 4.4 mg L^{-1}.

Discussion

There are two differences between *T. sparrmanii* MT coding sequence and *O. mossambicus* sequence. A base pair difference occurs at position 144, where G is modified to A. This is, however, a silent mutation, as the *O. mossambicus* and *O. aureus* triplet (GTG) and the *T.*

sparrmanii triplet (GTA) both code for histidine. Another base pair difference occurs at position 72 of the *T. sparrmanii* coding sequences, where C is modified to A. This causes both triplets (TCA from the *T. sparrmanii* sequence and TCC from *O. mossambicus* and *O. aureus*) to encode for serine (Mathews and Van Holde, 1995). There would thus be no structural and functional differences in MT protein of *T. sparrmanii* and *O. mossambicus*.

High copper concentrations cause physiological parameters such as specific oxygen consumption rate of *T. sparrmanii* to change when exposures are performed in Potchefstroom tap water (Van Aardt and Booysen, 2004). This experiment was designed to determine whether or not copper had an effect on MT gene expression in these two fish species and if the technique is sensitive enough to measure effects, high copper levels were used. The copper levels in this experiment is therefore much higher than environmental copper levels of 6.96 ● 1.97 μg L^{-1} and 5.83 ± 3.91 μg L^{-1} in Klerkskraal Dam and Boskop Dam, Potchefstroom respectively. According to results from water analysis during the experiments, no significant changes in water copper levels occurred during the course of the experiment (Figure 3).

In correspondence with data gathered when exposing rainbow trout to copper (Van Heerden et al. 2004), *T. sparrmanii* MT mRNA production in gills starts to increase within 4 h of copper exposure, although no significant copper accumulation could be observed, and recovers to levels close to those of control fish within 48 h in water with no added copper. MT induction does not correlate with copper levels in gill tissue from *T. sparrmanii* experiments. Unlike in rainbow trout, *T. sparrmanii* MT mRNA production is only increased to levels significantly higher than the control levels after 24 h of exposure, with no significant increases in copper accumulation. This slow accumulation of MT, indicate that tilapia is much less sensitive to

copper than rainbow trout. Indeed, the LC_{50} values for copper for rainbow trout range between 0.020 mg L^{-1} in soft acid water and 0.520 mg L^{-1} in hard alkaline water, in tests with hardness ranging from 30 to 360 mg L^{-1} as $CaCO_3$ and pH from 5 to 9 (Howarth and Sprague, 1978) whereas the values for *O. mossambicus* and *O. aureus* range from 0.2mg L^{-1} to 133.5 mg L^{-1} for in water with hardness ranging between 7 and 268 mg L^{-1} as $CaCO_3$ and pH 6.5 and 8.7 (for review see Straus, 2003).

In conclusion, specimens of *Tilapia sparrmanii* showed an increase in MT mRNA production after a short-term exposure to copper. However, the concentration of copper used was higher than that in local water bodies. Thus, although it is presently possible for us to carry out MT determinations on a species occurring in many South African rivers, and although MT accumulation occurs at high copper concentrations, causing changes in the physiological status of the species, field surveys are required to determine whether induction of the MT gene occurs in the species in natural conditions and whether the induction can be used as a biomarker of metal exposure in South African rivers.

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Figure legend

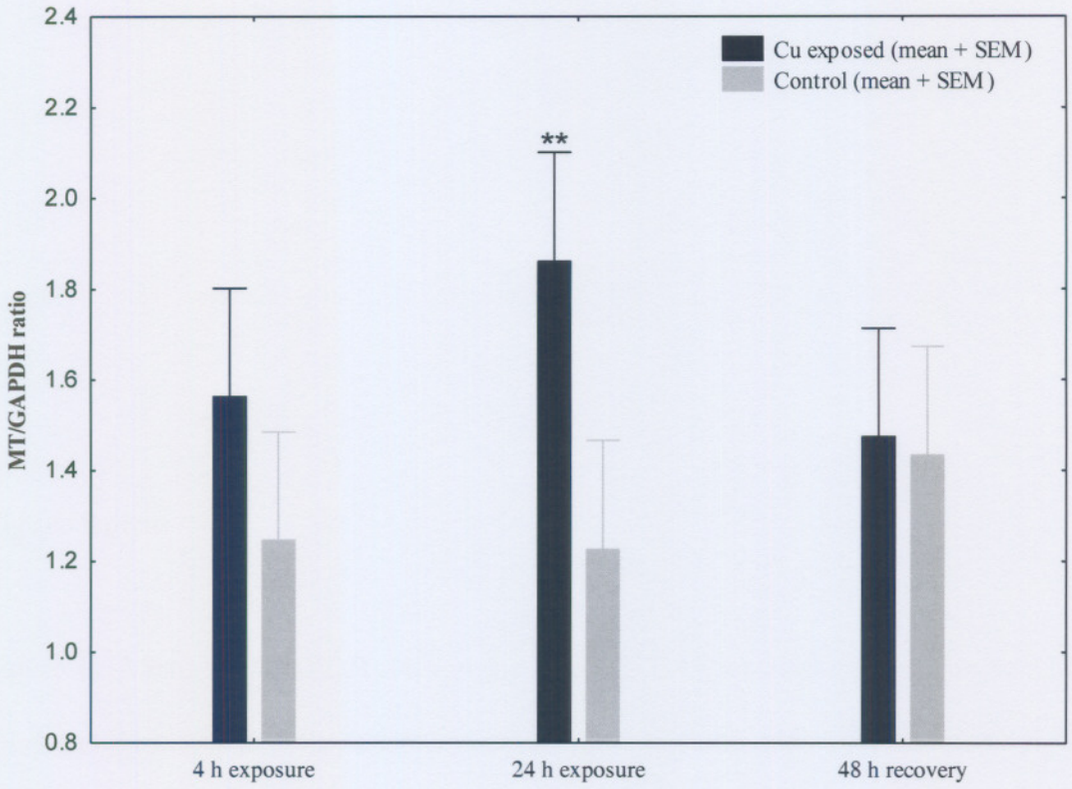
Figure 1. Mean MT/GAPDH ratio in the gills ± S.E.M for *T. sparrmanii* exposed to copper for 4 and 24 h respectively and recovered in water with no added copper for 48 h. Asterisks (**) indicate significant differences between copper-exposed and control fish at the same time point ($P < 0.01$). Two-way ANOVA, followed by Tukey's post-hoc test was used for comparisons (N = 6).

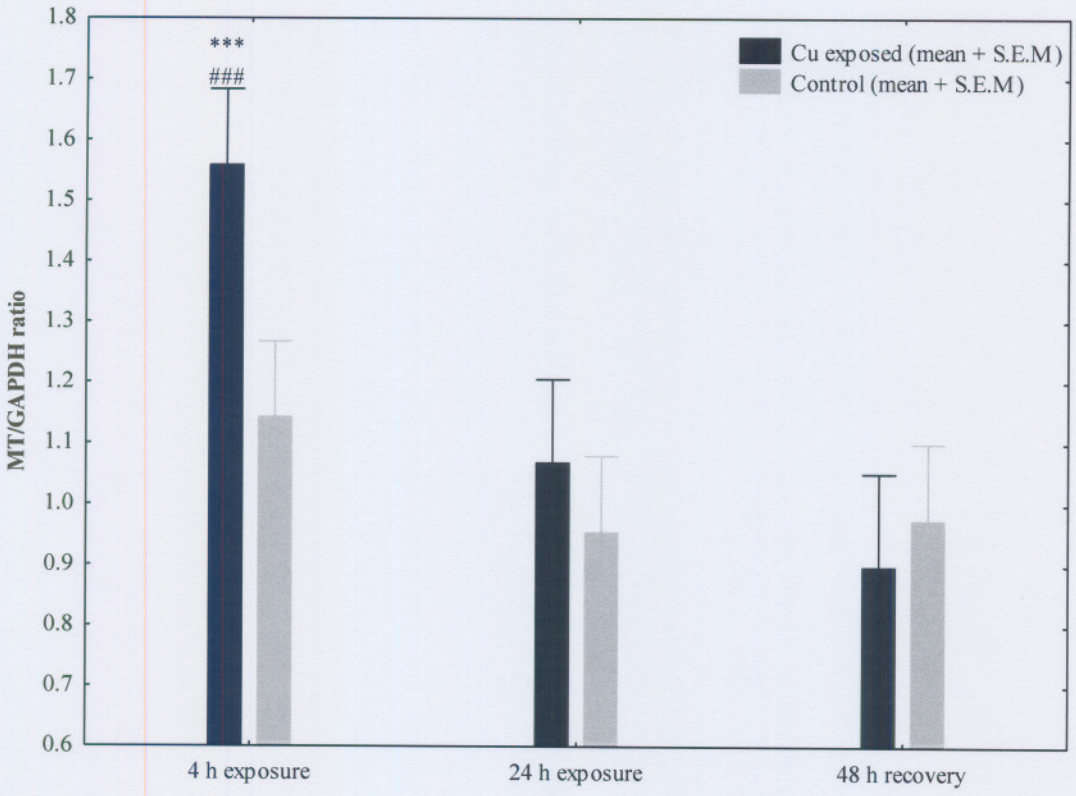
Figure 2. Mean MT/GAPDH ratio in the gills ± S.E.M for *O. mossambicus* exposed to copper for 4 and 24 h respectively and recovered in water with no added copper for 48 h. Asterisks(**) indicate significant differences between copper-exposed and control fish at the same time point ($P < 0.001$) and ### indicate significant differences between the MT/GAPDH ratio of fish exposed to copper for 4 and 24 h respectively ($P < 0.001$). Two-way ANOVA, followed by Tukey's post-hoc test was used for comparisons (N = 6).

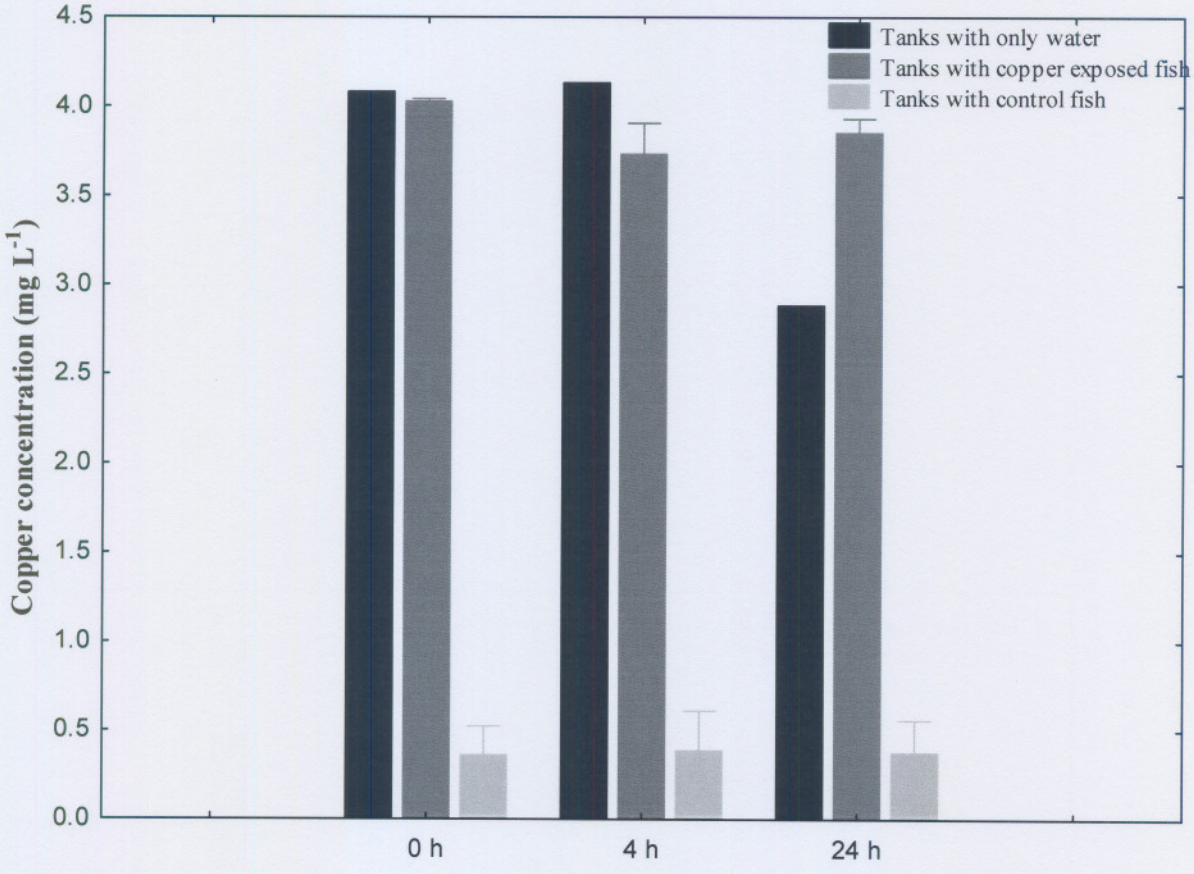
Figure 3. Water copper levels in experimental and control tanks immediately after copper was added to experimental tanks (T=0), 4 h after copper was added, when the first gill and liver samples were collected (T=4) and 24 h after copper was added (T=24).

Table legend

Table 1. Results from water analysis performed on water from holding systems for fish. *Tilapia sparrmanii* was held in Potchefstroom tap water and *Oreochromis mossambicus* in Stellenbosch tap water. Elements are given in $\mu g\ L^{-1}$ and water hardness in mg $CaCO_3\ L^{-1}$.


Cu exposed (mean + SEM)
Control (mean + SEM)
MT/GAPDH ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
**
4 h exposure
24 h exposure
48 h recovery


1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
MT/GAPDH ratio
***
###
Cu exposed (mean + S.E.M)
Control (mean + S.E.M)
4 h exposure
24 h exposure
48 h recovery


Tanks with only water
Tanks with copper exposed fish
Tanks with control fish
Copper concentration (mg L-1)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 h
4 h
24 h

Water elemental analysis	Potchefstroom tap water (µg L^{-1})	Stellenbosch tap water (µL^{-1})
Li	0.68	N.D.
Na	25000.00	2900.00
Mg	43000.00	N.D.
Si	2.80	1800.00
K	3000.00	91.00
Ca	14000.00	970.00
Sc	0.47	0.08
Cr	3.20	1.80
Fe	31.00	N.D.
Co	1.00	N.D.
Ni	2.40	1.00
Cu	13.00	19.00
Ge	0.20	N.D.
As	9.00	0.05
Se	6.40	N.D.
Br	39.00	0.61
Rb	3.60	0.23
Sr	21.00	6.60
Mo	0.40	0.05
Ag	0.05	N.D.
Sb	0.05	0.21
Cs	0.19	0.06
Hg	0.07	0.06
U	1.80	N.D.
Water hardness (mg CaC03 L^{-1})	139.87	12.75

N.D. = Not detected

Paper IV

Gill damage, metallothionein gene expression and metal accumulation in Tilapia sparrmanii *from selected field sites in Rustenburg and Potchefstroom, South Africa*

African Journal of Aquatic Science
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(**Updated**: 2003-05-15 08:38)

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Gill damage, metallothionein gene expression and metal accumulation in *Tilapia sparrmanii* from selected field sites in Rustenburg and Potchefstroom, South Africa

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ABSTRACT

Fish were collected from field sites in the mining and agricultural areas of Potchefstroom and Rustenburg, North-West Province, South Africa. Water and sediment samples from each site, together with fish muscle and gills were analysed by ICP-MS for Li, Na, Mg, Al, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Hg, Pb and U. All metal levels were within the levels set by the South African Department of Water Affairs and Forestry (DWAF), except for Cu, which is still within the levels set by other guidelines. Water hardness, pH, dissolved oxygen concentration (DO), and conductivity were measured at each site. Fish gills were analysed morphometrically for arithmetic mean epithelial thickness (H_{ar}) and with Reverse Transcriptase PCR for metallothionein gene expression levels. Fish liver was also analysed for MT expression. H_{ar} was found to increase with changing Cu levels in gills of fish, while gill and liver metallothionein levels corresponded to changes in sediment and gill cadmium levels respectively. There were highly significant differences in H_{ar} between different sampling sites while no significant differences in gill and liver MT expression were found.

KEYWORDS

Metal accumulation, South African rivers, Metallothionein, Gill damage

INTRODUCTION

South Africa is an arid to semi-arid region, with an average rainfall of less than 500 mm per annum. Areas with high demand receive little rainfall and together with a very high evaporation rate, this can cause water to be a very scarce resource in some parts of the country (O' Keeffe, Uys and Bruton 1994). Although we have large quantities of exploitable ground water, its distribution is limited and the quality of the available sources is often impacted by anthropological practices such as agriculture, urbanisation and industries (Rabie and Day 1994).

As awareness about and concern for environmental pollution increased in recent years, the demand for water quality monitoring, and especially biological monitoring, started to increase (Howells 1976, Stegeman and Hahn 1994). Routine monitoring programmes, however, only recently started to include biological monitoring techniques due to the following shortcomings in standard chemical and physical measurements: (1) it is both difficult and expensive to analyse every possible pollutant in a water sample, whereas biological monitoring is both cost effective and usually obtained easily; (2) physical and chemical analysis of the medium alone do not give an indication of the recent history of water quality, while biological sampling reflects both, as the exposure of aquatic animals to pollutants is continuous; (3) monitoring of biological communities allows integrated measures of the ecological integrity and over-all "health" of rivers, unlike chemical and physical stressors, which do not reflect the overall integrity of ecosystems (Chutter 1998, De La Rey, Taylor, Laas, Van Rensburg and Vosloo 2004, Eekhout, Brown and King 1996).

Another advantage of biological monitoring is it gives an indication of the fraction of the pollutant which is bioavailable. This alone cannot, however, give information on the effect of

toxicants on the organism, which is why it is important to use biomarkers, which could be biochemical, cellular, physiological or behavioural responses of animals to pollutants (Depledge 1993, Lam and Wu 2003). Biomarkers should be sensitive enough to react to pollutants, without the animal showing obvious signs of deterioration in health. If sensitive enough, these biomarkers can be short-term indicators of long-term effects, allowing human intervention in aquatic ecosystems before the effects of toxicants become irreversibly detrimental (McCarthy and Shugart 1990).

Aquatic organisms are targets of contaminated water from, amongst other, mining, industrial and agricultural activities. These waters usually contain heavy metals such as copper (Cu), zinc (Zn), cadmium (Cd) and mercury (Hg) (Zafarullah, Bonham and Gedamu 1988). When heavy metals from industrial activities are released into rivers, aquatic organisms can easily be exposed to metals at concentrations in excess of background levels normally encountered. Some organisms may be more tolerant to metal exposure than others, causing these animals to be in seemingly good health, even though they are exposed to exceptionally high metal levels. If these animals are used as food source, it could be detrimental for human health (Bryan 1976).

Fish gills are, due to their large surface area, the prime target for metals in water. Cu will be taken up through the apical sodium pathway in gills, inhibiting sodium uptake via gills. Cu has been reported to cause severe gill damage in fish, which included epithelial lifting, cell swelling, chloride and mucus cell proliferation and increased epithelial thickness caused by hypertrophy of epithelial cells (Cerqueira and Fernandes 2002, Van Heerden, Vosloo and Nikinmaa 2004, Mallat 1985). Gill damage can be linked to impaired physiological function (Woodward, Riley and Smith 1983) *e.g.* where increased epithelial thickness can cause an

increase in the barrier between blood and oxygen rich water (Lappivaara, Nikinmaa and Tuurala 1995), causing tissue hypoxia as indicated by an induction in Hypoxia inducible factor-1α (HIF-1α, Van Heerden, Vosloo and Nikinmaa 2004).

Animals in contaminated water act as natural accumulators of the toxicants they are exposed to, thereby reducing the detection limits required by analytical methods (STAP 2003). By measuring pollutant concentrations in animals, one can determine the fraction of the pollutant which is bioavailable and therefore can have an effect on the animals (Lam and Wu 2003). The accumulation of metals in animal tissue is often associated with an increase in metallothionein (MT) levels in both liver and gills of animals. MTs are small (6-7 kDa), cystein-rich, nonenzyme proteins. MTs are essential for the homeostasis of essential metals such as Cu and Zn, while it detoxifies non-essential metals such as Cd and Hg (Suzuki and Koiqumi 2000, Cousins 1985). They also act as scavengers of free radicals and reactive oxygen species (Kagi and Shaffer 1988).

The effect of metals on both accumulation of MT proteins and upregulation of MT gene expression have been studied extensively in different fish species, which include rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and zebrafish (*Danio rerio*) (Kling and Olsson 1995, De Boeck, Ngo, Van Campenhout and Blust 2002, Oslvik, Hindar, Zachariassen and Andersen 2001, Riggio, Filosa, Parisi and Scudiero 2003, Van Heerden, Vosloo and Nikinmaa 2004).

Because South Africa has an active mining industry, with an overall contribution of 12% to the GDP (South African Chamber of Mines 2004), it would be useful to find biomarkers in selected fish species to assess the impact of metal levels in rivers on aquatic fauna. Cichlid

fish were selected as model fish because they are widespread across Southern Africa and because they are omnivorous, they represent a trophic level high up in the food chain. The banded tilapia, *Tilapia sparrmanii*, is widely distributed northwards from the Orange River and Natal southcoast to southern Zaire, Lake Malawi and the Zambezi sytem. It has been translocated to the Cape, south of the Orange River in South Africa and was selected as target species during this study because it is an economically important species, being a common component of subsistence fisheries (Skelton 1993).

In this study we investigated if metal levels in water and sediment were associated with quantitative damage on gill structure, thus reducing the capacity for oxygen uptake in gills, with the consequent result that cellular oxygen levels are reduced. In addition, we investigated whether MT gene expression could be used to indicate the metal-induced effects in fish.

MATERIAL AND METHODS

Fish were collected from three sites in the Rustenburg area and two in the Potchefstroom area (Figure 1). In the Rustenburg area, one site was chosen close to the origin of the Hex River, one in the Hex River, downstream from Rustenburg town and one site downstream from where the Hex River and Leragane River flows into the Elands River. In the Potchefstroom area one site was chosen close to the origin of the Mooi River and one site downstream from mining, agricultural and industrial development. Fish were collected with nets, stunned by a blow to the head and the total length measured. The second left gill arch of each fish was clamped both dorsally and ventrally with forceps and dissected outside the forceps. The gill arch was immediately immersed in ice-cold Todd's fixative (Todd 1986) and stored at 4°C until analysis. The rest of the gill arches were cut from the gill chamber and tissue was

scraped from the cartilage before being snap-frozen in liquid nitrogen. Liver tissue was also collected and snap-frozen.

At each of the sites pH, dissolved oxygen concentration (DO) and temperature were measured (YSI 556 MPS, YSI Incorporated, USA). Water samples were collected in labelled plastic bottles, acidified to 0.1 mol L^{-1} with HNO_3 and stored until metal analysis. Water hardness (mg $CaCO_3$) was determined by means of EDTA titration (Clesceri, Greenberg, Eaton, 1998). Sediment samples were collected with a PVC corer from below the water surface. The sediment samples were stored in labelled plastic bags for transport to the lab.

Metal analysis

For the determination of tissue metal concentration frozen tissue samples were thawed, weighed and digested in a 5:1 mixture of 65% nitric acid and 70% perchloric acid. Samples were digested for 2 h (80°C in round bottom flasks with distillation condensers) after which digest were made up to a final volume of 20 mL with ultra pure deionized water.

Sediment samples were dried for at least 24 h at 70°C, ground with a mortar and pestle, passed through a 500 micron sieve and the larger particles discarded. The <500 micron fractions were stored in dry plastic containers until digestion and analysis. One gram fractions of sediment were digested in 12 mL of a 5:1 mixture of 65% nitric acid and 70% perchloric acid. Samples were digested for 2 h (80°C in round bottom flasks with distillation condensers) after which digests were filtered and made up to a final volume of 20 mL with ultra pure deionized water.

Metal concentration of the digested tissue, sediment and acidified water was determined by means of ICP-MS.

Gill morphometric studies

Gill samples of banded tilapia were treated at the Laboratory of Electron Microscopy, North-West University, South Africa. In short, after fixing with Todd's fixative, gill filaments were cut from the gill arch, post-fixed in 2% osmium tetroxide, stained with 2% uranile asetate and dehydrated in acetone. Filaments were then embedded in Spurr resin (Spurr 1969). Semithin (1 μm) saggital sections were cut from gill filaments and stained with 0.5% toluidine blue. Digital images were taken at 100 times magnification and analysed as previously described, with a grid constant of 16.5 μm (Van Heerden, Vosloo and Nikinmaa 2004).

Semi-quantitative analysis of metallothionein gene expression with RT-PCR

The *T. sparrmanii* MT coding sequence, obtained in a previous study, was used for designing the PCR-primers for this study (Daléne Van Heerden, André Vosloo and Mikko Nikinmaa, unpublished results). Liver and gill tissue of fish caught at the sampling sites were homogenized on ice (ULTRA-TURRAX, Ika Labortechnik, Germany) in Tri Reagent (Sigma-Aldrich Inc, USA). Total RNA was isolated using the Tri reagent method (Sigma Technical Bulletin MB-205). RNA integrity was determined by separation with 1.2 % agarose gel electrophoresis and both the yield and purity of RNA was determined by measuring absorbance at 260 and 280 nm. cDNA were synthesized from 2 μg of RNA using standard Oligo$(dT)_{15}$primer (Promega, Madison, WI, USA), together with avian myeoloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland). DNA was

amplified using polymerase chain reaction (PCR) with gene specific primers for a product size of 150 base pairs. An annealing temperature of 60°C was used for amplification. PCR product was separated with 1.2 % agarose gel electrophoresis in TBE buffer. Image analysis was performed under UV light with Chemi-Imager (Alpha Innotech Corp., San Leandro, CA, USA). Signals were normalized with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (GenBank accession no. AY140649; forward primer 5'gggtgtcaaccaa-aagt3', reverse primer 5'cgtcaaccgtcttctgtg3'). To confirm that the amplified sequence was indeed MT, PCR product was separated on 1.2% agarose gel in modified TAE gel extraction buffer (Millipore, Billerica, Massachusetts, USA) and isolated from the gel with Montage gel extraction kit (Millipore, Billerica, Massachusetts, USA). The extracted PCR product was sequenced by the Sequencing Center, Department of Biology, University of Turku, Finland by means of automated fluorescent sequencing to confirm that the PCR product was indeed MT.

Statistical analysis

A modified Kolmogorov-Smirnov (KS) test was used for determining normal distribution (Dallal and Wilkinson 1986).

From the ICP/MS results the following metals, accounting for 92 – 99% of the water and sediment metal content, were used in statistical analysis: Li, Na, Mg, Al, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Hg, Pb, U. Canonical Correspondence Analysis (CCA) were performed on the metal content of fish muscle and gills, together with MT induction in gills and liver as well as gill epithelial thickness (H_{ar}). Water and metal content as well as water pH, DO, conductivity and water hardness were used as environmental parameters. The Monte Carlo

Permutation Test (Bootstrapping) was used to determine the significance of the environmental parameters on the different variables (CANOCO program, Ter Braak and Prentice 1988).

Results for arithmetic mean epithelial thickness (H_{ar}) and MT levels at the different sampling sites were analysed with one-way analysis of variance (ANOVA), using Tukey HSD post-hoc test (Statistica version 7, STATSOFT inc.). All results were given as mean ± standard deviation (SD). Significant differences between data are indicated on graphs with either * or # (# or * indicates $P<0.05$, ## or ** indicates $P<0.01$ and ### or *** indicates $P<0.001$). The interpretation of * and # is explained in the caption of each graph.

Because Cu, Zn and Cd are known inducers of MT (Samson and Gedamu 1998), their influence on gill and liver MT levels were determined by means of stepwise multiple regression (Statistica version 7, STATSOFT inc.)

RESULTS

Physical parameters and water metal levels

Table 1 contains physical properties (pH, DO, hardness, temperature and conductivity) of the different field sampling sites: Water hardness was significantly higher in Boskop Dam, compared to all other sampling sites. pH in Vaalkop Dam was significantly lower and conductivity higher than other sites, while DO and temperature in Bospoort Dam was significantly higher than at other sites.

The metal levels in the water from all the field sites (Table 2) were compared to the South African Water Quality guidelines (Department of Water Affairs and Forestry 1996). Cu levels in both Klerkskraal (9.96 ± 1.965 μg L^{-1}) and Boskop Dam (5.83 ± 3.91 μg L^{-1}) are higher than the target water quality range (< 0.9 μg L^{-1}), the chronic effect value (0.15 μg L^{-1}) and the acute effect value (4.6 μg L^{-1}) of aquatic ecosystems set by the Department of Water Affairs and Forestry (DWAF) for aquatic ecosystems.

According to the ordination diagram in Figure 2A, fish parameters at Vaalkop Dam were influenced by the following water quality parameters: conductivity, As, Na, Cr and Mn levels. Fish parameters at Boskop Dam were influenced by water hardness, Co, Fe, Ni, Ca, Cu, Mg Li and to a lesser extent, pH; fish parameters at Klerkskraal Dam were influenced by Mn, Hg and temperature and fish parameters at both Bospoort and Olifantsnek Dams were mainly associated with DO.

Figure 2B shows that none of the potential biomarkers (liver MT, gill MT and H_{ar}) is associated with physical water parameters such as conductivity, hardness, pH, DO and temperature, because the biomarkers fall in the middle of the ordination diagram. The biological parameters such as gill Mn, Li, U and Cu were associated with water hardness, muscle Cu, Pb, Ni and Zn were associated with DO, gill Zn was associated with water temperature and gill Ni as well as muscle As were associated with conductivity.

Cu, Zn and Cd levels in water, sediment and their influence on biomarkers

Table 3 shows metal levels in water, sediment as well as fish muscle and gills. Water Cu levels were high in Klerkskraal and Boskop Dams while Cu was not detected in water

samples from the Rustenburg sites. Cu was detected in sediment, fish muscle and fish gills from all sampling sites. No Zn and Cd was detected in water samples from any of the sampling sites, but was detected in sediment, fish gills and fish muscle from all sampling sites.

Figure 3 gives the arithmetic mean epithelial thickness (H_{ar}) for *T. sparrmanii* at each of the sampling sites. At Boskop Dam, H_{ar} of 4.19 ± 0.69 μm was significantly higher ($P < 0.001$) than at any of the other sites (Klerkskraal Dam: 3.59 ± 0.59 μm, Olifantsnek Dam: 3.18 ± 0.49 μm, Bospoort Dam: 3.10 ± 0.50 μm and Vaalkop Dam: 3.38 ● 0.75 μm), while H_{ar} at Bospoort Dam was also significantly lower than H_{ar} at Klerkskraal Dam. Gill Cu levels (Table 3), which significantly influenced H_{ar} (Table 4, $P = 0.022$) were higher in fishes from Boskop Dam and Olifantsnek Dam, than fishes from the other sites.

Vaalkop Dam had the highest (although not significantly) gill MT induction, followed by Bospoort Dam, Boskop Dam, Olifantsnek Dam and Klerkskraal Dam (Figure 4). Gill MT was influenced by sediment Cd ($P = 0.08$, Table 4). The gill MT increased downstream from the origins of both rivers, with fish from Boskop Dam having a higher level of MT gene expression than fish from Klerkskraal Dam and fish from Vaalkop Dam a higher level than fish from Bospoort Dam, which in turn had a higher level of expression than fish from Olifantsnek Dam (Figure 4). There was no pattern in the liver MT gene expression in terms of distance from the origin of the river. The gene expression of liver MT was influenced by gill Cd levels, which was the highest at the sites furthest from the origin from each river (Table 3).

DISCUSSION

Because *T. sparrmanii* is used as a source of protein by local populations, it is very important to assess the influence of water quality and metal contamination on its health. *T. sparrmanii* is a hardy fish, tolerating a wide range of environmental disturbances. If the fish has a high tolerance to metal levels, without obviously being affected, this could cause people to ingest fish, which may seem healthy but accumulated metals and other chemicals from the water. It is therefore important to identify certain physiological or biochemical responses to toxicants to be assessed in monitoring programs.

Metal levels at all sites were well within the target water quality range (TWQR) set by DWAF (1996), except for Cu levels in Boskop Dam and Klerkskraal Dam exceeding even the acute effect value. The Mooi River flows through a mining and agricultural area, where Cu could be present in effluent and agricultural run-off water. According to a report by Heath and Claassens (1999), the TWQR value for Cu of 0.9 $\mu g\ L^{-1}$ is not a realistic range for South African Rivers. Because of the high Cu content of South African rivers, Heath and Claassens (1999) found that our rivers rather complied with guidelines set by Kempster, Hattingh and Van Vliet (1980), which is 200 $\mu g\ L^{-1}$ Cu. Measured by that standard, the Cu levels in Boskop Dam and Klerkskraal Dam was well within the guidelines set by Kempster, Hattingh and Van Vliet (1980) for the protection of aquatic ecosystems.

All the metal levels measured in fish muscle during this study were within the ranges of metal levels measured in fish muscle in a variety of water bodies around the world, including African inland waters, British rivers, Finnish lakes, The Gulf of Thailand and New Zealand (For summary: see Heath and Claassens 1999). In short, the ranges for metals from these

water bodies ranged between not detectable to 4.06 μg g^{-1} for Hg, not detectable to 0.9 μg g^{-1} for Cd, not detectable to 1.2 μg g^{-1} for Pb, not detectable to 4.43 μg g^{-1} for Cu and 0.3 to 58.4 μg g^{-1} for Zn.

Gill MT induction was influenced by sediment Cd concentrations while liver metallotionein levels are dependent on gill Cd levels (Table 4). Cd, along with Cu and Zn are known to cause induction in MT gene expression (Samson and Gedamu 1998). It has been shown that MT gene expression does increase during Cu exposure in *T. sparrmanii* (Daléne Van Heerden, Andre Vosloo and Mikko Nikinmaa, unpublished results). Cu, Zn and Cd were not detected in the water of the Rustenburg sites, with the result that no significant differences in MT gene expression from these sites were measured. Whatever inducers of MT gene induction present in the water, their levels were below the induction threshold for gill and liver MT responsive to metals. The MT/GAPDH ratio levels in all sampling sites were within range of the levels found for *T. sparrmanii* not exposed to elevated levels of Cu during laboratory exposures (Daléne Van Heerden, Andre Vosloo and Mikko Nikinmaa, unpublished results).

There should, however, be further investigation into the effects of other factors (including oxygen stress) on MT induction, especially where different concentrations of mixtures of metals are present at the various sites.

An increase in the arithmetic mean thickness of gill epithelium (H_{ar}) corresponded to an increase in gill Cu levels in fish from the various sampling sites (Figure 3, Table 3, Table 4). These results are in accordance with results obtained by Van Heerden, Vosloo and Nikinmaa

2004, who showed H_{ar} to increase with Cu exposure in *Oncorhynchus mykiss*. Similar results have been obtained for *Tilapia sparrmanii* and *Oreochromis mossambicus* (Van Heerden, Tiedt and Vosloo 2004).

Histopathology biomarkers is in general assumed to be useful as indicators of an array of anthropogenic pollutants, where the alteration caused by exposure to toxicants can persist even after the exposure has ceased, making it ideal in monitoring long-term effects of short-term exposure (Hinton, Baumann, Gardner, Hawkins, Hendricks, Murchelano, Okihiro 1992, Hinton 1994, Lease, Hansen, Bergman, Meyer 2003). Overall, it seems that H_{ar} could be a very sensitive, general biomarker of toxic exposure in fish. Although metal levels are in range of the quality guidelines, H_{ar} is still elevated at sites with higher metal levels (especially Cu).

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FIGURE LEGENDS

Figure 1. Maps of South Africa and the North-West Province indicating the field sites (Flags with arrows) selected for testing biomarkers on wild caught *Tilapia sparrmanii.* Samples were collected in both the Limpopo and Vaal River catchments. No other rivers and streams than those used for gathering samples were shown on maps.

Figure 2. Ordination diagrams indicating the water quality variables characterizing each field site. It also indicates which water quality variable determines which parameter measured in fish.

Figure 3. Arithmetic mean thickness of gill epithelium (H_{ar}). Asterisks indicate significant differences from Klerkskraal Dam and #'s represent significant differences from Boskop Dam. ***/### indicate $P < 0.001$, **/## indicate $P < 0.01$ and */# indicate $P < 0.05$.

Figure 4. Gill and Liver metallothionein levels as indicated by the ratio of MT and GAPDH. Black columns represent Liver MT and gray represents Gill MT. There were no significant differences in either Liver or Gill MT between the different sampling sites.

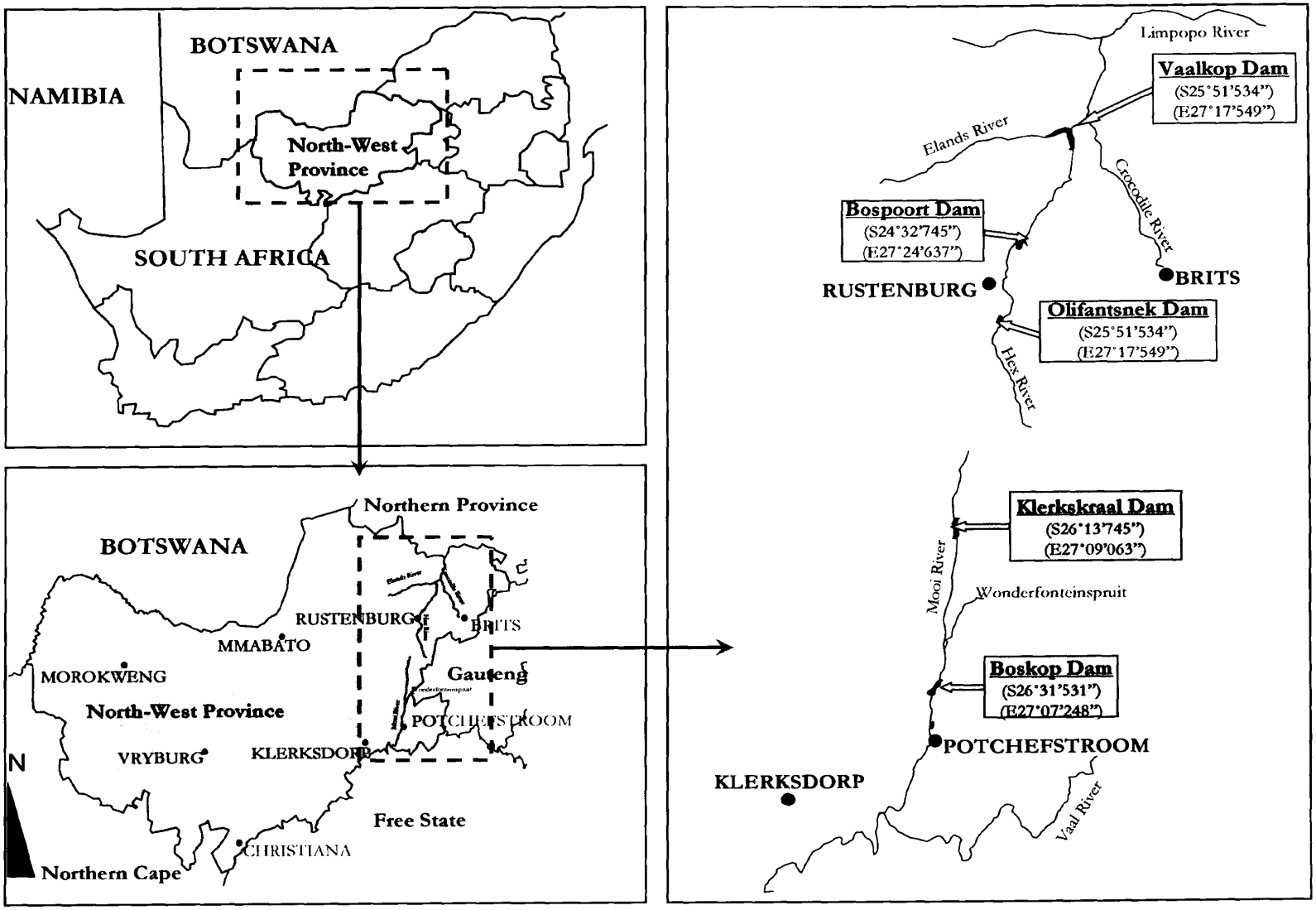
TABLE LEGENDS

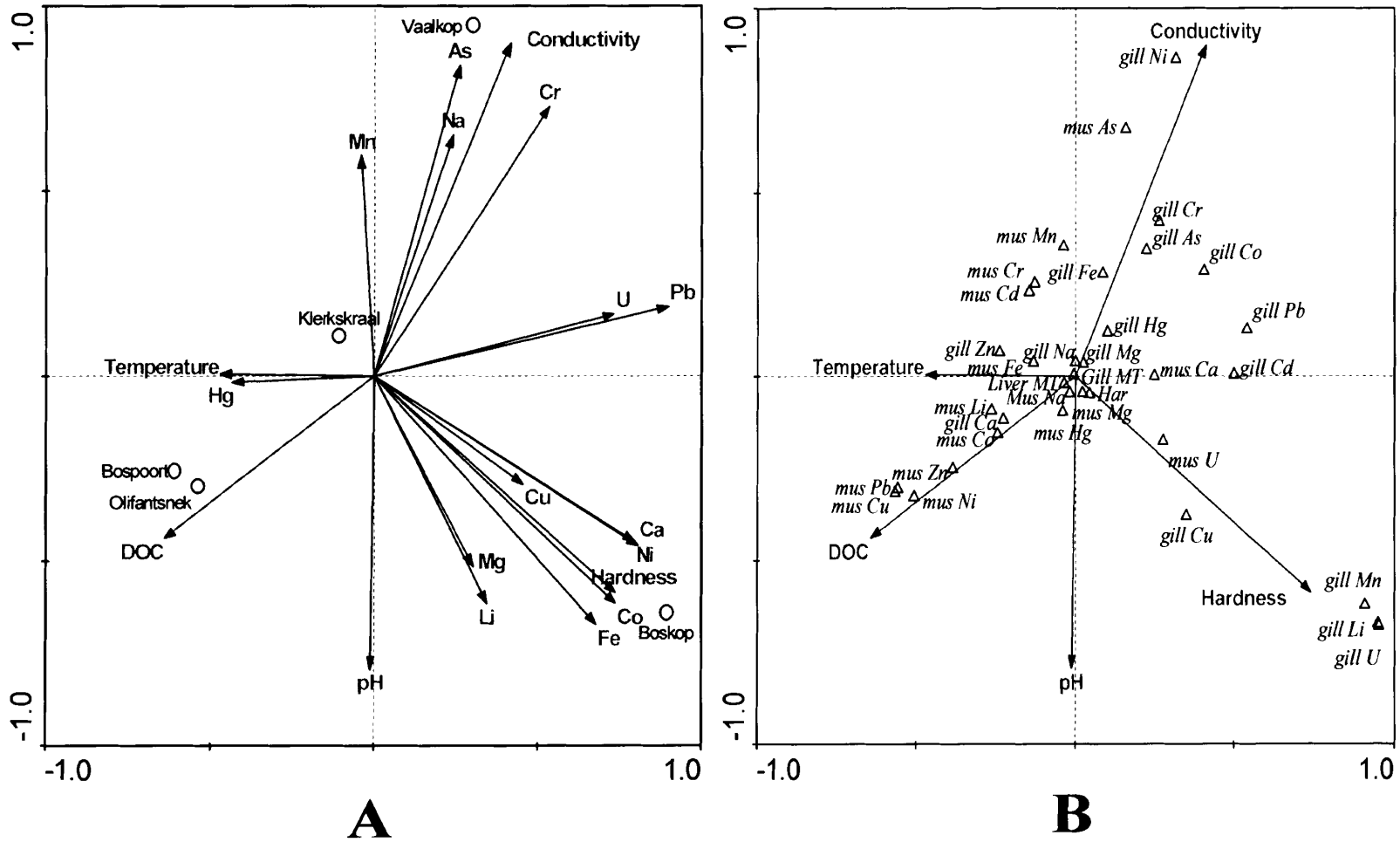
Table 1. Physical properties of the water from the different sampling sites. Hardness, pH, dissolved oxygen concentration (DO), temperature and conductivity were measured for each site. The target water quality ranges (TWQR) for aquatic ecosystems and domestic use, as determined by the South African Department of Water Affairs and Forestry (Department of Water Affairs and Forestry 1996), were included for comparison.

Table 2. Metal levels in water from the selected field sites showing the concentration of each metal present in the water in $\mu g\ L^{-1}$. The coloured blocks indicate where levels exceed the allowable metal concentrations as determined by the South African Department of Water Affairs and Forestry (1996). The target water quality ranges (TWQR) for aquatic ecosystems and domestic use, as determined by the South African Department of Water Affairs and Forestry (Department of Water Affairs and Forestry 1996), were included for comparison.

Table 3. Copper, zinc, cadmium and lead concentrations in water, sediment, fish muscle and fish gills from the respective field sampling sites. Averages are given in $\mu g\ L^{-1}$ for water samples and µg/g for sediment and fish tissue. N represents the amount of samples where measurable concentrations of metals occurred. Zinc and cadmium levels in water samples were below the detection limit of the ICP-MS.

Table 4. Variables influencing the arithmetic mean epithelial thickness (H_{ar}) of gills as well as gill and liver metallothionein induction. Only physical water properties and Copper, zinc, cadmium and lead levels in water, sediment, gills and muscle were considered.


BOTSWANA
NAMIBIA
North-West Province
SOUTH AFRICA
Northern Province
BOTSWANA
RUSTENBURG
BRITS
MMABATO
MOROKWENG
Gauteng
North-West Province
POTCHEFSTROOM
VRYBURG
KLERKSDORP
N
Free State
CHRISTIANA
Northern Cape
Limpopo River
Vaalkop Dam
(S25°51'534")
(E27°17'549")
Elands River
Crocodile River
Bospoort Dam
(S24°32'745")
(E27°24'637")
RUSTENBURG
BRITS
Olifantsnek Dam
(S25°51'534")
(E27°17'549")
Hex River
Klerkskraal Dam
(S26°13'745")
(E27°09'063")
Mooi River
Wonderfonteinspruit
Boskop Dam
(S26°31'531")
(E27°07'248")
POTCHEFSTROOM
KLERKSDORP
Vaal River


1.0
-1.0
-1.0
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Vaalkop
As
Conductivity
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Hg
Bospoort
Olifantsnek
DOC
Cu
Ca
Ni
Mg
Hardness
Li
Co
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pH
A
Conductivity
gill Ni
mus As
gill Cr
mus Mn
gill As
gill Co
mus Cr
gill Fe
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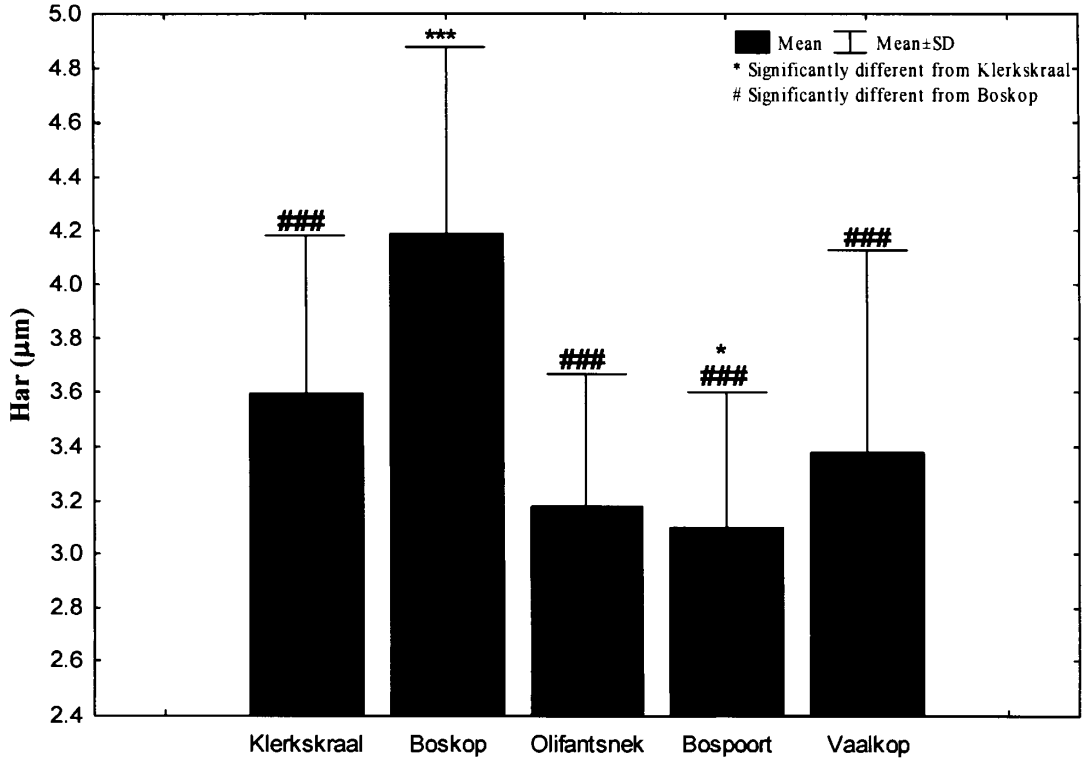
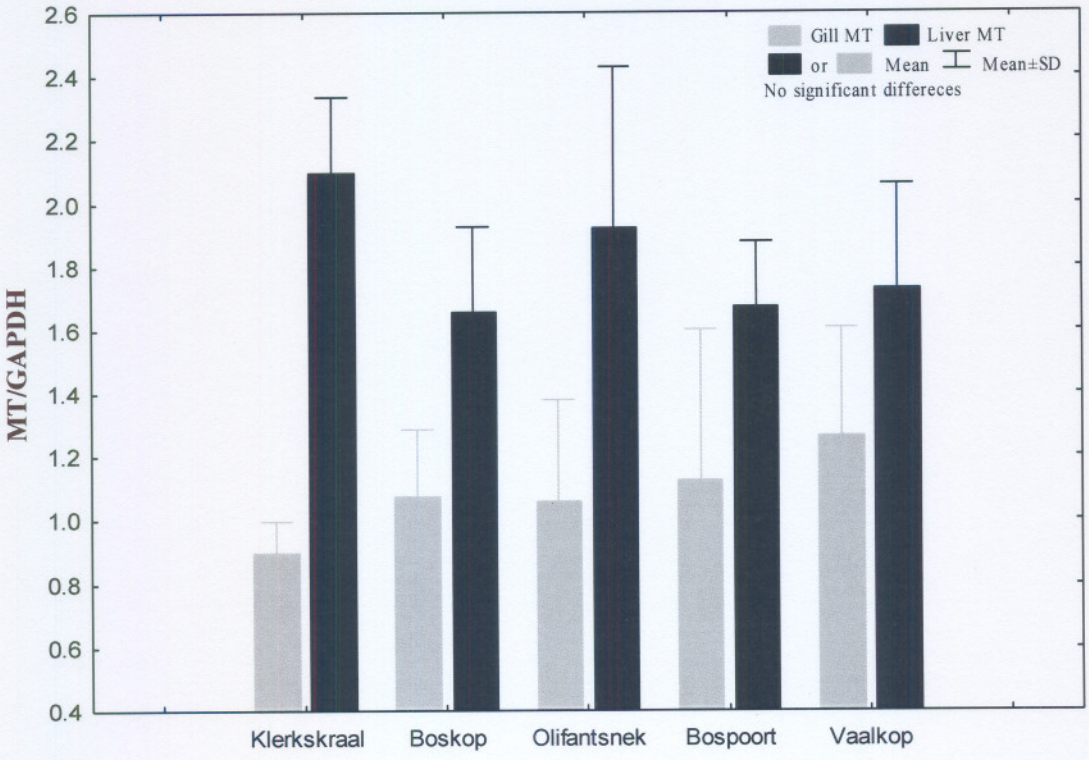

Mean
Mean±SD
* Significantly different from Klerkskraal
# Significantly different from Boskop
Har (µm)
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
###
***
###
*
###
###
Klerkskraal
Boskop
Olifantsnek
Bospoort
Vaalkop


Gill MT
Liver MT
or
Mean
Mean±SD
No significant differeces
MT/GAPDH
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Klerkskraal
Boskop
Olifantsnek
Bospoort
Vaalkop

	Klerkskraal Dam	Boskop Dam	Olifantsnek Dam	Bospoort Dam	Vaalkop Dam	Target water quality range (TWQR, DWAF 1996)	
						Aquatic ecosystems (µg L^{-1})	Human consumption (µg L^{-1})
Hardness (mg L^{-1})	129.80 ± 14.5 N = 5	212.42 ± 26.99 N = 6	141.83 ± 5.77 N = 6	102.61 ± 34.38 N = 6	111.76 ± 2.77 N = 5	Not available	Not available
pH	8.66 ± 0.03 N = 6	8.66 ± 0.03 N = 6	8.32 ± 0.04 N = 5	8.50 ± 0.2 N = 6	7.77 ± 0.08 N = 6	Not available	6 – 9
DO (mg L^{-1})	7.88 ± 0.24 N = 6	7.82 ± 0.05 N = 6	8.42 ● 0.17 N = 5	10.43 ± 2.13 N = 6	7.29 ± 0.35 N = 6	80 – 120% saturation	Not available
Temperature (°C)	21.16 ± 0.12 N = 6	21.11 ± 0.14 N = 6	21.54 ± 0.1 N = 5	28.86 ± 2.09 N = 6	22.89 ± 0.23 N = 6	Not available	Not available
Conductivity (µS cm^{-1})	550.33 ± 11.81 N = 6	514.17 ± 9.22 N = 6	491.20 ± 115.01 N = 5	456.83 ± 16.08 N = 6	683.50 ± 3.39 N = 6	Not available	Not available

	Klerkskraal Dam ($\mu g\ L^{-1}$)	Boskop Dam ($\mu g\ L^{-1}$)	Olifantsnek Dam ($\mu g\ L^{-1}$)	Bospoort Dam ($\mu g\ L^{-1}$)	Vaalkop Dam ($\mu g\ L^{-1}$)	Target water quality range (TWQR) (DWAF 1996)	
						Aquatic ecosystems ($\mu g\ L^{-1}$)	Human consumption ($\mu g\ L^{-1}$)
As	0.16 ± 0.03 N = 2	0.11 ± 0.06 N = 4	0.00003 N = 1	0.196 ± 0.11 N = 5	0.48 ± 0.16 N = 5	≤ 10	0 - 10
Ca	7260 ± 1631.87 N = 5	11000 ± 5059.64 N = 6	4716.67 ± 803.53 N = 6	6666.67 ± 2125.72 N = 6	6100 ± 141.42 N = 5	Not available	0 - 3200
Co	0.05 ± 0.03 N = 3	0.88 ± 0.1 N = 6	0.20 ± 0.13 N = 6	0.13 ± 0.04 N = 6	0.11 ± 0.04 N = 5	Not available	Not available
Cr	N.D.	1.01 ± 0.27 N = 2	N.D	N.D	5.5 N = 1	≤ 7	0 - 50
Cu	**6.96 ± 1.97 N = 5**	**5.83 ± 3.91 N = 6**	N.D	N.D	N.D	≤ 0.3	0 - 100
Fe	31.13 ± 22.45 N = 4	99 ± 60.82 N = 6	66.5 ± 48.11 N = 4	22 ± 12.53 N = 3	16.25 ± 5.80 N = 4	Not available	0 - 100
Hg	0.06 N = 1	N.D	N.D	0.06 N = 1	N.D	≤ 0.04	0 - 1
Li	0.3 ± 0.05 N = 5	0.365 ± 0.06 N = 6	0.25 ± 0.04 N = 6	N.D	0.02 ± 0.004 N = 2	Not available	Not available
Mg	16400 ± 547.72 N = 5	23000 ± 1095.45 N = 6	24000 ± 632.46 N = 6	11033.33 ± 4394.03 N = 6	15000 ± 0 N = 5	Not available	0 - 30 000
Mn	7.17 ± 6.92 N = 3	8.48 ± 7.96 N = 6	51 ± 25.93 N = 6	5.18 ± 5.81 N = 5	65.4 ± 14.14 N = 5	≤ 180	0 - 50
Na	1780 ± 44.72 N = 5	11833.33 ± 752.77 N =6	13166.67 ± 408.25 N = 6	12666.67 ± 3614.78 N = 6	34600 ± 894.43 N = 5	Not available	0 – 100 000
Ni	0.46 ± 0 N = 1	2.18 ± 2.03 N = 6	0.25 ± 0.31 N = 4	0.68 ± 0.31 N = 6	0.62 ± 0.17 N = 5	Not available	Not available
Pb	N.D	0.23 N = 1	N.D	N.D	0.17 N = 1	≤ 0.2	0 - 10
U	0.034 ± 0.02 N = 3	0.42 ± 0.05 N = 6	0.21 ± 0.03 N = 6	0.05 ± 0.05 N = 6	0.42 ± 0.06 N = 5	Not available	Not available

Exceed allowable concentrations for aquatic ecosystems (where N ≥ 3)

N.D. = Not Detected

	Klerkskraal Dam	Boskop Dam	Olifantsnek Dam	Bospoort Dam	Vaalkop Dam
Water (μg L^{-1})					
Cu	6.96 ± 1.97, N = 5	5.83 ± 3.91, N = 6	N.D.	N.D.	N.D.
Sediment (μg g^{-1})					
Cu	6.01 ± 1.85, N = 6	3.30 ± 0.73, N = 6	8.67 ± 1.00, N = 6	3.26 ± 1.43, N = 6	2.2 ± 0.52, N = 6
Zn	10.8 ± 2.74, N = 6	4.84 ± 1.41, N = 6	6.4 ± 0.98, N = 6	0.85 ± 0.06, N = 3	3.34 ± 0.47, N = 6
Cd	0.08 ± 0.04, N = 6	0.02 ± 0.01, N = 5	0.02 ± 0.003, N = 5	0.01 ± 0.003, N = 4	0.01 ± 0.01, N = 6
Fish muscle (μg/g)					
Cu	0.07 ± 0.005, N = 2	0.34 ± 0.45, N = 3	132.70, N = 1	0.04 ± 0.03, N = 4	0.004, N = 1
Zn	1.72 ± 0.37, N = 6	1.32 ± 0.15, N = 6	25.06 ± 42.60, N = 3	0.83 ± 0.29, N = 6	0.96 ± 0.79, N = 3
Cd	0.01, N = 1	0.005, N = 1	0.03 ± 0.004, N = 2	0.08 ± 0.004, N = 2	0.02 ± 0.002, N = 2
Fish gills (ng/g)					
Cu	1.79, N = 1	2.30 ± 3.78, N = 3	3.07, N = 1	0.25, N = 1	0.45, N = 1
Zn	6.26 ± 2.56, N = 3	0.003, N = 1	1.997, N = 1	0.90 ± 0.45, N = 6	1.57, N = 1
Cd	0.11 ± 0.07, N = 5	0.50 ± 0.60, N = 3	0.05, N = 1	0.03, N = 1	0.41 ± 0.41, N = 2

N.D. = Not detected

Variable influenced	Influenced by	Multiple R	Multiple R-square	R-square change	F	P
H_{ar}	gill Cu	1.000	1.000	0.042	833.873	0.022
Gill MT	Sed Cd	0.807	0.651	0.651	9.313	0.028
Liver MT	gill Cd	1.000	1.000	0.011	414.529	0.031

Chapter 4

Outlines of the original papers and discussion

The present study evaluated the effects of copper exposure on Cu accumulation, gill structure, MT gene expression and HIF-1α protein accumulation on selected fish species.

Rainbow trout, Mossambique tilapia and banded tilapia were exposed to Cu for a 24 h period, where gill and liver samples were taken at 4 and 24 h respectively. Fish were then transferred to water with no added Cu. Another set of samples were taken after 48 h of recovery.

The study on rainbow trout proved that the measured effects changed in response to Cu exposure (Paper I). For this reason and because SA has a lot of potential sources of metal pollution (*e.g.* mining industry and agriculture practices), these effects were also measured

in two Southern African fish species, Mossambique tilapia (*O. mossambicus*) and banded tilapia (*T. sparrmanii*).

Because *T. sparrmanii* is used as a source of protein by rural communities in South Africa, it is very important to assess the influence of water quality and metal contamination on its health. *T. sparrmanii* is a hardy fish, tolerating a wide range of environmental parameters. If the fish has a high tolerance to metal levels, without obviously being affected, this could cause people to ingest fish, which may appear healthy but had accumulated metals and other chemicals from the water. It is therefore important to identify certain physiological or biochemical responses to toxicants to be assessed.

T. sparrmanii were collected from selected field sites in the Rustenburg and Potchefstroom areas in order to evaluate the use of the selected structural and physiological changes that occur during Cu exposure in field sampling. Arithmetic mean epithelial thickness in gills, metal accumulation in tissue (gills and muscle) and MT gene expression were measured in fish caught at selected field sites (Figure 2.4). The MT gene expression, as portrayed by the MT/GAPDH ratio in fish gills, as well as metal levels in gill and muscle tissue, was compared with the occurrence of metals in sediment and water samples taken from each site (Paper IV).

A synopsis of the results obtained from all these analyses will now be given and discussed:

3.1 Laboratory exposures

3.1.1 Gill structure

The studies on rainbow trout showed an increased degree of gill damage as indicated by an increase in the mean arithmetic epithelial thickness (H_{ar}) of secondary gill lamellae. H_{ar} values were significantly higher than control samples after only 4 h of exposure to Cu ($P<0.001$). The thickening remained for the entire 24 h of Cu exposure. After 48 h of recovery in water with no added Cu, H_{ar} values decreased to levels still higher, though not significantly higher, than the control values (Paper I, Figure 3, Figure 4).

The arithmetic mean thickness of the gill epithelium of *O. mossambicus*, exposed to Cu, increased only after 24 h of exposure to levels significantly different from 4 h exposure and control levels (Paper II, Figure 1).

When exposed to Cu r for 24 h, the arithmetic mean epithelial thickness (H_{ar}) of *T. sparrmanii* was significantly higher than corresponding control values after only 4 h of exposure. This increased H_{ar} remained for the whole 24 h of exposure (Paper II, Figure 2).

There are differences between basal H_{ar} values between different fish species. *O. mossambicus* has a mean basal H_{ar} value of 3.03 μm while *T. sparrmanni* has a mean basal H_{ar} value of 4.98 μm (Paper II). Studies done by Van Heerden *et al.* (2004, Paper I) and Lappivaara *et al.* (1995) showed basal H_{ar} values for *O. mykiss* of 3.73 μm and 4.2 μm respectively. In another study on the effects of Cu on gill structure of *O. mykiss*, the mean basal H_{ar} value was found to be 3.9 μm (Daléne Van Heerden and André Vosloo, unpublished results). Since there was no significant difference between the basal H_{ar} in three separate studies on *O. mykiss*, measuring mean arithmetic epithelial thickness indicates that this technique could successfully be used to compare the changes in epithelial thickness within species from different experiments or caught from different locations during field based studies. Although sublethal, the concentrations of Cu used during these experiments were enough to cause severe gill damage in both *O. mossambicus* and *T. sparrmanii* as indicated by the highly significant increase in gill epithelial thickness during the 24 h Cu exposure. An increase in gill epithelial thickness leads to a decrease in the relative diffusion capacity of gill epithelium for oxygen (Lappivaara *et al.* 1995), which in turn could cause tissue hypoxia, as indicated in rainbow trout by an accumulation of HIF-1α proteins (Paper I).

Cerqueira and Fernandes (2002) found thickening in gill epithelium as result of hypertrophy in both pavement and chloride cells as well as lamellar telangiectasis (both as seen in Paper I, Fig. 3B) in *Prochilodus scrofa* as a result of Cu exposure. Changes in gill morphology due to exposure to metals are found to be a compensatory response to

prevent metals from entering through gill cells. These changes include cell proliferation, mucus secretion and hypertrophy (Mallat, 1985; Dang, Balm *et al.* 2000). Although hypertrophy of chloride cells (Paper I, Figure 3B) is considered a compensatory response, the increased chloride cell turnover, resulting from hypertrophy, can result in increased numbers of necrotic and apoptotic cells (Dang, Balm *et al.* 2000). Although it is indicated that the effects of Cu exposure on gills could be reversed to a certain extent (Cerqueira and Fernandes 2002, Paper I, Figures 3C and 4), permanent damage, for instance an increase in the number of necrotic and apoptotic cells, due to hypertrophy, could occur (Dang, Lock *et al.* 2000). Since changes in the mean arithmetic occurred early during Cu exposure, gill morphometric measurements could be used to indicate toxic exposure. The samples are quite easily collected and although time consuming, the analysis is easily carried out.

We have found other physiological effects associated with the thickening of gill epithelium due to Cu exposure in rainbow trout, including an accumulation of hypoxia inducible factor-1α (HIF-1α) (Paper I) , as well as decrease in both pH_a and P_aO_2 (Daléne Van Heerden and André Vosloo, unpublished results) after 4 h exposure to Cu.

3.1.2 Metallothionein gene expression

Induction of MT gene expression in rainbow trout gills occurred after only 4 h of Cu exposure (Paper I, Figures 1 and 2A) and remained for the full 24 h exposure period. There were no significant changes in liver MT gene expression in rainbow trout (Paper I, Figure 2B).

Because the MT coding sequence of *O. mossambicus* was already known, this fish species was included in laboratory experiments to assess the effects of copper on the selected structural and physiological parameters. After only 4h of exposure to Cu, *O. mossambicus* gill MT expression increased to levels significantly different from control levels and decreased to a level close to that of control samples after 24 h of exposure (Paper III, Figure 2).

The cloned MT coding sequence of *T. sparrmanii* showed a 98 % correspondence to the coding sequences of both *O. mossambicus* and *O. aureus* from GenBank (Paper III). The MT gene expression in *T. sparrmanii* gills were elevated (although not significantly) after 4 h of exposure to Cu and significantly elevated, compared to control animals, after 24 h of exposure (Paper III, Figure 1). The 98% similarity between *T. sparrmanii* MT coding sequence and *O. mossambicus* and *O. aureus* sequences occurs because of a one base pair difference at position 144, where G is modified to A. This is, however a silent mutation, as the *O. mossambicus* and *O. aureus* triplet (GTG) and the *T. sparrmanii* triplet (GTA) both code for histidine. Another base pair difference occurs at position 72 of the *T. sparrmanii* and *O. mossambicus* coding sequences, where C is modified to A. This causes the triplet from the *T. sparrmanii* sequence (TCA) and that of *O. mossambicus* (TCC) to code for serine (Mathews and Van Holde 1995). There would thus be no structural or functional difference in MT protein of *T. sparrmanii* and *O. mossambicus.*

The high Cu exposure concentrations were selected becaue they are known to cause physiological disturbances, such as changes in oxygen onsumption rates, when fish were exposed to metals in Potchefstroom water (Van Aardt and Booysen 2004). The water quality is given in Paper III, Table 1. The Cu concentration in the *O. mossambicus* experiments were within the ranges of 96 h LC_{50} for this species. Because these experiments were designed to determine whether or not Cu had an effect on MT in these two fish species and if the technique is sensitive enough to measure effects, high Cu levels were used. The Cu levels in these experiments are therefore much higher than environmental Cu levels of 6.96 ± 1.97 μg L^{-1} and 5.83 ± 3.91 μg L^{-1} in Klerkskraal Dam and Boskop Dam, Potchefstroom, respectively. According to results from water analysis during the experiments, no significant changes in water Cu levels occurred during the course of the experiment (Paper III, Figure 3).

In correspondence with data gathered when exposing rainbow trout to Cu (Paper I), *T. sparrmanii* MT mRNA production is increased after only 4 h of exposure to Cu, with no Cu accumulation, and recovers to levels close to that of control fish after 48 h in water

with no added Cu. Unlike in rainbow trout, *T. sparrmanii* MT mRNA production is only increased to levels significantly higher than the control levels after 24 h of exposure. This could possibly be attributed to cichlids being less sensitive to Cu than rainbow trout. This is depicted by the Cu LC_{50} values for rainbow trout ranging between 0.020 mg L^{-1} in soft acid water and 0.520 mg L^{-1} in hard alkaline water, in tests with hardness ranging from 30 to 360 mg L^{-1} as $CaCO_3$ and pH from 5 to 9 (Howarth and Sprague 1978). This is lower than the LC_{50} values of 0.2 mg L^{-1} to 133.5 mg L^{-1} for *O. mossambicus* and *O. aureus* in water with hardness ranging between 7 and 268 mg L^{-1} as $CaCO_3$ and pH 6.5 and 8.7 (review Straus 2003). The sensitivity of both *T. sparrmanii* and *O. mossambicus* to Cu being less than that of *O. mykiss* during lab exposures could also be attributed to the fact that *T. sparrmanii* and *O. mossambicus* was held in tap water, containing 13 and 19 μg L^{-1} of Cu, respectively, before the onset of the experiments. Grosell *et al.* (2001) found that Cu-acclimated rainbow trout show reduced Cu accumulation in plasma without the branchial Cu uptake being affected. The reduced plasma Cu levels were explained by an increased rate in Cu clearance and biliary Cu excretion where Cu acclimation preceded Cu exposure. This could also explain why MT gene expression was initially (after 4h of exposure) high in *O. mossambicus* and decreased after 24 h of exposure.

Since no MT gene upregulation was observed in the liver tissue of any of the experimental species, the changes in Cu level in this tissue must have been below the induction threshold for liver MT responsiveness to Cu.

3.1.3 Hypoxia inducible factor-1α (HIF-1 α)

Changes in rainbow trout HIF-1α levels occurred after only 4 h of Cu exposure (Paper I, Figures 2A, 4, 5), and decreased to levels close to control levels within the next 20 h of exposure.

During preliminary studies on HIF-1α proteins in *O. mossambicus* and *T. sparrmanii* it was found that the available HIF-1α antibody did react with *O. mossambicus* HIF-1α, but not with *T. sparrmanii.* Investigations into HIF-1α protein induction in *O. mossambicus* suggests

that an effect (probably indirect because of gill damage) can be observed after 24 h of Cu exposure. Further investigation is needed to obtain reliable results.

Lappivaara *et al.* (1995) found decreases in both the relative diffusion capacity and arterial oxygen tension of *O. mykiss* when exposed to Zn. We did not measure arterial oxygen tension and relative diffusion capacity. However, an increase in mean arithmetic distance between water and blood indicates that relative diffusion capacity is decreased. In accordance with this, we found evidence of hypoxia in gill tissue: HIF-1α protein levels in gill cells of exposed fish were higher than those of control fish after only 4 h of exposure. HIF-1α protein levels returned to normal within the next 20 h - a reduction of HIF-1α protein level appears to occur after the initial, early peak in rainbow trout cells exposed to hypoxia (Eeva Rissanen and Mikko Nikinmaa, unpublished results). Mouse liver, kidney and brain cells (Stroka *et al.*, 2001) and human lung epithelium (Uchida *et al.* 2004) also has an initial accumulation of HIF-1α protein, with a decrease in HIF-1α during prolonged hypoxia. This could be attributed to the destabilization of the HIF-1α protein during prolonged times of hypoxia (Uchida *et al.* 2004). The physiological consequences of the initial accumulation and consequent destabilization of HIF-1α are unknown at present.

3.2 Field studies

Paper IV, Table 1 contains physical properties (pH, DO, hardness, temperature and conductivity) of the different field sampling sites: Water hardness was significantly higher in Boskop Dam, compared to all other sampling sites. pH in Vaalkop Dam was significantly lower and conductivity higher than other sites, while DO and temperature in Bospoort Dam were significantly higher than at other sites.

The metal levels in the water from all the field sites (Paper IV, Table 2) were compared to the South African Water Quality guidelines (Department of Water Affairs and Forestry 1996). Cu levels in both Klerkskraal (9.96 ± 1.965 μg L^{-1}) and Boskop Dam (5.83 ± 3.91 μg L^{-1}) are higher than the target water quality range (< 0.9 μg L^{-1}), the chronic effect

value (0.15 μg L^{-1}) and the acute effect value (4.6 μg L^{-1}) of aquatic ecosystems set by the Department of Water Affairs and Forestry (DWAF) for aquatic ecosystems.

According to the ordination diagram in Figure 2A, fish parameters at Vaalkop Dam were influenced by the following water quality parameters: conductivity, As, Na, Cr and manganese (Mn) levels. Fish parameters at Boskop Dam were influenced by water hardness, cobalt (Co), iron (Fe), Ni, calcium (Ca), Cu, magnesium (Mg), lithium (Li) and to a lesser extent, pH; fish parameters at Klerkskraal Dam were influenced by Mn, Hg and temperature and fish parameters at both Bospoort and Olifantsnek Dams were mainly associated with DO.

Paper IV, Figure 2B shows that none of the potential biomarkers (liver MT, gill MT and H_{ar}) is associated with physical water parameters such as conductivity, hardness, pH, DO and temperature, because the biomarkers fall in the middle of the ordination diagram. The biological parameters such as gill Mn, Li, uranium (U) and Cu were associated with water hardness, muscle Cu, Pb, Ni and Zn were associated with DO, gill Zn was associated with water temperature and gill Ni as well as muscle As were associated with conductivity.

Paper IV, Table 3 shows metal levels in water, sediment as well as fish muscle and gills. Water Cu levels were high in Klerkskraal and Boskop Dams while Cu was not detected in water samples from the Rustenburg sites. Cu was detected in sediment, fish muscle and fish gills from all sampling sites. No Zn and Cd was detected in water samples from any of the sampling sites, but was detected in sediment, fish gills and fish muscle from all sampling sites.

Paper IV, Figure 3 gives the arithmetic mean epithelial thickness (H_{ar}) for *T. sparrmanii* at each of the sampling sites. At Boskop Dam, H_{ar} of 4.19 ± 0.69 μm was significantly higher ($P < 0.001$) than at any of the other sites (Klerkskraal Dam: 3.59 ± 0.59 μm, Olifantsnek Dam: 3.18 ± 0.49 μm, Bospoort Dam: 3.10 ± 0.50 μm and Vaalkop Dam: 3.38 ± 0.75 μm), while H_{ar} at Bospoort Dam was also significantly lower than H_{ar} at Klerkskraal Dam. Gill Cu levels (Paper IV, Table 3), which significantly influenced H_{ar}

(Paper IV, Table 4, P = 0.022) were higher in fishes from Boskop Dam and Olifantsnek Dam, than fishes from the other sites.

Vaalkop Dam had the highest (although not significantly) gill MT induction, followed by Bospoort Dam, Boskop Dam, Olifantsnek Dam and Klerkskraal Dam (Paper IV, Figure 4). Gill MT was influenced by sediment Cd (P = 0.08, Paper IV, Table 4). The gill MT increased downstream from the origins of both rivers, with fish from Boskop Dam having a higher level of MT gene expression than fish from Klerkskraal Dam and fish from Vaalkop Dam a higher level than fish from Bospoort Dam, which in turn had a higher level of expression than fish from Olifantsnek Dam (Paper IV, Figure 4). There was no pattern in the liver MT gene expression in terms of distance from the origin of the river. The gene expression of liver MT was influenced by gill Cd levels, which was the highest at the sites furthest from the origin from each river (Paper IV, Table 3).

Because *T. sparrmanii* is used as a source of protein by local populations, it is very important to assess the influence of water quality and metal contamination on its health. *T. sparrmanii* is a hardy fish, tolerating a wide range of environmental disturbances. If the fish has a high tolerance to metal levels, without obviously being affected, this could cause people to ingest fish, which may seem healthy but accumulated metals and other chemicals from the water. It is therefore important to identify certain physiological or biochemical responses to toxicants to be assessed in monitoring programs.

Metal levels at all sites were well within the target water quality range (TWQR) set by DWAF (1996), except for Cu levels in Boskop Dam and Klerkskraal Dam exceeding even the acute effect value. The Mooi River flows through a mining and agricultural area, where Cu could be present in effluent and agricultural run-off water. According to a report by Heath and Claassens (1999), the TWQR value for Cu of 0.9 $\mu g\ L^{-1}$ is not a realistic range for South African Rivers. Because of the high Cu content of South African rivers, Heath and Claassens (1999) found that our rivers rather complied with guidelines set by Kempster *et al.* (1980), which is 200 $\mu g\ L^{-1}$ Cu. Measured by that standard, the Cu levels in Boskop Dam and Klerkskraal Dam were well within the guidelines set by Kempster *et al.* (1980) for the protection of aquatic ecosystems.

All the metal levels measured in fish muscle during this study were within the ranges of metal levels measured in fish muscle in a variety of water bodies around the world, including African inland waters, British rivers, Finnish lakes, The Gulf of Thailand and New Zealand (For summary: see Heath and Claassens 1999). In short, the ranges for metals in fish muscle from these water bodies ranged between not detectable to 4.06 $\mu g\ g^{-1}$ for Hg, not detectable to 0.9 $\mu g\ g^{-1}$ for Cd, not detectable to 1.2 $\mu g\ g^{-1}$ for Pb, not detectable to 4.43 $\mu g\ g^{-1}$ for Cu and 0.3 to 58.4 $\mu g\ g^{-1}$ for Zn.

Gill MT induction was influenced by sediment Cd concentrations while liver MT levels are dependent on gill Cd levels (Paper IV, Table 4). Cd, along with Cu and Zn are known to cause induction in MT gene expression (Samson and Gedamu 1998). It has been shown that MT gene expression does increase during Cu exposure in *T. sparrmanii* (Daléne Van Heerden, Andre Vosloo and Mikko Nikinmaa, unpublished results). Unfortunately there were no Cu, Zn and Cd detected in the water of the Rustenburg sites, with the effect that no significant differences in MT gene expression from these sites were measured. Whatever inducers of MT gene induction present in the water, their levels were below the induction threshold for gill and liver MT responsive to metals. The MT/GAPDH ratio levels in all sampling sites were within range of the levels found for *T. sparrmanii* not exposed to elevated levels of Cu during laboratory exposures (Daléne Van Heerden, Andre Vosloo and Mikko Nikinmaa, unpublished results).

There should, however, be further investigation into the effects of other factors (including oxygen stress) on MT induction, especially where different concentrations of mixtures of metals are present at the various sites.

An increase in the arithmetic mean thickness of gill epithelium (H_{ar}) corresponded to an increase in gill Cu levels in fish from the various sampling sites (Paper IV, Figure 3, Table 3, Table 4). These results are in accordance with results obtained by Van Heerden, Vosloo and Nikinmaa 2004, who showed H_{ar} to increase with Cu exposure in *O. mykiss*. Similar results have been obtained for *T. sparrmanii* and *O. mossambicus* (Van Heerden, Tiedt and Vosloo 2004).

Histopathology biomarkers is in general assumed to be useful as indicators of an array of anthropogenic pollutants, where the alteration caused by exposure to toxicants can persist even after the exposure has ceased, making it ideal in monitoring long-term effects of short-term exposure (Hinton *et al.* 1992, Hinton 1994, Lease *et al.* 2003). Overall, it seems that H_{ar} could be a very sensitive, general biomarker of toxic exposure in fish. Although metal levels are in range of the quality guidelines, H_{ar} is still elevated at sites with higher metal levels (especially Cu).

3.3 Conclusion

The present results indicate that sublethal concentrations of Cu are already enough to result in gill damage and induction of MT in gills of *O. mykiss*, *O. mossambicus* and *T. sparrmanii* during laboratory Cu exposures, without Cu being accumulated. Furthermore, there is a clear difference in HIF-1α levels in exposed rainbow trout between 4 and 24 h exposure, whereas similar difference is not observed in control animals.

During short-term exposure to Cu, sublethal effects such as gill damage, tissue hypoxia and induction of MT gene expression can be observed. It can thus be concluded that gill morphometric analyses, HIF-1α protein accumulation and upregulation of MT gene expression in gills could be more sensitive as biomarkers of short-term Cu or other metal exposures than the mere accumulation of the metal in tissue.

Gill morphometric analysis of the epithelial thickness of secondary gill lamellae could be a very sensitive, general biomarker of toxic exposure in fish. Although metal levels at field sampling sites are in range of the quality guidelines, H_{ar} is elevated at sites with higher metal levels (especially Cu).

There should, however, be further investigation into the effects of other factors (including oxygen stress) on MT induction, especially where different concentrations of different metals are present at the various sites. There should also be further investigation into the preliminary results suggesting HIF-1α protein accumulation in *O. mossambicus*,

while another antibody for immunodetection of HIf-1α in *T. sparrmanii* tissues should be considered.

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Conference Contributions

ZSSA/SASAqS Conference, University of Cape Town, 30 June- 4 July 2003

3rd ICCPB in Africa, Ithala Game Reserve, South Africa, 7 – 13 August 2004

The influence of waterborne Copper on Metallothionein mRNA induction and gill structure in rainbow trout (*Oncorhynchus mykiss*)

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INTRODUCTION

Although copper is an essential nutrient to fish it becomes toxic when fish are exposed to high concentrations.

Because copper is known to accumulate in liver of fish and because gills play an important role in metal uptake, these two organs were chosen for this study. Cerqueira and coworkers (2002) found gill damage in *Procilodus scrofa* exposed to sub lethal concentrations of copper for 96 hours. Recovery of gill damage occurred after 7 to 15 days. Metallothionein protein concentration is known to increase with exposure to copper (Kling & Olson 1995). Kaplan *et al.* (1995) found it more sensible to use Metallothionein mRNA levels rather than protein concentrations because other post transcriptional processes could influence he expression or activity of proteins. Figure 1 shows the process of Metallothionein gene expression under basal an metal induced conditions. Hypoxia inducible factor (HIF-1α) protein increases during hypoxia in mammalian (Wang *et al.* 1995) and fish cells (Soitamo *et al.* 2001) and could thus serve as indication of gas exchange restriction. HIF-1α induction in human HeLaS3 cells occurs after only one hour of hypoxia (Jewel 2000).

The aim ot the study was to assess if (1) Metallothionein was upregulated, (2) the blood-barrier distance between water and gills increased during short term copper exposure, (3) these changes could be reversed by recovery in copper-free water and (4) HIF-1α levels in gills increased because of hypoxia in gill cells due to an increase in epithelial thickness of gill lamellae.

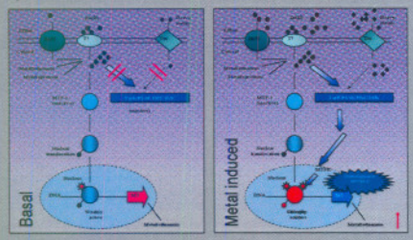

Figure 1 Under basal conditions zinc and other heavy metals will enter cells through known (Tm) and unknown (T?) metal transporters and will be metabolised by various metalloproteins, including Metallothionein. Somet metal-MTF-1 (metallothionein transcriopiton factor 1) complexes will be transported into the nucleus and activated to bind to DNA. In excessive concentrations, zinc and/or other heavy metals activate MTF-1 and MTFK (metallothionein fosfokinase) will cause a strong induction in Metalloprotein gene expression (Adapted from La Rochelle *et al.* (2001)).

MATERIAL AND METHODS

Adult rainbow trout (*O. mykiss*) with a body mass of 107-220 g were obtained from the Finnish Institute for Fisheries en Environment (Kirjala, Finland). Fish were acclimated for 1 week at 14°C (12h:12h night:day cycle) prior to experiments. Experimental fish were exposed to 1.65 µM copper.

After 4 and 24 houers of exposure and 48 hours of recovery, 6 copper exposed fish and 6 control fish were stunned with a blow to the head. The second left gill arch of each fish was clamped, dissected and fixed in 2.5 % glutharaldehyde. Liver and gills were dissected on ice and snap frozen in liquid nitrogen.

Metallothionein mRNA induction was determined by means of Reverse Transcriptase PCR. Product was run on an agarose gel and photographed under UV light. Intensity of the bands formed were measured with Chemi-Imager (Alpha Innotech, USA) (Airaksinen 2003). Simultaneous GAPDH mRNA expression was used to normalise the signal.

Hypoxia inducible factor-1α (HIF-1α) proteins were determined with Western Blot Immunodetection using C-terminal rabit-anti-trout HIF-1α as primary antibody. HRP-conjugated anti rabbit was used as secondary antibody. Autoradiography was performed and analysis were made with Chemi-Imager (Alpha Innotech, USA) (Airaksinen 2003). Alpha-tubulin was used to normalise the signals.

Gills were embedded in Epon and semithin sagital sections were made (Tuurala 1983). Morphometric analyses were carried out and arithmetic mean thickness of the epithelium (H_a) calculated for the secondary gill filaments of each fish (Weibel & Knight 1964). The arithmetic mean thickness of the epithelium gives an account of the blood-barrier distance between gills and water.

RESULTS AND DISCUSSION

Gill structure

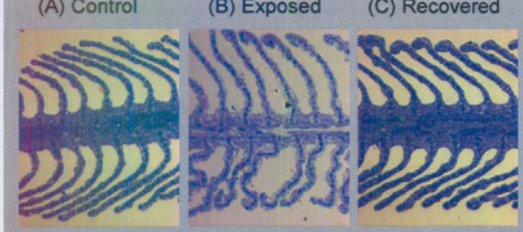

Figure 2 Light micrographs of sagital sections through secondary lamellae of rainbow trout gills. Micrograph A shows gills of control fish while micrograph B shows gills of fish exposed to 1.65µM coppper. Thickening of the epithelium because of hypertrophy of pavement cells (▶) and chloride cells (→) as well as lamellar telangiectasis (*) can clearly be seen in micrograph B. After 48 hour recovery some epithelial thickening and telangiectasis are still visible (C).

Gill damage occurred in gills of fish exposed to copper for only 4 hours (Figure 2B). Recovery took place to a certain extent after 48 hours. Thickening in epithelium as result of hypertrophy in both pavement and chloride cells as well as lamellar telangiectasis was also seen in the gills of the other copper exposed fish (Cerqueira *et al.* 2002). Hypertrophy of chloride cells is considered a compensatory response to maintain ion balance during metal exposure but Dang (2000) found that increased chloride cell turnover can result in increased numbers of necrotic and apopotic cells.

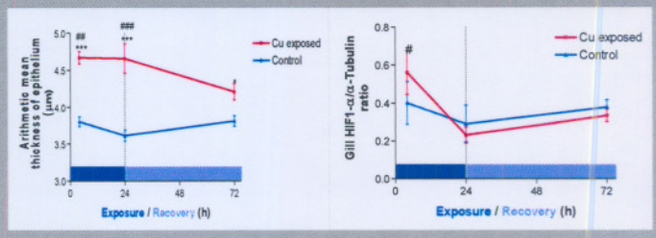

Figure 3 The arithmetic mean thickness of the epithelium (Har) increased significantly afgter only 4 hours of exposure to sublethal concentrations of copper. Har did not change significantly after a further 20 hours of exposure. After 48 hours of recovery, Har decreased significantly, but was still significantly higher than in control fish.

Figure 4 Increased levels of Hypoxia inducible factor-1α was detected after 4 hours copper exposure. After 24 hours HIF-1α levels had returned to normal and stayed constant after 48 hours recovery.

Changes in gill morphology due to exposure to metals help to keep the metals from entering through gill cells of fish. These changes in morphology induce cell proliferation and mucus secretion (Mallat 1995). In this study the arithmetic mean thickness of the gill epthelium increased with exposure to copper (Figure 3), which increased the blood-barrier distance. This increase in blood-barrier distance resulted from hypertrophy in pavement and chloride cells in the epithelium of secondary gill lamellae (Figure 2, Cerqueira *et al.* 2002).

Lappivaara *et al.* (1995) found a decrease in the arterial PO_2 of fish exposed to zinc because of an increase in the epithelial thickness. We found evidence of hypoxia in gill tissue based on the fact that HIF-1α protein levels in gill cells were found in significantly higher levels after 4 hours of exposure than control fish. The function of HIF-1α is to increase erythropoietin gene expression resulting in increased oxygen carrying capacity of red blood cells (Wang 1995). Jewel (2000) found human HeLaS3 cells to have peaked HIF-1α protein concentrations after only 1 hour of metal exposure and was maintained for 4 hours. After reoxygenation, nuclear HIF-1α protein concentrations decreased below detection within 32 minutes.

Metallothionein induction and copper accumulation in gill and liver tissue

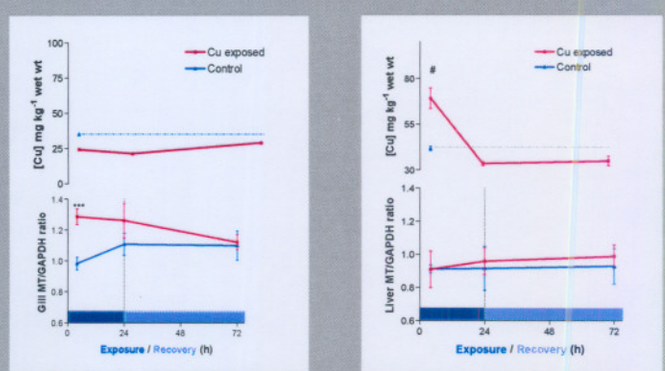

Figure 5 Metallothionein gene expression in gill tissue was induced within 4 hours of the start of the copper exposure. After 48 hours of recovery, gene expression decreased. Copper did not accumulate in gills (there were no significant differences in gill copper concentration in gill cells). There was no significant Metallothionein gene expression in liver tissue. Copper levels in liver tissue were significantly higher than control levels after only 4 hours of exposure. No differences in liver copper levels were detected between fish exposed for 24 hours and control values.

Copper enters gills through cells via apical sodium-channels (Grosell & Wood 2002). Metallothionein gene expression in gill tissue is induced. Gill cells in reaction to copper exposure will produce Metallothionein proteins. Copper will not accumulate in gills (Figure 5) as Metallothionein levels were high enough to bind all incoming copper. Because the Cu-thionein is transported to the liver, liver copper levels will be high at 4 hours. As the Cu-thionein is metabolised by lysosomes and excreted in bile (Cousins 1985), copper levels in the liver wil decrease, as is seen after 24 hours exposure (Figure 5). Metallothionein mRNA expression will not be induced in the liver because copper reaches the liver as Cu-thionein and not as free copper.

This study shows that care should be taken when interpreting tissue metal levels and even metallothionein protein concentration data.

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Metallothionein gene expression in two freshwater fish species during short-term exposure to copper

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INTRODUCTION

Amongst other effects, high copper levels can cause fast generation of reactive oxygen species (Harris and Gitlhin 1996). It also binds histidine-, cystein and methonine-containing proteins, resulting in dysfunction (Grosell and Wood 2002). Significant gill damage caused by high levels of copper has been documented in Rainbow Trout (Van Heerden *et al.*, 2004) and *Prochiladus scrofa* (Cerqueira and Fernandez 2002). An increase in the arithmetic mean epithelial thickness due to copper insult causes subsequent tissue hypoxia as indicated by an increase in HIF-1α protein levels in Rainbow Trout (Van Heerden *et al.* 2004).

Metallothioneins are small, non-enzymatic proteins that play a role in metal homeostasis and metal detoxification in fish. They are also induced by a number of mitogens and cytokines as well as oxidative stress (Samson *et al.* 2001). Of al the agents inducing MT synthesis, metals are the most common inducers (Cheung *et al.* 2004).

The aim of this study was to determine if an expected induction in metallothionein gene expression could be detected in Mozambique Tilapia (*Oreochromis mossambicus*) and Rainbow Trout (*Oncorynchus mykiss*) after short term copper exposure.

EXPERIMENTAL ANIMALS AND METHODS

Rainbow Trout
(*Oncorhynchus mykiss*)

Mozambique Tilapia
(*Oreochromis mossambicus*)

Rainbow Trout and Tilapia were exposed to a calculated copper concentration of 105 µg L^{-1} and 600 µg L^{-1} respectively for 24 hours. After both 4 and 24 hours of exposure 6 fish from exposure tanks and 6 fish from control tanks (with no added copper) were stunned, gill and liver tissue were collected and snap-frozen in liquid nitrogen. After the 24 hour exposure all fish, including control fish, were transferred to aquariums with clean water with no added copper. A similar sampling regime as during copper exposure was performed after 48 hours of recovery in clean water.

Total RNA was isolated using the TRI-Reagent method (Sigma Technical Bulletin MB-205). cDNA was synthesized with standard oligo(dT)15primer and AMV-Reverse Transcriptase. PCR amplification of cDNA was performed with primers designed from the respective metallothionein coding sequences available in GenBank for Rainbow Trout and Mozambique Tilapia. PCR-product was resolved on 1.2 % agarose gel and image analysis performed under UV light with Chemi-Imager (Alpha Innotech Corp., San Leandro, CA, USA). RT-PCR was also performed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and the MT/GAPDH ratio calculated.

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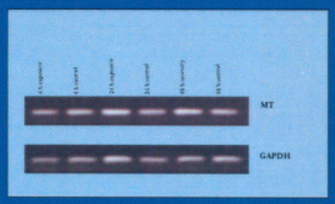
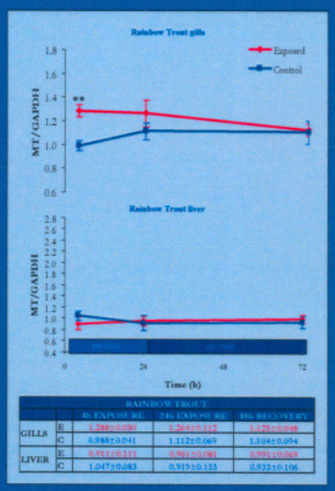
RESULTS AND DISCUSSION

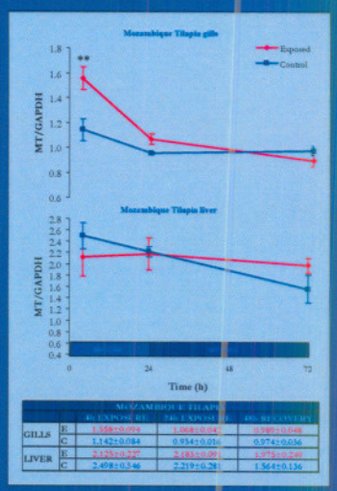


An example of the images obtained by photographing the resolved PCR product under UV light. Light intensity values for each fragment was obtained and the ratio between MT and GAPDH calculated.



RAINBOW TROUT		4h EXPOSURE	24h EXPOSURE	48h RECOVERY
GILLS	E	1.288±0.050	1.264±0.112	1.125±0.048
	C	0.988±0.041	1.112±0.069	1.104±0.094
LIVER	E	0.851±0.111	0.961±0.081	0.991±0.065
	C	1.047±0.083	0.919±0.133	0.935±0.106

MT/GAPDH of Rainbow trout (*O. mykiss*). in both gill and liver tissue of fish exposed to copper for 4 and 24 hours and 48 hours after transfer to water with no added copper. Asterisks indicate statistically significant differences (P < 0.01)



MOZAMBIQUE TILAPIA		4h EXPOSURE	24h EXPOSURE	48h RECOVERY
GILLS	E	1.558±0.094	1.068±0.042	0.980±0.048
	C	1.142±0.084	0.954±0.016	0.974±0.036
LIVER	E	2.145±0.042	2.183±0.097	1.974±0.036
	C	2.498±0.346	2.219±0.281	1.564±0.136

MT/GAPDH of Tilapia (*O. mossambicus*) in gill and liver tissue of fish exposed to copper for 4 and 24 hours and 48 hours after transfer to water with no added copper. Asterisks indicate statistically significant differences (P < 0.01)

Exposure of Rainbow Trout and Mozambique Tilapia to 105 µg L^{-1} and 600 µg L^{-1} copper respectively, caused a significant copper load in the animals as indicated by the induction of gill metallothionein in both fish at 4 hours. Although the induction remained for the entire 24 hour exposure in Rainbow Trout, downregulation was detected in Tilapia at the end of the 24 hour exposure. Within 48 hours after transfer to water with no added copper, metallothionein gene expression was downregulated to levels close to those of control fish as indicated by the MT/GAPDH ratio. The fact that no metallothionein gene upregulation was observed in the liver tissue of both fish species, indicates that whatever copper present was below the induction threshold for liver metallothionein responsive to copper. The thickening of gill epithelium and subsequent accumulation of HIF-1α proteins as indicated by Van Heerden *et al.* 2004 during copper exposure could also be partially responsible for an upregulation of metallothionein gene expression.

CONCLUSION

During short-term exposure to copper, gill metallothionein induction occurs within the first four hours of exposure. This proves that metallothionein gene expression in the gills of both *Oncorhynchus mykiss* and *Oreochromis mossambicus* is a sensitive biomarker of short term metal exposures.

Banded Tilapia (*Tilapia sparrmanii*) shows a wider distribution than *O. mossambicus* in Southern Africa (Skelton 1993). For this reason we recently sequenced the metallothionein coding sequence for *T. sparrmanii* to be used in similar studies. Future studies will include the validation of metallothionein as biomarker of environmental metal contamination in Southern African rivers.

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