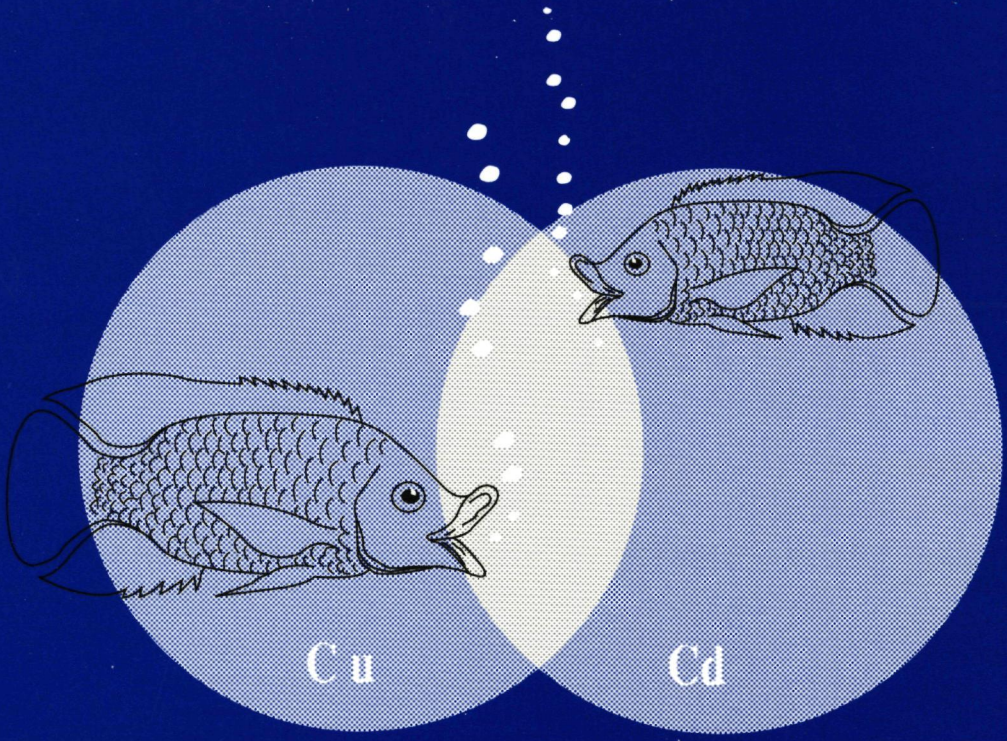
Interactions between Copper and Cadmium in Fish

metal accumulation, physiology and endocrine regulation



Sylvia Pelgrom

Interactions between copper and cadmium in fish

-metal accumulation, physiology and endocrine regulation

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op het gebied van de Natuurwetenschappen

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Ter nagedachtenis aan mijn vader
Aan mijn moeder
Voor Bert

GENERAL INTRODUCTION

Heavy metals, such as copper (Cu) and cadmium (Cd), are commonly present in freshwaters The natural background concentrations of Cu and Cd in aquatic environments is generally below 5 and 0 1 μg l^{-1}, respectively (Hodson *et al* , 1979, Bewers *et al* , 1987) In polluted freshwaters such as the rivers Rhine and Meuse, however, concentrations up to 70 μg Cu l^{-1} and 100 μg Cd.l^{-1} have been measured, mainly as a result of industrial waste (Department of Public Works of the Netherlands, 1988) Since many years there is concern about heavy metal pollution of freshwaters, because increased body concentrations of metals contribute to disturbed physiological homeostasis, which will lead to increased susceptibility to diseases, reduced performance and ultimately, death (Friberg *et al* , 1974, Frieden, 1979) Originally, toxicological studies have focused on lethality studies From these, safe concentrations of heavy metals as Cu and Cd in freshwaters were deduced, and maximal acceptable concentrations of Cu and Cd in drinking water have been published (Dutch Department of Housing, Physical Planning and Environmental Management, VROM, 1991, Dutch Department of Welfare, Health and Culture, WVC, 1992) More recently, the need to supplement these data with the effects of sublethal pollutant exposure on fish, and in particular the effects of the metals on reproduction and physiological homeostasis, has been recognized (Sprague, 1987) Such studies have shown that the gills are the primary target organ during waterborne heavy metal exposure Metals are not only taken up via the gills, but also interfere with branchial ion regulation This is illustrated by the observations of metal-induced disturbed plasma ion balance in fish exposed to sublethal concentrations of waterborne Cu or Cd (Giles, 1984, Fu *et al* , 1989) It became apparent that physiological homeostasis is disturbed in fish exposed to low concentrations of heavy metals, although these animals possess compensatory mechanisms to offset the adverse effects of metal exposure (Laurén and McDonald, 1987, Fu *et al* , 1989)

As in other vertebrates, exposure to environmental challenges generally induces a stress-response in fish, characterized by the sympathetic activation of the chromaffin cells as well as by activation of the hypothalamus-pituitary interrenal axis (HPI axis) The head kidney contains the tissue that is the teleost equivalent of the adrenal gland of the terrestrial vertebrates It has been

argued that compensating mechanisms during sublethal heavy metal exposure may depend on activation of the HPI-axis Studies on Cd (Fu *et al* , 1989) and Cu (Donaldson and Dye, 1975) have shown an increased release of the interrenal hormone cortisol during sublethal exposure to these metals This hormone potentially plays a role in the restoration of metal-induced disturbed ion-balance, by decreasing the branchial efflux and increasing the uptake capacity of ions partly by means of proliferation of ion-transporting cells in the gills (Perry *et al* , 1992, McDonald and Wood, 1993) The studies on Cu and Cd have increased the knowledge about the mechanisms by which Cu or Cd interfere with physiological systems in fish (see Figure 1), and the mechanisms involved in accommodating the adverse effects of sublethal waterborne heavy metal exposure

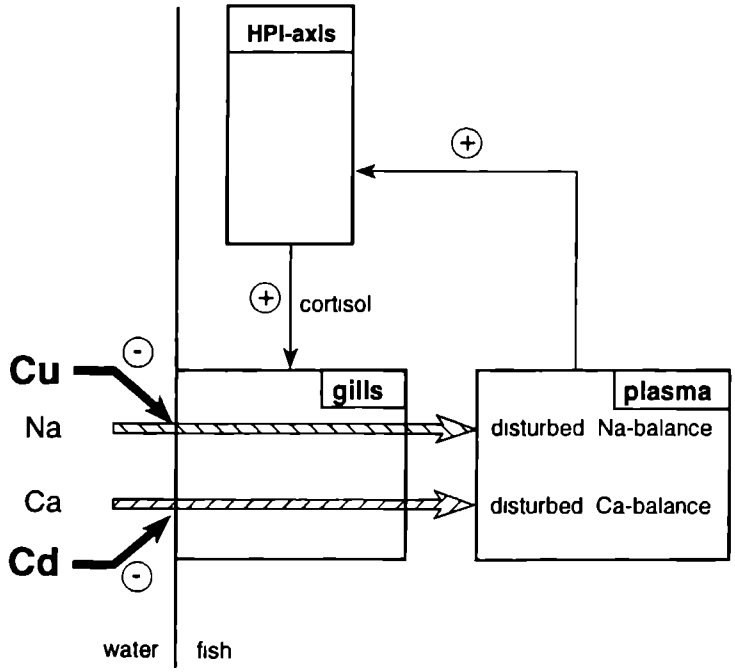

Figure 1. *Summary of Cu and Cd action on gill function, as described in the literature Action of waterborne Cu or Cd (⊖ arrows) include negative effects on processes in the total gill tissue (chloride cells and respiratory cells, apical as well as basolateral) Cu mainly affects Na-homeostasis whereas Cd impairs Ca balance in fish Disturbance of plasma ion homeostasis activates the HPI axis (Hypothalamus-Pituitary-Interrenal-axis) Subsequently, cortisol, released by the interrenal tissue, initiates adaptive regulation aimed to restore the ion balance*

Pollution of natural freshwaters, however, is not limited to one contaminant, but consists of mixtures of contaminants, which likely have interactive effects *In vitro* studies with mammalian cell cultures have demonstrated that the effects of combined Cu/Cd exposure are not simply a summation of the effects observed with the metals separately (Kaji *et al* , 1986, Meshitsuka *et al* , 1987), but resulted in an increased toxicity for the cells This points to interactions between Cu and Cd during combined exposure, and interactive effects have also been observed in other animal models Since fish are frequently faced with combined exposure to Cu and Cd in polluted freshwaters insight into the effects of combinations of these metals may contribute to more adequately defined safety levels for these metals in freshwaters In addition to *in vitro* studies, *in vivo* studies on metal accumulation and tissue distribution, and on physiological and endocrinological responses are needed to extend our knowledge of Cu/Cd exposure and accumulation in relation to branchial ion-transport (Na^+/K^+ ATPase activity and Ca^{2+}-transport, structural changes in gill tissue) and on compensatory mechanisms regulated by the HPI-axis (cortisol from the interrenal tissue and POMC- (pro-opiomelanocortin) derived peptides, such as ACTH (adrenocorticotropin hormone) and α-MSH (α-melanocyte stimulating hormone), from the pituitary gland) To this end, Cu/Cd interactions during waterborne metal exposure were studied in a euryhaline fish, the Mozambique tilapia *Oreochromis mossambicus*, since previous studies (exposure to acid water or to waterborne Cd) have led to insight in both physiological and endocrine responses to stressors in this species (Balm, 1986, Fu, 1989)

Outline of the present study

To analyze possible Cu/Cd interactions *in vivo* on metal accumulation, whole body Cu and Cd concentrations in tilapia exposed to a range of concentrations of waterborne Cu, Cd and to combinations of both metals were investigated (Chapter 1) Subsequently, organ (re-)distribution of Cu and Cd was determined in mature fish, exposed to these metals singly and in combination (Chapter 2)

In two previous studies, the effects of Cd exposure on cellular Ca transport mechanisms in the gills (Verbost, 1989) and the hormonal responses to Cd (Fu, 1989) were investigated in this species To complement these studies on Cd exposure with studies on the effects of waterborne Cu on physiological and biochemical parameters in tilapia, such as chloride cell numbers and diameter, branchial Na^+/K^+-ATPase activity and Ca^{2+}-transport activity, plasma Cu and ceruloplasmin levels, were studied (Chapter 3)

In Chapter 4, the effects of combined Cu/Cd exposure on whole body and plasma ion composition and the effects on plasma cortisol levels are described

Whole body calcium flux measurements (Chapter 5) show the effects of combined Cu/Cd exposure on Ca-influx, and are compared with the effects of either Cu or Cd exposure.

Chapter 6 describes the structural and physiological changes in the gills of Cu/Cd exposed fish in comparison with the effects observed in Cu or Cd exposed fish

In addition to waterborne heavy metals, the physiological homeostasis of fish is adversely affected by stressors such as predators and capture. Heavy metals as well as other stressors challenge branchial ion regulation, and have been demonstrated to activate the HPI-axis The effects of exposure of tilapia to various concentrations of waterborne Cu and to (non-metal) stressors on melanotrope function (MSH release from the pituitary) were studied in Chapter 7.

Chapter 8 describes the physiological response of tilapia exposed to stressful stimuli during exposure to waterborne metals. Also, the effects of waterborne Cu/Cd on compensatory mechanisms in response to stressful stimuli were studied

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CHAPTER 1

INTERACTIONS BETWEEN COPPER AND CADMIUM DURING SINGLE AND COMBINED EXPOSURE IN JUVENILE TILAPIA *OREOCHROMIS MOSSAMBICUS*

-Influence of feeding condition on whole body metal accumulation and the effect of the metals on tissue water and ion content

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ABSTRACT

Juvenile tilapia (*Oreochromis mossambicus*) were exposed for 96 h to ranges of sublethal concentrations of Cu or Cd, under both fed and non-fed conditions Exposure to one metal (Cu or Cd) not only resulted in an increased whole body content of the metal exposed to, but also influenced the concentration of the other metal present in the fish Furthermore, the total amount of Cu and Cd accumulated during exposure to heavy metals was influenced by the nutritional state of the fish

Besides exposure to either Cu or Cd, fish were also exposed to mixtures of Cu and Cd Results indicated that accumulation during Cu/Cd co-exposure cannot be predicted by simple addition of the effects of of single metal exposures Obviously, complex interaction mechanisms are involved, as was concluded e g from the significantly decreased whole body Cd-content of Cu/Cd co-exposed fish compared to the Cd content of Cd exposed fish This phenomenon was observed in both fed and non fed fish

Because ionic homeostasis is known to be affected by heavy metals, in this study also whole body water, calcium and sodium content in Cu and/or Cd exposed fish were determined The results indicate that also with respect to these parameters the two metals interact The effects on water and ions appear to be dissociated The data reveal previously unrecognized effects of the metals on whole body metal content, water and ion regulation

INTRODUCTION

As a result of increased industrialization, contamination of natural freshwaters by heavy metals, such as copper (Cu) and cadmium (Cd), has become a global problem In the case of essential trace elements, e g copper, zinc and nickel, the optimal concentration range for growth and reproduction are narrow, and both excess and deficiency are harmful to fish Some non essential trace metals suchs as mercury, lead and cadmium are toxic at concentrations observed in natural waters (Leland and Kuwabara, 1985, McKim, 1985) To assess the toxic impact of ambient levels of Cu or Cd on fish, the effects on several parameters have been studied For example, Cu affects the sodium balance (Reid and McDonald, 1988), growth (Lett *et al* , 1976), and swimming performance in rainbow trout (Waiwood and Beamish, 1978), and reproductive success in bluntnose minnow (Horning and Nieheisel, 1979), whereas Cd affects calcium balance in rainbow trout (Verbost *et al* , 1987) and induces damage in gill structure of zebrafish, rainbow trout and tilapia (Karlsson-Norrgren *et al* , 1985, Pratap and Wendelaar Bonga, 1993) It is evident from these studies that increased tissue concentrations of heavy metals induce significant stress in the animals The key to predict important toxic effects will be the understanding of the relation between accumulation of metals and their physiological or biochemical actions (Sprague, 1971, Grahl *et al* , 1985)

In most natural waters heavy metals are present as mixtures The effects of mixtures of metals on aquatic organisms are complex (Hamilton *et al* , 1987) As a reflection of this complexity, conflicting results have been published on the interaction between Cu and Cd concerning their accumulation and, consequently, their toxicity Westernhagen *et al* (1979) reported an additive effect of Cu and Cd on embryonic survival and hatching success in seawater herring, whereas Eisler and Gardner (1979) observed synergistic actions on mortality of Cu/Cd/Zn mixtures in the killifish, *Fundulus heteroclitus*

Since there is no clear insight in the interaction between Cu and Cd that determine the accumulation of the metals during combined exposure, in the present study we investigated the influence of Cu-exposure on whole body Cd content and vice versa in the tilapia *Oreochromis mossambicus*, after single and combined exposure to sublethal concentrations of these metals in the water Since it has been demonstrated that the feeding condition of the fish affects the adaptation to heavy metals (Collvin, 1985, Segner, 1987, Handy and Eddy, 1990), this study was performed with both fed and non-fed fish Finally, in order to investigate effects of altered whole body metal content on the ion balance of the fish, we related whole body Cu and Cd concentrations to body water, calcium and sodium content

MATERIALS AND METHODS

Fish and control water conditions

One-month old tilapia (*Oreochromis mossambicus*) were obtained from own laboratory stock. From 9 days after hatching the fish were kept in 'artificial freshwater' to ensure well-defined control water conditions, with undetectable Cu and Cd levels (detection levels below 0.10 and 0 01 $\mu g.l^{-1}$ respectively). The artificial freshwater consisted of demineralized water supplemented with: 1 3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$; pH 7.8. The composition and preparation of the water was based on the EEC instructions for artificial water for use in toxicity studies in fish (EEC Directive 84/449 /EEC Annex 5 method c1: Acute toxicity for fish) The ion concentrations were based on those in Nijmegen tapwater. The water was continuously aerated and filtered. The light/dark regime was 12/12 hours, and the water temperature 26°C Fish were fed at about 2% ($^{dw}/_{ww}$) of their body weight per day with commercial tropical fishfood (Micromin™). The Cu and Cd contents of the food were : Cu 5.90 ± 0 21 $\mu g.g^{-1}$ dry food; Cd 0.130 ± 0.005 $\mu g.g^{-1}$ dry food, (means ± SE; n=6)

Experimental design

Three days before the start of the experiment, 15 groups of 10 or 15 fish (average weight 1 gram) were placed randomly in polyethylene 4 5 l aquaria filled with artificial freshwater Depending on the experiment, fish were fed daily (2% $^{dw}/_{ww}$; all food was eaten within $^{1}/_{2}$ minute) or not fed During the acclimation period, the water in the aquaria was continuously refreshed by means of a flow-through-system (flow rate: 0.24 $l.h^{-1}$) The experiments with fed fish were performed prior to those with non-fed fish. The experiments started with the connection, by means of a 16 channel peristaltic pump (Watson Marlow), of each aquarium to its own water-supply (stock-solution of metal) with a well-defined concentration of Cu and/or Cd (added as nitrate; Spectrosol, BDH, England) in artificial freshwater During the first 6 h of exposure, the flow rate was 0 66 $l.h^{-1}$, followed by 90 h of exposure at a flow rate of 0 24 l h^{-1} Cu and Cd concentrations in the stock-solutions were monitored daily During the first 6 hours of exposure, Cu- and Cd-concentrations in the aquaria were monitored every hour, and at least once a day during the rest of the exposure period After 96 h of exposure, the fish were rinsed in artificial freshwater and immediately killed in icecold CO_2/aceton.

Determination of water- and tissue-concentrations of Cu and Cd, and of tissue water, sodium and calcium content

Water samples were acidified with nitric acid in a final concentration of 0 1%(v/v) Cu and Cd concentrations in the water samples were determined with a flameless atomic absorption spectrometer (AAS, Philips PU 9200), connected with an electrothermal atomiser (Philips PU 9390X)

Fish were put in vials, weighed and lyophilised After determination of the dry weight, the tissues were completely destroyed by the following procedure 1 h at 40° C after addition of 150 µl 65% HNO_3 , 1 h at 75° C after addition of 200 µl 65% HNO_3 and subsequently overnight at 110° C After complete destruction the samples were dissolved in 4 ml 0 1% HNO_3 (final concentration) and stored at 4° C until metal- and ion-analysis Whole body sodium and calcium contents in non-fed fish were determined by means of an inductive coupled plasma (ICP) atomic emission spectrometer (Plasma IL 200, Thermo Electron USA)

Several control procedures were performed No Cu or Cd could be detected in blank destruction samples, indicating that the vials did not release any of these metals The contribution to the total body metal content of metal adsorption to the body surface of the fish was negligible rinsing of metal-exposed fish in a 2mM EDTA-solution did not change the metal content of fish in comparison with fish rinsed in artificial freshwater (data not shown) No interference between Cu and Cd during the measurements could be detected in water and tissue samples, as determined by adding known amounts of Cu or Cd solutions to the samples Recovery of the spikes was nearly 100%, whereas no changes in the concentrations of Cu or Cd originally in the samples could be observed Cu and Cd determinations were performed under standard matrix conditions The accumulation factor (A F) was calculated according to (Holwerda, 1991)

$$\frac{[Me]_{fw\ exp} - [Me]_{fw\ control}}{[Me]_{water}}, \text{ where}$$

$[Me]_{fw\ exp}$ = metal concentration in the experimental group in µg g^{-1} fresh weight

$[Me]_{fw\ control}$ = metal concentration in the control group in µg g^{-1} fresh weight

$[Me]_{water}$ = metal concentration in the water in µg l^{-1}

Statistical analysis

Data are presented as means ± SE. Differences between groups were tested for significance by the Student's *t*-test for unpaired observations. Each metal concentration was tested in at least 2, generally 3, separate experiments. Before combining the results of the separate experiments, each group of fish was tested for significance against the corresponding control group within the experiment. Controls did not differ significantly between similar experiments and were therefore pooled.

The metal accumulation in feeding and non-feeding fish were fitted by applying regression analysis. Statistical significance is indicated as follows: * $P \leq 0.05$; ** $P \leq 0.02$; *** $P \leq 0.01$ and **** $P \leq 0.001$.

RESULTS

Metal concentrations in the aquaria gradually increased during the first 18 h of exposure, whereafter the metal concentrations remained constant during the exposure period (Fig. 1). No difference between the water metal concentrations before and after filtration over a 0.45 μm millipore filter in aquaria with fed and non-fed fish was observed. During the experiments, no mortality or changes in food intake, temperature and pH occurred. Exposure to Cu and/or Cd did not result in significant differences in total body weights between control and metal-exposed fish (results not shown).

Cu and Cd accumulation

Metal accumulation in fed fish after 96 h exposure to a Cu range of 0 - 400 $\mu g.l^{-1}$ (Fig. 2) could be described by the linear function $y=0.23x+9.44$ ($R=0.963$), where x=Cu concentration in the water (plateau) in $\mu g.l^{-1}$ and y=whole body Cu content at t=96h in $\mu g.g^{-1}$ dry weight). Cu accumulation in non-fed fish followed a different pattern. In particular at low Cu concentrations, non-fed fish accumulated significantly more Cu than the fed fish. In non-fed fish Cu-accumulation was fitted best by the non-linear function $y=13.65x^{0.31}$ ($R=0.915$). The difference in Cu accumulation between feeding and non-feeding fish was also noticeable in the accumulation factor (A.F.): in fed fish the A.F. was more or less constant whereas in non-fed fish the A.F. was very high at low Cu-concentrations and decreased with increasing Cu concentrations in the water. The A.F. in 100, 200 and 400 $\mu g\ Cu.l^{-1}$ exposed fish was similar in fed and non-fed fish (insert Fig. 2).

Cd-accumulation in non-fed fish was best described by the non-linear function $y=1.84x^{0.70}$

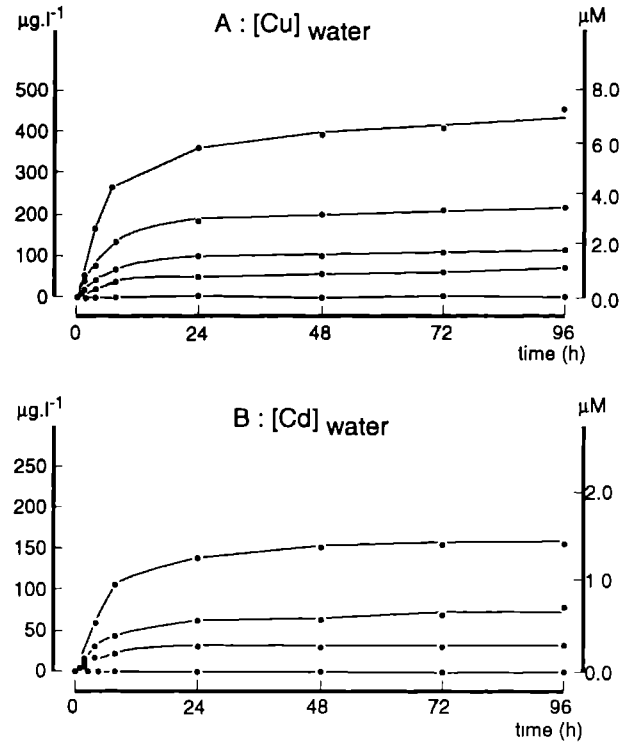

Figure 1. *Cu (A) and Cd (B) concentrations in the water (in µg.l^{-1} and µM) during an experiment with fed fish.*

(R=0.974). Cd-accumulation in fed fish did not differ from accumulation in non-fed fish at the concentrations tested (Fig. 3). The A.F. decreased slightly in the non-fed fish with increasing Cd-concentrations in the water (insert Fig. 3).

Interactions between Cu and Cd

-fed

Two types of interactions between Cu and Cd were studied. Firstly, the effect of Cu-exposure on the Cd-content of fish and of Cd-exposure on the Cu-content of fish (type I). Secondly, the effects of combined exposure on whole body Cu and Cd content (type II). Both types of interactions were studied in fed and food restricted fish.

Type I interactions in fed fish are shown in Fig. 4(A,B). Exposure during 96h to 20 or 35 µg

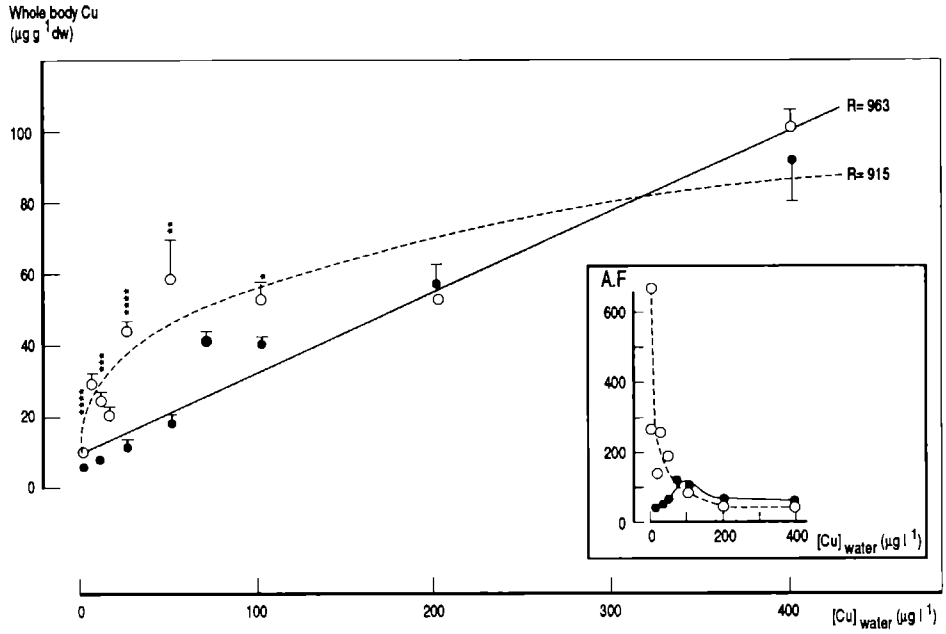

Figure 2. Whole body Cu content of fed and non-fed juvenile tilapia after 96h exposure to a range of Cu-concentrations In fed fish (•), Cu-accumulation can be described by y=0 23x+9 44, in non-fed fish (○), Cu-accumulation can be described by $y=13\ 65x^{0\ 71}$, with y=whole body Cu content (in µg g⁻¹ dw) and x=Cu concentration in the water (in µg l⁻¹) Asterisks indicate statistical differences in whole body Cu concentrations between fed and non-fed fish (n≥10) Insert Cu accumulation factor (A F) after 96h exposure to a range of Cu-concentrations

Cd l⁻¹, resulted in a significant decrease of the Cu content (Fig. 4A) Fig 4B shows that exposure to Cu resulted in a significant increase in the whole body Cd-content at all concentrations used

Type II interactions in fed fish are illustrated by Fig 5(A,B) Cu/Cd co-exposure had no consistent effect on whole body Cu-content (Fig. 5A) Compared to the fish exposed to Cu alone, two Cu/Cd combinations (70 µg Cu.l⁻¹ + 20 µg Cd l⁻¹ and 400 µg Cu l⁻¹ + 70 µg Cd.l⁻¹) decreased the Cu-content in fish while the combination 400 µg Cu g⁻¹ + 35 µg Cd g⁻¹ increased the Cu content Exposure to Cu/Cd combinations resulted in a significant decreased whole body

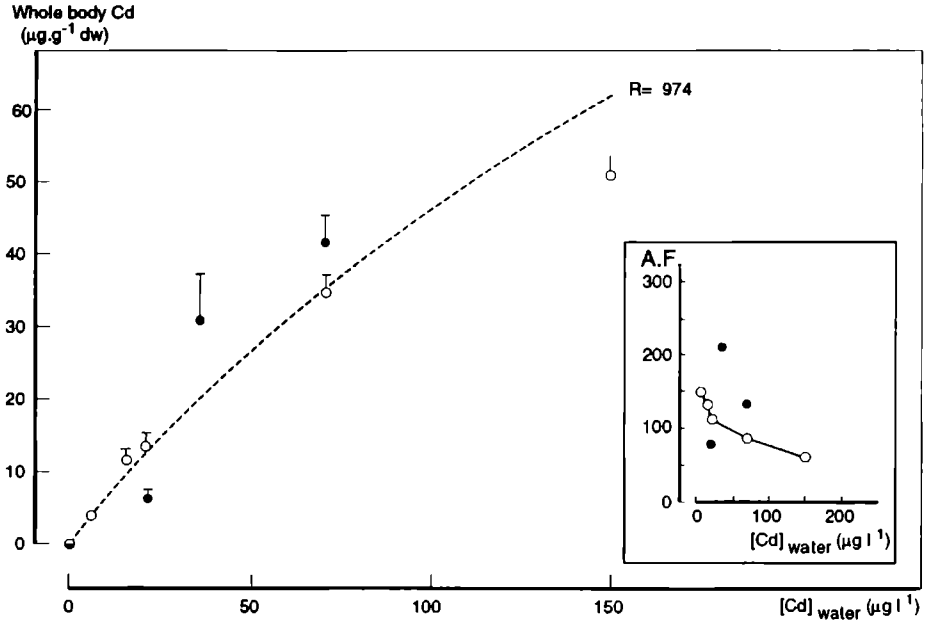

Figure 3. *Whole body Cd content of fed and non-fed juvenile tilapia after 96h exposure to a range af Cd-concentrations Cd-accumulation in non-fed fish (○) can be described by* $y = 1.84x^{0.70}$, *with y = whole body Cd-content (in µg g⁻¹ dw) and x = Cd-concentration in the water (in µg l⁻¹) Cd-accumulation in fed fish (●) is not significantly different (n ≥ 10) Insert Cd accumulation factor (A F) after 96h exposure to a range of Cd-concentrations*

Cd-content when compared to single Cd exposure, with the exception of exposure to the lowest Cd concentration tested (fig. 5B)

-non-fed

Type I interactions in non-fed fish are shown in Fig 6 Except for exposure to 70 µg Cd l⁻¹ (fig. 6A), Cd-exposure had no effect on the Cu-content Fig 6B shows that, in contrast to the results obtained with fed fish, exposure to Cu concentrations above 100 µg l⁻¹ resulted in a significant decrease of the Cd-content, whereas lower concentrations (with exception of 5 µg

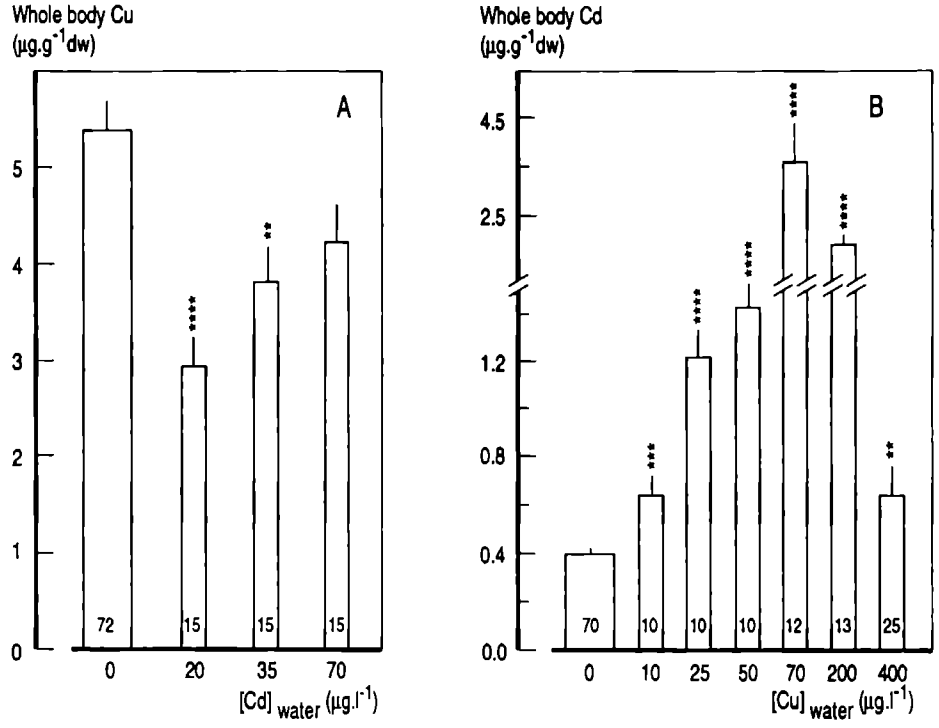

Figure 4. *Whole body Cu content of Cd-exposed fed fish (A) and whole body Cd content in Cu-exposed fed fish (B) after 96h of exposure. Asterisks indicate significant differences between control and metal exposed groups. The number of fish per group is indicated in the bars.*

$Cu.l^{-1}$) had no effect.

When compared to single Cu exposure, co-exposure to Cu and Cd had no consistent effect on whole body Cu in non-fed fish (Fig. 7A). In contrast, Fig. 7B shows that Cd-exposure in the presence of Cu had a very pronounced effect on the whole body Cd-content. Exposure to nearly all combinations of Cu and Cd used resulted in a significant decrease of the Cd-content in fish when compared to fish exposed to Cd alone.

Effects of Cu and Cd on water- and ion content

In fed fish, exposure to Cu concentrations higher than 70 $\mu g.l^{-1}$ resulted in a significant, dose-dependent, decrease of their water content (Fig. 8A). Exposure to Cd and combinations of Cu and Cd had a similar effect (Fig. 8B,C). In food-restricted fish, exposure to low Cu concentrations

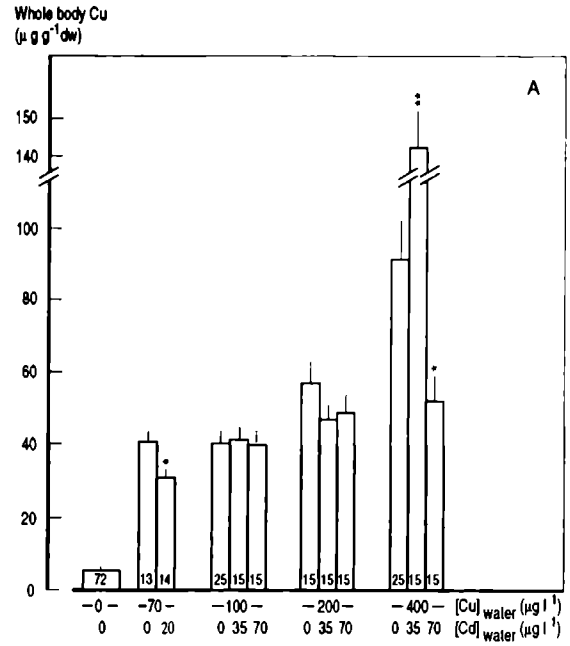

Figure 5A. *Whole body Cu content of fed fish after exposure to combinations of Cu and Cd during 96h Asterisks indicate significant differences between Cu contents of fish exposed to Cu alone and fish co-exposed to Cu+Cd. The number of fish per group is indicated in the bars*

resulted in an increased water content whereas the opposite was found after exposure to higher Cu-concentrations (Fig. 9). In contrast to the decreased water content observed in fed fish after Cd-exposure, in non-fed fish exposure to Cd concentrations between 10 and 70 $\mu g.l^{-1}$ resulted in an increase of the water content of the fish. Fig. 9 illustrates the effect of interaction of the metals on whole body water content. For example, exposure to 100 μg $Cu.l^{-1}$+70 μg $Cd.l^{-1}$ resulted in significantly decreased water content However, exposure to 100 μg $Cu.l^{-1}$ had no effect on water content of fish while exposure to 70 μg $Cd.l^{-1}$ resulted in a significant increased water content.

Exposure to Cu and Cd, alone or in combination, did not result in consistent effects on sodium and calcium content of non-fed fish (data not shown). In the groups with altered average body sodium or calcium content (either expressed as dry or wet weight), these parameters were not necessarily correlated with whole body water content. To illustrate this, in the fish exposed to

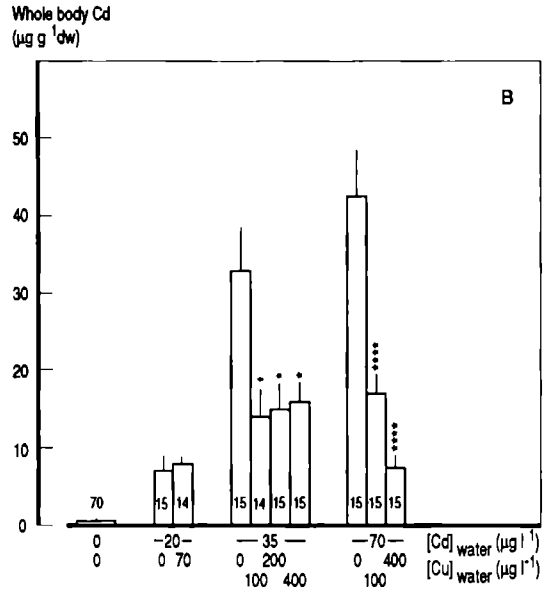

Figure 5B. Whole body Cd content of fed fish after Cu+Cd co-exposure during 96h Asterisks indicate significant differences between Cd content of fish exposed to Cd alone and fish co-exposed to Cu+Cd The number of fish per group is indicated in the bars

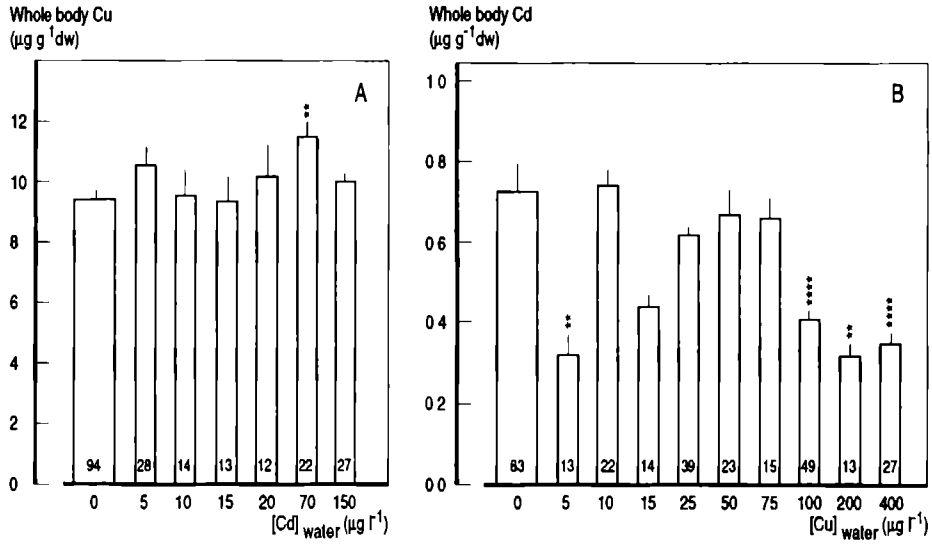

Figure 6. Whole body Cu content of Cd-exposed non-fed fish (A) and whole body Cd content in Cu-exposed non fed fish (B) after 96h of exposure Asterisks indicate significant differences between control and metal exposed groups The number of fish per group is indicated in the bars

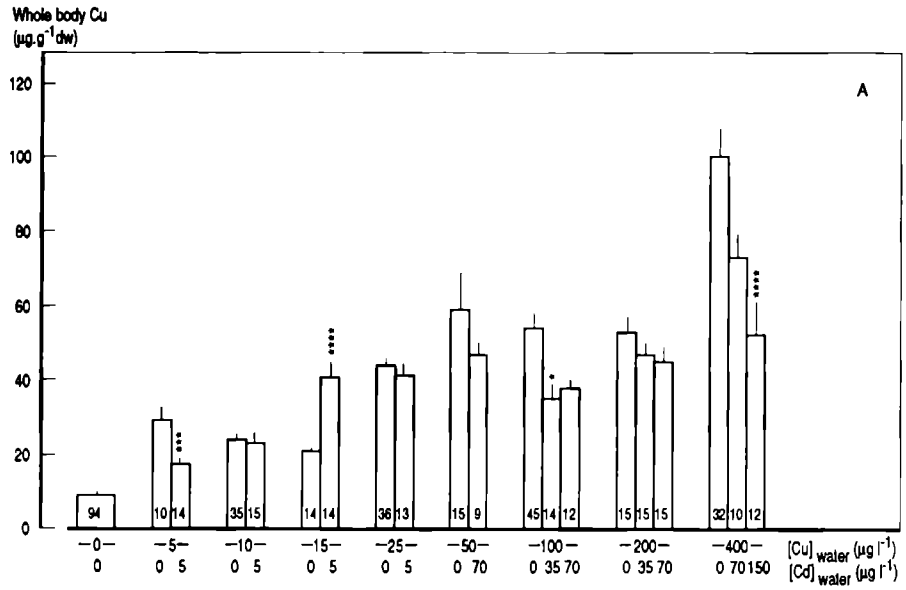

Figure 7A. *Whole body Cu content of non-fed fish after exposure to combinations of Cu and Cd during 96h Asterisks indicate significant differences between Cu content of fish exposed to Cu alone and fish co-exposed to Cu+Cd The number of fish per group is indicated in the bars*

400 µg $Cu.l^{-1}$ + 70 µg $Cd.l^{-1}$ (Fig. 10C), water content and whole body calcium were significantly decreased. In other groups of fish with a changed water content, no significant changes in whole body sodium or calcium were observed (Fig. 10A,B).

DISCUSSION

Two major conclusions can be drawn from the present study. Firstly, interactions between Cu and Cd present in the fish are noticeable both during single and combined exposure to the metals. The interactions result in changed metal and water content of the fish. Secondly, metal-induced effects are also determined by the feeding condition of the fish.

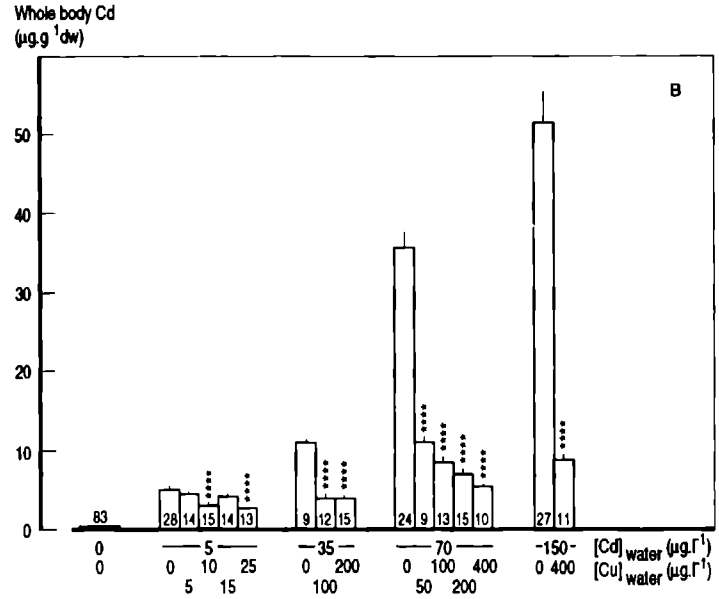

Figure 7B Whole body Cd content of non-fed fish after exposure to combinations of Cu and Cd during 96h Asterisks indicate significant differences between Cd content of fish exposed to Cd alone and fish co-exposed to Cu+Cd The number of fish per group is indicated in the bars

Cu accumulation in Cu-exposed fish

In accordance with our results with fed fish, Buckley *et al* (1984) found a positive relationship between the Cu concentration in the water and that in the gills and liver of *Oncorhynchus kisutch* More complex results are described by Stokes (1979) and Segner (1987) Stokes reported less Cu in tissues of fish exposed to high Cu levels than in fish exposed to intermediate levels of Cu

The accumulation rate, at least in the lower concentration range, was higher in non-fed fish Two explanations for this phenomenon may be suggested Firstly, the physiological consequences of food restriction may determine the ability to handle exogenous toxicants This is corroborated by data from a study with fed and non-fed roach exposed to 80 µg Cu l^{-1} during 7 days Only liver tissue of non-fed fish showed significant accumulation of Cu (Segner, 1987) The author suggested that food-deprived fish lack the ability to regulate transfer of Cu within the body Diet-related differences in Cu-tolerance were also reported for rainbow trout and perch, resulting in an increased tolerance to waterborne Cu due to available dietary carbohydrate (Dixon and Hilton,

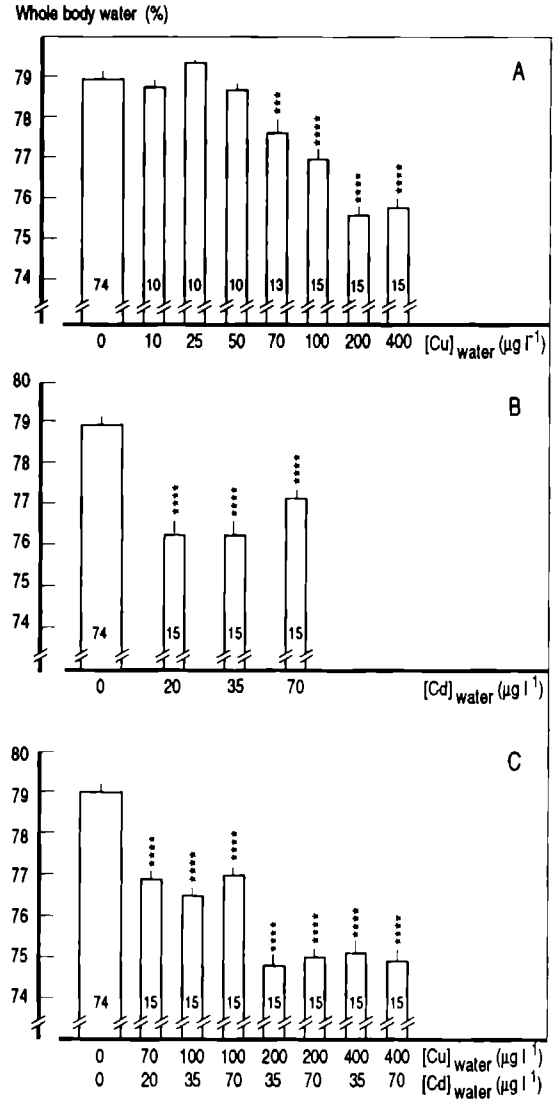

Figure 8. Water content of fed fish after 96h of exposure to a range of Cu concentrations (A), Cd concentrations (B) and combinations of Cu+Cd concentrations (C). Asterisks indicate significant differences in % water between control and metal exposed fish. The number of fish per group is indicated in the bars.

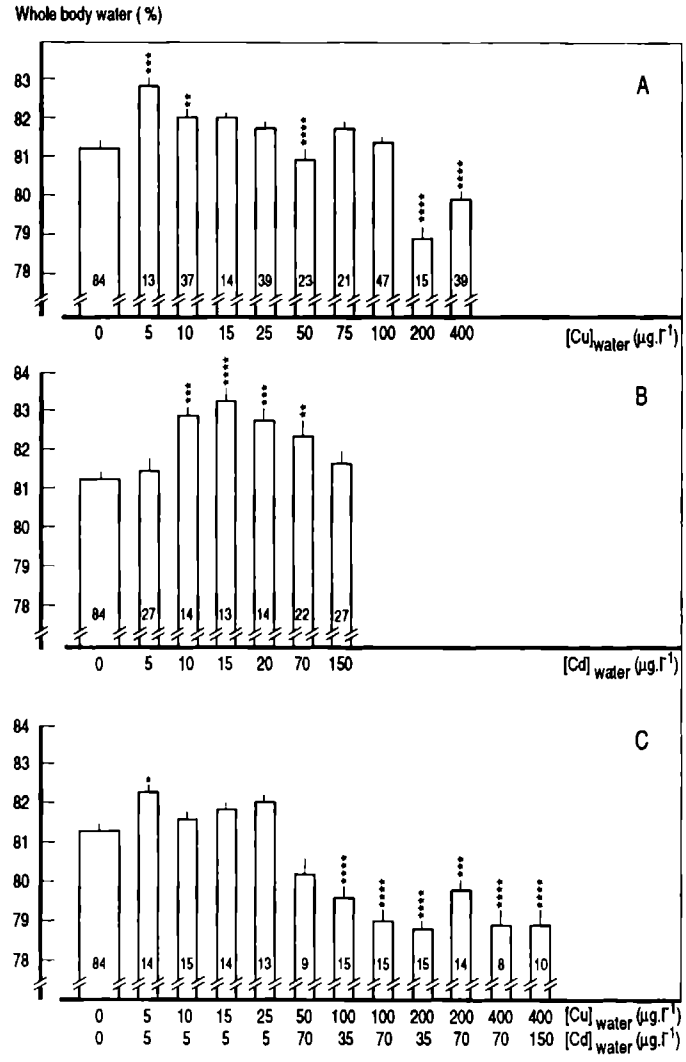

Figure 9. Water content of non-fed fish after 96h of exposure to a range of Cu concentrations (A), Cd concentrations (B) and combinations of Cu+Cd concentrations (C). Asterisks indicate significant differences in % water between control and metal exposed fish. The number of fish per group is indicated in the bars.

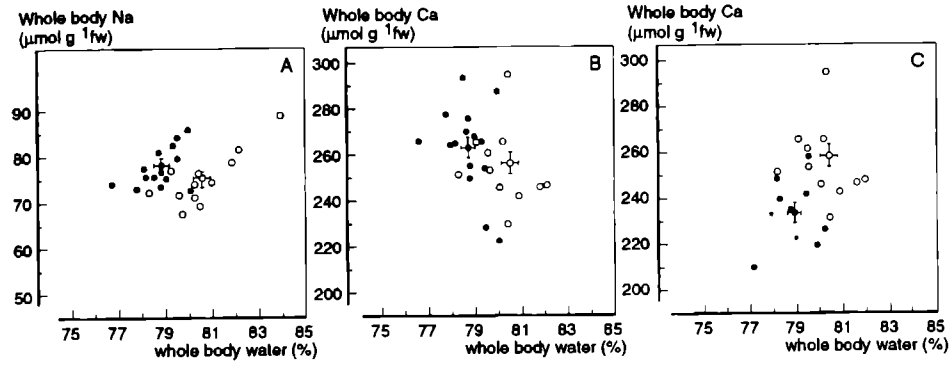

Figure 10. *A Relationship between whole body sodium and water content in non fed control fish (○) and Cu-exposed fish (400 µg l⁻¹) (●) after 96h In the Cu-exposed group, water content was significantly decreased, whereas whole body sodium content was unchanged*

B Relationship between whole body calcium and water content in non fed control fish (○) and Cu-exposed fish (400 µg l⁻¹) (●) after 96h In the Cu-exposed fish, water content was significantly decreased, whereas whole body calcium content was unchanged

C Relationship between whole body (fw=fresh weight) calcium and water content in non-fed control fish (○) and fish exposed to 400 µg l⁻¹ Cu + 70 µg l⁻¹ Cd (●) after 96h In the metal-exposed fish water content and calcium content were significantly decreased

1981, Collvin, 1985) Handy and Eddy (1990) demonstrated that gill tissue and body mucus of non-fed fish accumulated zinc quicker than tissues of fed fish They suggested that the difference could be related to the observed decreased ion-content of mucus of non fed rainbow trout A second explanation for the difference we observed between fed and non-fed fish might be the formation of Cu complexes in the water of fed fish (Boyle, 1979, Leckie and Davis, 1979) as a result of Cu-complexation to faeces The amount of dissolved Cu might therefore be lower than that in aquaria of non-fed fish Consequently, the amount of available Cu could be significantly reduced, especially at low environmental Cu concentrations However, this second mechanism seems less likely under the present conditions because we could measure no differences in metal concentration after filtration over a 0 45 µm filter

Cd accumulation in Cd-exposed fish

Our results show that Cd uptake was non-linear, and the accumulation factor decreased with increasing water Cd concentrations. Similar results were reported after exposing rainbow trout alevins (Beattie and Pascoe, 1978) and eggs of Atlantic salmon (Rombough and Garside 1982) to Cd. A mechanism explaining non-linear Cd accumulation in largemouth bass and bluegill was hypothesized by Cearly and Coleman (1974). They suggested modulation of active transport mechanisms for Cd, which was dependent on the ambient Cd concentration. However, we are unaware of active Cd transport mechanisms. It subsequently has been demonstrated that Cd is transported via calcium transport mechanisms (Verbost *et al.*, 1987). An alternative explanation, based on the metal binding capacity of mucus might be considered. The amount of mucus on the body- and gill-surface is negligible under control conditions, but increases during metal exposure (Lock *et al.*, 1981; Handy and Eddy, 1990, 1991). It has not been demonstrated that this increase is concentration-dependent. Therefore, it may be that the contribution of metal-accumulation in mucus is most pronounced at low water Cd-concentrations. At higher Cd concentrations mucus will be more saturated with Cd, and is less readily to bind more Cd at higher environmental Cd concentrations.

Effects of single Cd-exposure on whole body Cu content and of single Cu-exposure on whole body Cd content (type I)

In this study we showed that exposure to Cd affected the total body Cu concentration and vice versa. As a reflection of the fact that Cu is an essential element, the Cu concentration in fish is normally maintained within narrow limits by coordinated uptake, transport and excretion mechanisms (Grahl *et al.*, 1985). Unlike Cu, Cd is a non-essential metal. Once absorbed from the water, Cd is eliminated slowly, as has been shown for Cd-exposed rainbow trout and lake whitefish (Harrison and Klaverkamp, 1989) upon transfer to Cd-free water. Our data indicate that exposure to low levels of Cd does not only result in Cd accumulation but also in elimination of Cu from fed (but not from non-fed) fish. This is the first demonstration that Cd exposure has effects on the whole body Cu content of fish and this may contribute to the toxicity of Cd.

Cu-exposure of fed fish resulted in a significant increase in whole body Cd content at all Cu concentrations tested. This is difficult to explain. Dallinger and Kautzky (1985) demonstrated for rainbow trout that, under control conditions, most of the Cd derived from food is excreted. Cu exposure could therefore affect the fecal excretion of Cd. However, the increase in whole body Cd after Cu exposure cannot be explained completely by the amount of Cd originating from the food ingested during the experiment. It can further be excluded that this Cd-increase is an artifact introduced by the method of Cd detection used. Addition of nitric acid to vials (control

destruction, see M & M section) did not result in any detectable Cu and Cd Interaction of Cu and Cd during measurement of Cd is unlikely, because absorption spectra of the metals do not overlap. Besides, Cd-increase after Cu exposure is only found in fed fish Thus, the effect is related to the feeding conditions of the fish during the experiment The explanation of this phenomenon needs further study.

In contrast to the results obtained with fed fish, Cu-exposure of non-fed fish resulted in a significant decrease of the whole body Cd content, especially at higher concentrations (>75 μg Cu.l^{1}). This decrease is most likely the result of clearance of previously accumulated Cd and not of the removal of absorbed Cd from the skin- and gill-surface because EDTA-rinsed fish contained as much Cu and Cd as water-rinsed fish (not shown). This observation has not been reported earlier for fish, but is in line with findings of Holwerda (1991) on clams, who reported an accelerated elimination of previously accumulated Cd during Cu-exposure Release of Cd as a result of Cu exposure *in vitro* was also reported for a human carcinoma cell line by Meshitsuka *et al* (1983) A possible explanation may lie in the involvement of metallothioneins (MT's) Increased levels of MT's are associated with increased tolerance to toxic metals (Klaverkamp *et al* , 1984). The amount of MT's also increases during sublethal exposure and MT's are important for the recovery of physiological homeostasis (Giles, 1984) Both Cu and Cd are bound to MT's (Day *et al.*, 1984, Cousins, 1985) However, Cu can displace Cd from MT's (Scheuhammer and Cherian, 1986). Therefore, during Cu exposure, the Cd-binding capacity of MT's decreases (Laurén and McDonald 1987) and this could explain the observed Cd elimination The physiological effect of the Cd-release by Cu is not clear It is possible that this forms an additional burden during exposure to Cu, because the released Cd will be more harmful to the organism than MT-bound Cd.

Interactions between Cu and Cd during combined exposure

-Cu accumulation

The effects observed on Cu content after Cu/Cd co-exposure are not simply comparable to results obtained after single Cd exposure This suggests two types of interaction (I and II) Firstly, interaction between Cu already present in the body and the Cd taken up as a result of Cd exposure. Secondly, Cu/Cd interaction during the uptake of Cu as a result of Cu/Cd co-exposure In addition, our results can also be taken as evidence for the fact that the kind of interaction may, at least in part, depend on the ratio of the metals. This is also demonstrated by the results of Finlayson and Verrue (1982) and Gill *et al* (1992) Exposure of the American eel (Anguilla rostrata) to 75 μg l^{1} Cd resulted in an increased Cu concentration in the kidney, while exposure to 150 μg l^{1} Cd decreased the Cu content of the kidney (Gill *et al* , 1992).

-Cd accumulation

In both fed and non-fed tilapia, Cu/Cd co-exposure resulted in a significant reduction of the whole body Cd content compared to the Cd content of fish exposed to Cd alone. For non-fed fish, this is in line with our results obtained with exposure to Cu alone. For the fed fish, this seems to contradict the increased whole body Cd content obtained after single Cu exposure. However, this increase was limited when compared to the amounts of whole body Cd after Cu/Cd co-exposure. Our data corroborate the results of Elliot *et al.* (1986), who reported reduced Cd-accumulation in clams in the presence of 10 or 20 $\mu g.l^{-1}$ Cu, when Cd was present at 20, but not at 10 $\mu g.l^{-1}$. Our results also show no effect of Cu co-exposure at the lowest Cd concentration used The notable decrease in whole body Cd during Cu/Cd co-exposure compared to accumulation during Cd exposure alone, will likely be a combination of increased elimination and decreased uptake of Cd. It is difficult to imagine that the reduced Cd content of fish exposed to Cu+Cd is the result of elimination alone, because in that case the amount of Cd excreted would be inconceivably high.

Water and ion content

Both single and combined exposure to Cu and Cd had an effect on the whole body water content, demonstrating osmoregulation as one of the targets of the metals In fed fish, these conditions reduced the water content of the fish However, the effects in non-fed tilapia on water content seem to be complex. For example, exposure to 100 μg Cu l^{-1} had no effect while 70 μg Cd.l^{-1} resulted in increased water content Moreover, exposure to a combination of both metals at these concentrations resulted in a significant decrease of the water content This apparent complexicity can also be found in the literature, although information on effects on water content after e.g. Cd exposure is scarce. Increase (in goldfish, McCarthy and Houston, 1976), no effect (in embryos of *Salmo salar*) and inhibition of net water uptake (alevins of *Salmo salar*, Rombough and Garside, 1984) have been reported

Overall, our results indicate that changes in water content, observed after metal exposure, are not directly related to metal-induced changes in whole body sodium- and/or calcium-concentration On the basis of comparable results, observed in *Salmo salar*, Rombough and Garside (1984) also concluded that there was no involvement of ion-regulatory processes in the observed changes in whole body water content In tilapia co-exposed to 400 μg Cu l^{-1} and 70 μg Cd l^{-1}, decreased whole body water content was accompanied by a decreased whole body calcium content compared to controls These results could imply a relation between ionic- and osmoregulation during Cu/Cd co-exposure, as was suggested by McCarthy and Houston (1976) after Cd exposure of goldfish However, in other groups of fish, metal exposure only resulted in

changes in water content, whereas whole body sodium and/or calcium were not affected or vice versa.

In vitro Cu/Cd co-exposure of cell cultures resulted in an increased toxicity of these metals, as concluded from the protein content of trout hepatocytes (Denizeau and Marion, 1990) and KB cells (Meshitsuka *et al.*, 1983, 1987). Meshitsuka and coworkers suggested that the toxicity of Cd did not depend solely on the amount of Cd absorbed by the cells but also on cofactors such as Cu. An explanation for increased toxicity due to exposure to metal mixtures is given by Irons & Smith (1976), who suggested MT-aggregation as a result of combined exposure, which prevented Cd-sequestration.

From this study can be concluded that the nutritional state of fish influences the amount of metal accumulated during exposure to heavy metals. Furthermore, accumulation and toxicity during Cu/Cd co-exposure cannot be predicted by simple addition of the effects of single metal exposure. Obviously, the complex interaction mechanisms underlying the observed effects, concern previously unrecognized toxic effects of the metals. Also at the cellular level, Meshitsuka and coworkers (1983, 1987) suggested that the toxicity of Cd did not depend solely on the amount of Cd absorbed by the cells but also on cofactors such as Cu.

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CHAPTER 2

INTERACTIONS BETWEEN COPPER AND CADMIUM MODIFY METAL ORGAN DISTRIBUTION IN MATURE TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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ABSTRACT

Sexually mature female tilapia were exposed to sublethal concentrations of waterborne Cu and/or Cd during 6 days, and subsequent concentrations of these metals were determined in several organs. The results show that the distribution of Cu and Cd was metal and organ specific. This is for example demonstrated by the observation that in tilapia, Cu exposure did not result in Cu accumulation in the liver, whereas in the intestinal wall notably high concentrations of Cu and Cd were measured in metal-exposed fish.

In addition to single-metal-exposed fish, we also determined Cu and Cd body distribution in Cu/Cd co-exposed fish. The observed interactions in metal accumulation were most pronounced in the organs of fish exposed to low, environmentally realistic, metal concentrations.

INTRODUCTION

Heavy metals such as copper (Cu) and cadmium (Cd) are frequently present at elevated concentrations in freshwaters, generally as a result of industrial pollution. As a consequence, aquatic organisms, including fish, are exposed to elevated levels of these metals. Cu, as an essential metal, plays an important role in cellular metabolism (Cousins, 1985) and its concentration is well regulated. However, exposure of fish to increased Cu concentrations results in Cu accumulation (Brungs *et al.*, 1973; Buckley *et al.*, 1982). As a result, various blood parameters (McKim *et al.*, 1970; Christensen *et al.*, 1972), enzyme activities in blood (Christensen and Tucker, 1976) and reproduction (Horning and Nieheisel, 1979) are affected. In contrast to Cu, a biological function for Cd is unknown, and the metal is toxic to organisms at

very low concentrations (Chmielnicka and Cherian, 1986). For example, exposure to Cd resulted in reduced growth, reproduction and survival in flagfish *Jordanella floridae* (Spehar, 1976). Also, Cd produces a variety of pathological effects in various organs in fish after acute exposure (Hawkins *et al.*, 1980; Karlsson-Norrgren *et al.*, 1985).

No clear picture exists concerning the accumulation of Cu and Cd in fish tissues, partly because of differences in species, analytical techniques and experimental designs of the published studies (McCracken, 1987; Douben, 1989). More importantly however, each of the metals Cu and Cd has been studied separately, not taking into account a possible concomitant influence of other metals. Few investigations have been made concerning the effect of one heavy metal on the accumulation of another metal in fish (Gill *et al.*, 1992; Pelgrom *et al.*, 1994[a,b]). Nevertheless, many of the toxic effects of Cd have been suggested to be the result of induced secondary deficiencies of essential trace elements, such as Zn and Cu, since the uptake of Cd both modulates, and is modulated by, the uptake of these metals (Bremner, 1974). Since heavy metals often occur together in polluted areas, it is of importance to study metal-metal interactions in fish at environmentally relevant concentrations.

In a previous paper (Pelgrom *et al.*, 1994[a]), interactions between Cu and Cd on whole body metal accumulation in juvenile tilapia during waterborne metal exposure were demonstrated. The present study examines Cu-Cd interactions in mature tilapia, paying particular attention to organs with diverse biological functions, because it is anticipated that differences in metal accumulation between organs will be related to their functions. Organ metal concentration or metal-metal interaction at this level may be a link to toxicity (Foulkes, 1990; Landrum *et al.*, 1992). In keeping with this, it has recently been suggested that metal concentrations in the organs of fish, rather than the metal concentrations in the ambient water, could be used as a biomonitor for water pollution in natural freshwaters (Handy, 1992).

We have investigated the effects of two concentrations of Cu and Cd, single as well as in combination, during 6 days on the Cu and Cd concentration of organs associated with osmoregulation (gills), metal detoxification (liver, kidney), digestion (intestine), neuro-endocrine regulation (brain, head kidney), locomotion (muscle) and reproduction (gonads). At one intermediate concentration of Cu and Cd, gills and liver were compared after 6 and 11 days of exposure, to compare effects of a more prolonged exposure on metal accumulation and interaction.

MATERIALS AND METHODS

Fish and control water conditions

Tilapia (*Oreochromis mossambicus*) were obtained from our own laboratory stock. Fish were kept, from 9 days after hatching, in artificial freshwater with undetectable Cu and Cd concentrations (detection levels below 0.1 and 0.01 $\mu g.l^{-1}$ respectively). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8. The composition and preparation of the water was based on the EEC instructions for artificial water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1· Acute toxicity for fish) Water was continuously aerated, filtered and flow-through, resulting in water of constant quality and with a stable pH (pH=7.6). The light/dark regime was 12/12 h and the water temperature 26°C Fish were fed commercial tropical fishfood Tetramin™, 2% $^{dw}/_{ww}$ per day. The food was eaten within one minute. The Cu and Cd contents of the food were: 9.86 ± 0.16 μg Cu.g^{-1} dry food and 0.22 ± 0.01 μg Cd.g^{-1} dry food (means ± SE; n=10)

Experimental design

Six weeks before the start of the experiments, sexually mature female fish (mean weight 20 g) were divided into 4 groups of 14 fish each, and kept in 80 l aquaria with continuously filtered and refreshed artificial freshwater by means of flow-through. The experiment started with the connection (by means of a 16 channel peristaltic pump; Watson Marlow) of each of the aquaria to its own reservoir filled with artificial freshwater with or without (controls) a well-defined metal concentration (added as nitrate; Spectrosol, BDH, England). During the first 6 h the flow rate was 4 5 l h^{-1}, followed by a flow rate of 1.5 l h^{-1} In this way, the metal concentrations in the aquaria were gradually raised, reaching a plateau after 18 h The measured concentrations deviated maximally 5% from the nominal concentrations (Pelgrom *et al.*, 1994). Two experiments were performed, with LOW and HIGH Cu and Cd concentrations. The experiment with the HIGH metal concentrations was performed first, followed by experiments with LOW concentrations. The experimental period lasted 6 days. In an additional experiment with intermediate metal concentrations (50 μgCu l^{-1} and 20 μgCd l^{-1}), fish were exposed during 6 and 11 days

6 DAYS	[Cu]	[Cd]	[Cu]+[Cd] ($\mu g.l^{-1}$)
LOW	20	5	20+ 5
HIGH	100	35	100+35
6 and 11 DAYS			
	50	20	50+20

TABLE 1. Cu and Cd concentrations in the aquaria of the metal-exposed fish.

Feeding was ended the day prior to sacrifice. Cu and Cd concentrations in both stock solutions and aquaria were monitored every hour during the first 6 h, and at least once a day during the rest of the exposure period. Water samples were acidified with nitric acid in a final concentration of 0.2% (v/v). Water Cu and Cd concentrations were determined with a flameless Atomic Absorption Spectrometer (AAS, Philips PU9200) connected with an electrothermal atomiser (Philips PU9390X). After exposure, the fish were killed by spinal dissection. Gills, head kidney, brain, liver, intestine (after removal of contents), kidneys, gonads (LOW experiment only) and white muscle were dissected carefully. The tissues were weighed, lyophilized and, after determination of the dry weights, digested with nitric acid (65% HNO_3, ultrapur, Merck). Finally, the samples were dissolved in 0.2% HNO_3, and stored at 4°C until metal analysis by means of AAS. Cu and Cd determinations in the tissues were performed under standard matrix conditions, with the exception of Cd determination in the gonads and muscle. The Cd concentrations in the latter tissues were determined in the presence of a matrix modifier (AAS matrix modifier, Merck). Interference between Cu and Cd during measurements can be excluded, as has been demonstrated previously (Pelgrom *et al.*, 1994). From the (wet) weights of the gonads and the total body weight, the Gonad Somatic Index (GSI) was determined, with the gonad weight expressed as % of the total body weight.

Statistics

Data are presented as means ± SE. For statistical evaluation the Student's *t*-test was applied, and significant differences between control and metal exposed groups are indicated by asterisks, whereas significant differences between single metal and Cu/Cd co-exposed groups are indicated by circles: * or ○: $P < 0.05$; ** or ○○: $P < 0.02$; *** or ○○○: $P < 0.01$, and **** or ○○○○ :$P < 0.001$.

RESULTS

During all experiments, no mortality occurred, and no differences in the feeding behaviour and body weights between the experimental and control groups were observed. The water in the aquaria was continuously refreshed by means of flow-through, resulting in water of constant quality, as demonstrated by the constant pH, and no changes in ammonia concentrations (data not shown) The results of the 6 day metal exposures on organ metal concentrations are presented in Figures 1 to 4A, with LOW· 5 μg Cd.l^{1} and/or 20 μg Cu.l^{1}, and HIGH. 35 μg Cd.l^{1} and/or 100 μg Cu.l^{1} Throughout the organs and concentrations, only incidental differences in the % water content were observed (data not shown). Therefore, Cu and Cd concentrations in the organs are expressed as dry weights

Tissue Cu concentration

I LOW

-Effects of Cu

Cu-exposure resulted in increased Cu concentrations in the intestine, muscle and gonads.

-Effects of Cd

Compared to control fish, an increased Cu concentration in the liver and intestine was observed in fish exposed to Cd singly

Compared to fish exposed to Cu singly, significantly more Cu accumulation was observed in the liver, kidneys and intestine of Cu/Cd co-exposed fish In contrast, the Cu concentrations in the head kidney and muscle of Cu/Cd co-exposed fish were lower than those observed in Cu-exposed fish As a result, the Cu concentration in the head kidney of fish co-exposed to Cu and Cd was not statistically different from the controls

II HIGH

Effects of Cu

Exposure to 100 μg Cu l^{1} resulted in accumulation of Cu in the gills, kidneys, intestine and muscle

-Effects of Cd

The Cu concentration in the kidneys and intestine were significantly lower in Cd-exposed fish than in control fish.

Combined Cu/Cd exposure resulted in a lower Cu concentration in the liver when compared to the liver Cu concentration of fish exposed to Cu singly

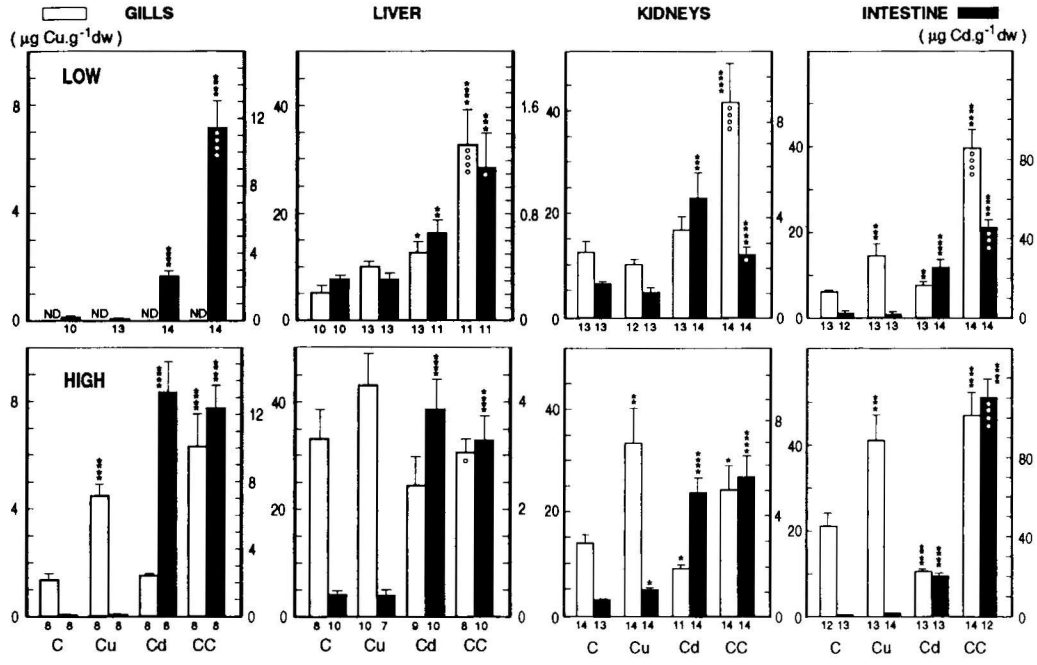

Figure 1. *Concentrations of Cu (open bars) and Cd (closed bars) in the gills, liver, kidneys and intestine of fish exposed during 6 days to LOW (5 µg Cd.l^{-1} and/or 20 µg Cu.l^{-1}; upper panels) or HIGH (35 µg Cd.l^{-1} and/or 100 µg Cu.l^{-1}; lower panels) metal concentrations. The Cu concentration is expressed on the left axis and the Cd concentration on the right axis. The Cu and Cd concentrations in the organs are given of the controls (C), Cu exposed (Cu), Cd exposed (Cd) and Cu+Cd co-exposed (CC) fish successively. Asterisks indicate significant differences between control and experimental fish, circles indicate significant differences between single metal exposed fish and Cu+Cd co-exposed fish. The number of fish per group is indicated under the bar. In the LOW group, Cu was not determined (ND) in the gills.*

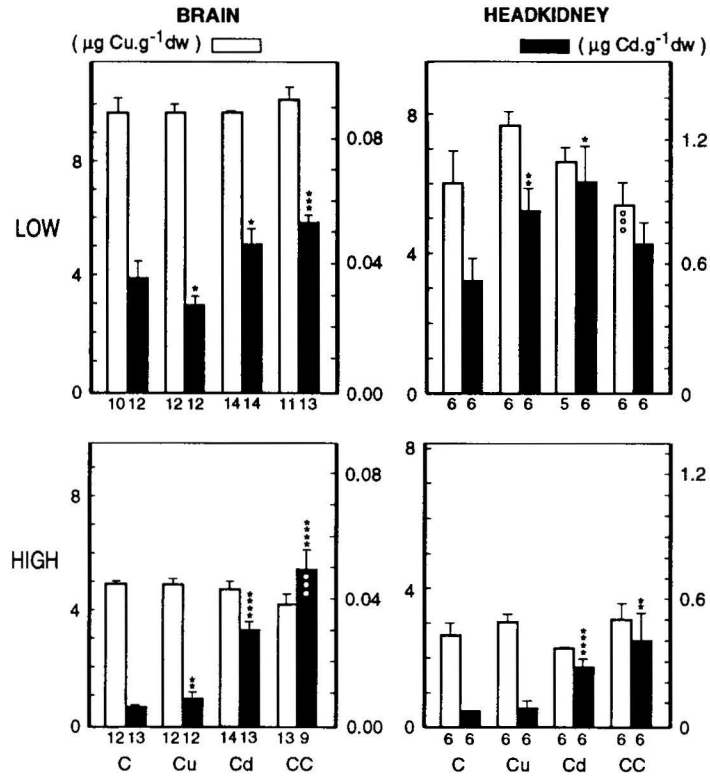

Figure 2. Concentrations of Cu (open bars) and Cd (closed bars) in brain and head kidney of fish exposed during 6 days to LOW or HIGH Cu and Cd concentrations. Symbols are used in the the same way as described in Figure 1.

Tissue Cd concentration

I. LOW

-Effects of Cd

Exposure to 5 µg $Cd.l^{-1}$ resulted in increased Cd concentrations in all tissues examined.

-Effects of Cu

In Cu-exposed fish, the Cd concentration in the brain and gonads were significantly lower, whereas in the head kidney we observed an increase in the Cd concentration compared to control fish.

Combined Cu/Cd exposure resulted in an increased Cd accumulation in the gills, liver,

intestine and gonads when compared to fish exposed to Cd singly. The Cd concentration in the kidneys of Cu/Cd co-exposed fish was lower than in Cd-exposed fish.

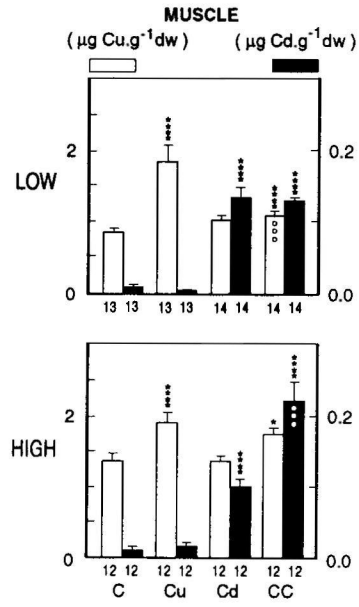

Figure 3. *Concentrations of Cu (open bars) and Cd (closed bars) in muscle of fish exposed during 6 days to LOW or HIGH Cu and Cd concentrations. Symbols are used in the same way as described in Figure 1.*

II. HIGH

-Effects of Cd

The Cd concentration was increased in all tissues examined of Cd-exposed fish.

-Effects of Cu

In singly Cu-exposed fish, the Cd concentration was higher in the kidneys and brain compared to controls.

Combined Cu/Cd exposure resulted in an increased accumulation of Cd in the intestine, brain and muscle when compared to fish exposed to Cd singly.

Table 2 shows the Cu and Cd concentrations in the gills and liver of fish exposed to 20 μg $Cd.l^{-1}$ and/or 50 μg $Cu.l^{-1}$ after 6 or 11 days of exposure. No differences were observed between the Cu concentrations of both gills and liver of fish exposed to Cu during 6 or 11 days. In the liver, 11

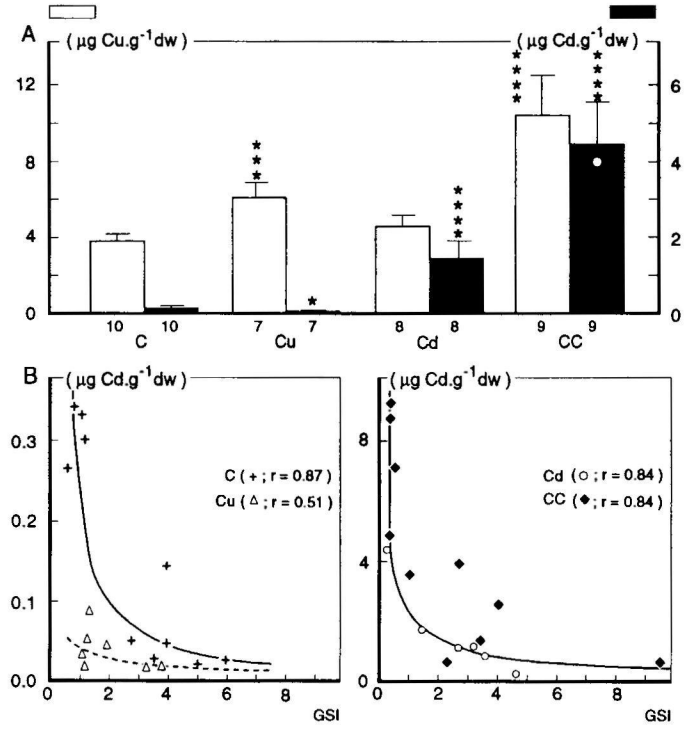

Figure 4. *Panel A shows the Cu (open bars) and Cd (closed bars) concentrations in gonads of fish exposed during 6 days to 5 µg Cd.l^{-1} (Cd), 20 µg Cu.l^{-1} (Cu) or co-exposed to 5 µg Cd.l^{-1}+20 µgCu.l^{-1} (CC). Symbols are used in the same way as described in Figure 1.*

Panel B shows the relation between the Cd concentration in the gonads and the GSI of the exposed fish described in panel A. In control and Cu exposed fish, the relation between the GSI and the Cd concentration is best described by $y=0.23x^{-1.17}$ (****) and $y=0.04x^{-0.51}$ (ns) respectively. In the Cd and Cu/Cd co-exposed fish, the GSI and the Cd concentration in the gonads are related in a comparable way, and can be best described by the function $y=2.21x^{-0.73}$ (****), with y=the Cd concentration in the gonads and x=the GSI.

days of Cd exposure resulted in a significantly decreased Cu concentration. Compared to the Cu concentration in the liver of fish exposed to Cd during 6 days, the Cu concentration was lower in the liver of fish exposed to Cd during 11 days ($p<0.05$). The Cd concentration in the gills of Cd exposed fish doubled between days 6 and 11 of exposure ($p<0.05$). In the liver of Cu exposed fish, a significantly decreased Cd concentration was observed after both 6 and 11 days of

exposure. Prolonged Cu/Cd exposure resulted in a significant increase in both Cu and Cd concentrations in the gills compared to single Cu or Cd exposure. In Cu/Cd co-exposed fish, significant differences between 6 and 11 days of exposure were observed in the Cu and Cd concentrations of the gills ($p < 0.001$ and $p < 0.05$ respectively) and the Cd concentration in the liver ($p < 0.05$).

			control	Cu	Cd	CC
[Cu] ($\mu g.g^{-1}dw$)						
Gills	6d	(n=5)	1.65 ± 0.17	3.19 ± 0.18 ***	1.64 ± 0.19	2.79 ± 0.17 ***
	11d	(n=9)	1.59 ± 0.19	3.22 ± 0.23 ****	1.81 ± 0.18	5.69 ± 0.60 :::
Liver	6d	(n=10)	73.1 ± 12 3	100.6 ± 1.26	66.6 ± 9 2	134 1 ± 18.6 **
	11d	(n=9)	86.2 ± 17.0	119.6 ± 24.2	44.5 ± 6.1 *	149.9 ± 26.7
[Cd] ($\mu g.g^{-1}dw$)						
Gills	6d	(n=5)	0.06 ± 0.01	0.07 ± 0.01	2 67 ± 0.54 ****	7.41 ± 1.52 :::*
	11d	(n=9)	0.12 ± 0.04	0.11 ± 0.02	6.13 ± 1.24 ****	20.98 ± 4 99 ::
Liver	6d	(n=10)	0.31 ± 0.04	0.19 ± 0.04 *	8.96 ± 1.50 ****	12.89 ± 2.85 ****
	11d	(n=9)	0.39 ± 0.12	0.20 ± 0.03 *	15.59 ± 5.23 ***	7 07 ± 0.93 ****

Table 2. *Concentrations of Cu and Cd in the gills and the liver of fish exposed during 6 or 11 days to 20 μg $Cd.l^{-1}$ (Cd), 50 μg $Cu.l^{-1}$ (Cu) or co-exposed to 20 μg $Cd.l^{-1}$ + 50 μg $Cu\ l^{-1}$ (CC). Asterisks indicate significant differences between control and metal exposed fish, while circles indicate significant differences between single metal exposed fish and Cu+Cd co-exposed fish*

The relation between GSI and the Cd concentration in the gonads of control, Cd and Cu/Cd co-exposed fish is best described by non-linear functions (Figure 4B). For the Cu exposed fish, however, no significant relation exists between GSI and the Cd concentration in the gonads. The observed higher Cd concentrations in the gonads of Cd and Cu/Cd exposed fish compared to the non-Cd exposed fish (Fig. 4A) appeared consistent throughout the GSI range (Fig. 4B; compare left and right panels).

DISCUSSION

The results demonstrate organ specific Cu/Cd interactions after single and co-exposure of tilapia via the water. Four major conclusions can be drawn from the present study. Firstly, compared to single metal exposed fish, Cu/Cd co-exposure resulted in significantly different Cu and/or Cd concentrations in the gills, liver, kidneys, intestine and gonads. Secondly, these metal interactions were most pronounced in the LOW group. Thirdly, in the gills of Cu/Cd co-exposed fish, accumulation and impact of the interactions were time dependent. Finally, Cu and Cd accumulated in notable amounts in the intestinal wall.

Cu/Cd accumulation and interactions were studied after 6 and 11 days of exposure, and therefore conclusions on Cu/Cd accumulation and interactions are restricted to the exposure regimes studied. However, it has been demonstrated that accumulation and toxicity of the metals are mainly critical during an exposure period of days rather than weeks (Gill *et al.*, 1992; Carbonell and Tarazona, 1994).

Water content

Exposure of the fish to Cu and Cd, both singly and combined, did not affect the water content of any of the organs studied, which contrasts with previous results on juvenile tilapia exposed to identical metal regimes (Pelgrom *et al.*, 1994[a]). This may relate to an increased sensitivity of younger life-stages to osmoregulatory disturbances, i.e. water balance, since the weight-specific surface area of the gills in juvenile fish is nearly two times higher than that of mature fish (Morgan, 1971).

Gills

Cu or Cd exposure resulted in a significant increase of either metal concentration in the gills. Only for Cd, this accumulation was concentration- and time-dependent. Increased metal concentrations were also observed in Cu exposed carp, brown bullhead and roach (Yamamoto *et al.*, 1977; Stagg and Shuttleworth, 1982; Segner, 1987) but not in rainbow trout (Laurén and McDonald, 1987), and in Cd exposed rainbow trout, pike and American eel (Brown *et al.*, 1986; Norey *et al.*, 1990; Gill *et al.*, 1992). Metal-metal interactions after single metal exposure were shown by Gill *et al.* (1992) who reported an increased Cu concentration in the gills of eels after Cd exposure. Previous experiments with single metal exposed fish have shown effects of sublethal concentrations of Cu or Cd on the number and function of ion transporting cells in the gills (Baker, 1969; Verbost *et al.*, 1987; Reid and McDonald, 1988; Pratap and Wendelaar Bonga, 1993; Pelgrom *et al.*, 1995). Exposure to low Cd concentrations resulted in an unexpectedly high increase in metal concentration in the gills. However, the free available ionic

metal concentration is not necessarily equivalent to the total water metal concentration to which the gill is exposed (Playle *et al.*, 1992), since: a) the pH at the gill surface is lower than the water, due to local release of carbon dioxide, and this facilitates the release of metal ions from complexes (Cusimano *et al.*, 1986), and b) the amount of mucus on the gill surface increases during metal exposure (Handy and Eddy, 1991), which may contribute to higher metal concentrations at the gill surface (Reid and McDonald, 1991). Both phenomena might be relatively more important at low water metal concentrations. In this study, co-exposure to Cu/Cd resulted in an even greater accumulation of Cd than when compared to single Cd exposure. At present it is not clear to what extent these increased metal concentrations affect the function of ion-transporting cells.

Liver and kidneys

Exposure to Cu during 6 as well as 11 days had no effect on the Cu concentration in the liver, whereas the Cu concentration in the kidney increased significantly after exposure to 100 μg Cu. l^{-1}. Opposing effects have been reported in most other fish species. Cu has been shown to accumulate in both liver and in the kidneys of catfish (Brungs *et al.*, 1973) and carp (Yamamoto *et al.*, 1977), whereas Stagg and Shuttleworth (1982) registered a decreased Cu concentration in the liver of flounder after Cu exposure in seawater. Starved, but not fed, roach accumulated significant amounts of Cu in the liver (Segner, 1987). Our experiments were performed with fed tilapia, and this may partly account for the absence of Cu accumulation in this study. In contrast to other species, however, tilapia accumulated extremely high amounts of Cu in the intestinal wall, which may partly explain the absence of Cu accumulation in the liver after Cu exposure. This will be discussed in the following section.

Cd exposure resulted in an increased Cd content of both liver and kidneys, and this agrees with the findings of Gill *et al.* (1992) on eel. The liver and the kidneys play a crucial role in detoxification and excretion of toxicants mainly through the induction of metal-binding proteins such as metallothioneins (MT's; Klaverkamp *et al.*, 1984; Cousins, 1985). Relatively few *in vivo* studies concerning both metal accumulation and MT-induction investigated the effects of Cu/Cd interactions during single and combined exposures. Both in liver and kidneys, the lowest concentrations used in combination, 5 $\mu g.l^{-1}$ Cd and 20 $\mu g.l^{-1}$ Cu, resulted in significantly increased Cu concentrations when compared to single Cu exposure. This suggests a disturbance of the Cu metabolism which might involve interactions between Cu and Cd during binding to MT. Changes of the Cu content of the liver and kidney following single Cd exposure are known from studies on mammals (Suzuki *et al.*, 1983; Chmielnicka *et al.*, 1985), and eels (Gill *et al.*, 1992). Our results demonstrate similar interactions in combined Cu/Cd exposed fish which occur in mixtures at very low, environmentally relevant, metal concentrations.

Intestine

Exposure to waterborne Cu or Cd resulted in a high increase of both metals in the intestinal wall. This is surprising because freshwater fish are known to drink very little (Potts *et al.*, 1967). In Cu/Cd co-exposed fish, the Cd increase was even more pronounced than in Cd exposed fish. After intravenous Cd administration, significantly increased Cd concentrations were found in the intestinal wall of rats and mice (Berlin and Ullberg, 1963; Stonard and Webb, 1976; Barański, 1987). Stonard and Webb (1976) reported that the Cd in the intestinal mucosa could be recovered in the fraction containing MT. Few reports are available on the effects of waterborne metal exposure on the intestinal wall of freshwater fish. Exposure of freshwater fish to high concentrations of Cu (Yamamoto *et al.*, 1977) or Cd (Gill *et al.*, 1992) resulted in a significant increase of these metal in the intestine. In our study, compared to single metal exposed fish, combined Cu/Cd exposure resulted in even higher Cu (LOW) and Cd concentrations. In line with this, Gill *et al.* (1992) observed indications for interaction between Cd and Cu in the intestine of Cd exposed eels. The Cu and Cd content of the food can not account for the amounts of metals found in the intestinal wall of our tilapia, since the contribution of Cd from the food can maximally account for 2.5% of total Cd concentration of the intestine. Also drinking could not explain this phenomenon (Gill *et al.*, 1992), since it would imply an exceptionally high drinking rate (at least 60 $ml.h^{-1}$ in the LOW group, assuming that 100% of the Cu in the water is taken up). It is therefore more likely that in tilapia the intestine wall serves as a storage organ and possibly excretion route for heavy metals, as has earlier been suggested for rats (Stonard and Webb, 1976). A negative consequence of this mechanism might be, that the high metal concentrations may affect the transport functions of the intestine. The results of *in vitro* experiments with tilapia intestinal basolateral plasma membrane preparations have shown that Cd^{2+} inhibits the active uptake of calcium (Schoenmakers *et al.*, 1992).

The metal accumulation in the intestinal wall might be a specific mechanism for tilapia, because in general in fish about 95% of the whole body Cu accumulation was allocated into the liver (Stagg and Shuttleworth, 1982; Laurén and McDonald, 1987). Perhaps an efficient accumulation and excretion route via the intestine contributes to the metal tolerance of tilapia, which is higher than other species studied.

Brain and head kidney

Because Cd, but not Cu, accumulated significantly in both brain and head kidney after Cd exposure, the blood-brain barrier might function better for Cu than for Cd. Generally, brain and head kidneys are not considered to be a storage place for metals during exposure (Pelgrom *et al.*, 1994[b]). In stone loach and carp, waterborne Cu exposure respectively increased (Solbé and Cooper, 1976) and decreased (Yamamoto *et al.*, 1977) Cu concentrations in the brain. Although

increased Cd concentrations in fish brains were observed previously, this effect does not appear to be related to the ambient Cd concentration (Gill *et al* , 1992) Effects of Cd exposure on the Cu content of the brain was observed in studies with rats (Chmielnicka *et al* , 1985) and eel (Gill *et al* , 1992) Cu ions may interfere with the control of GnRH (gonadotrophin-releasing hormone) release, whereas Cu deficiency leads to infertility in rats and guinea-pigs (Burrows and Barnea, 1982, Barnea *et al* , 1986)

To our knowledge, there are no other studies concerning Cu, Cd or Cu/Cd co-exposure on the metal content of head kidney of fish In mammals, the adrenal is the endocrine organ most sensitive to chemical induced lesions (Ribelin, 1984) In fish, the cortisol producing interrenal tissue, located in the head kidney, plays a key role in the regulation of ion-homeostasis and in the stress response, via the release of cortisol (Donaldson, 1981) The amount of metal accumulated in the head kidney tissue is remarkably lower than in the kidney This is in keeping with observations in tilapia that renal and interrenal tissue, despite their common origin, are two separate compartments with different functions (Nandi and Bern, 1960)

Muscle

In contrast to our data, other researchers found no detectable metal concentrations in fish muscle after waterborne Cu (Yamamoto *et al* , 1977) or Cd exposure (Hawkins *et al* , 1980) This probably relates to the difficulty of detecting metals in muscle tissue, because its high protein content influences metal measurements Such problems can be solved by applying a matrix-modifier during metal detection (Dabeka and Ihnal, 1987), as used presently Other authors reporting detectable Cu or Cd concentrations found no differences in metal content between control and metal exposed fish It is important to note that we have reared fish under metal free water conditions, which is reflected in low metal levels in the control fish In none of the experimental groups did metal concentrations in the muscle exceed the values stated in the directives of the Food and Drug Act for edibility of fish From these directives, the norm of safe levels of toxic metals in freshwaters have been deduced (Dutch Department of Welfare, Health and Culture, WVC, 1992) Results from the present study and from the study of Gill *et al* (1992) demonstrate that it is difficult to relate tissue Cu and Cd concentrations to water metal concentration or exposure time, which in particular applies to Cu/Cd co exposed fish

Gonads

It is remarkable that, in this tissue, at the low water concentrations used, significantly higher concentrations of Cd were found after co-exposure than after single metal exposure In a concomitant study on male tilapia, Cu/Cd co exposure resulted in an even more pronounced difference in the concentrations in the gonads, a 3- and 8-fold additional increase of Cu and Cd, respectively, compared to single metal exposure (our unpublished observations) Although no

significant effects on the average GSI were noticeable after 6 days of exposure, in all groups of fish there was a negative relationship between the Cd concentration and the GSI It is of interest, that the gonadal Cd concentration of the Cd and Cu/Cd co-exposed fish was higher than in controls over the entire GSI range, but particularly so at the lower GSI This indicates that Cd accumulation is predominantly associated with the connective and endocrine tissues The observed higher Cd concentrations at high GSI indicates, that in the mature ovaria, Cd also accumulated in the eggs Increased metal concentrations in the gonads likely implicate a direct burden for reproduction and/or for young fish in addition to indirect effects on regulatory systems at other sites Studies of Eaton (1973) and McFarlane and Franzin (1978) showed that exposure to mixtures of trace metals decreased the reproductive success of fathead minnows and white suckers

Previously, we demonstrated Cu/Cd interactions on whole body metal accumulation during waterborne metal exposure of juvenile tilapia (Pelgrom *et al* , 1994[a]) Data in the present study show that interactions observed are organ specific and therefore data for most organs are not representative for the whole organism Furthermore, Cu/Cd interactions already occur at low, environmentally relevant metal concentrations in the water The data in the present study substantiate the observations of Handy (1992) that metal concentrations in the organs of fish, rather than the metal concentrations in the water, are suitable for environmental monitoring, especially when trying to relate the toxicity of metals to the biological function of specific organs

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CHAPTER 3

PHYSIOLOGICAL RESPONSES OF TILAPIA, *OREOCHROMIS MOSSAMBICUS*, TO SUBLETHAL COPPER EXPOSURE

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ABSTRACT

Tilapia were exposed during 6 days to a range of sublethal Cu concentrations, to examine the physiological mechanisms underlying the toxic effects of waterborne Cu exposure To get an integrated picture of acclimation processes taking place in the gills of tilapia after sublethal Cu exposure, we studied several parameters. In the gills, Cu exposure not only resulted in Cu accumulation, but also induced chloride cell proliferation Whole body flux measurements demonstrated a decrease in the Na influx, whereas Ca influx was not affected. Na^+/K^+-ATPase activity in the gills was decreased in the crude homogenate, and not in a purified vesicle preparation. In the blood plasma, ceruloplasmin and glucose concentrations were increased, whereas Na and Cl concentrations were lower than in control fish These results emphasize the complicated effects of Cu on gills

INTRODUCTION

Copper (Cu) plays an important role in cellular metabolism (Prasad, 1984; Cousins, 1985). At elevated levels, however, it becomes toxic and affects physiological processes. In most fish, as well as in mammals, the liver is an important storage organ for Cu (Buck, 1978; Shearer, 1984). However, the gills make an important difference in the uptake route for Cu between fish and mammals Therefore, Cu accumulation in the liver and other organs of fish can only become crucial if the gills have been able to cope with elevated water Cu concentrations (Laurén and McDonald, 1985, 1987) In addition to their role in the regulation of ion homeostasis (Eddy, 1982, Perry and Flik, 1988), the gills of freshwater fish are also the primary organ for the uptake of waterborne heavy metals (Stagg and Shuttleworth, 1982[a], Norey *et al* , 1990; Battaglini *et al.*,

1993) Cu is rapidly taken up by the gills (Buckley *et al* , 1982), from where the metal is partially transferred into the blood The blood is the main pathway for the transport of metals in the body of the vertebrates, with ceruloplasmin as the major Cu transport protein of the plasma, donating Cu to the organs (Weiss and Linder, 1985, Cousins, 1985) The Cu concentration in plasma is generally elevated in Cu exposed fish (Stagg and Shuttleworth, 1982[a,b])

Following exposure to waterborne Cu, the metal is unevenly distributed over the different organs (Pelgrom *et al* , 1995) The organ with the highest metal accumulation is not necessarily the critical organ from the point of view of metal toxicity (Task group on metal accumulation, 1973, Laurén and McDonald, 1987) Therefore, to understand the effects of exposure to sublethal metal concentrations, more knowledge is required of the biochemical and physiological actions of the metal in key organs (Stagg and Shuttleworth, 1982[a])

This study focusses on the gills, the primary site of toxic insult during heavy metal exposure (Laurén and McDonald, 1987), and on the blood plasma Cu affects the active transport of ions in the gills (Stagg and Shuttleworth, 1982[b], Lauren and McDonald, 1985, 1986, Reid and McDonald, 1988), and therefore restoration of ion regulatory mechanisms will be an essential part of the acclimation of fish to this metal (Stagg and Shuttleworth, 1982[b], Laurén and McDonald, 1987) Cu exposure has been shown to disturb several blood parameters such as osmolality (McKim *et al* , 1970), plasma Na, Cl (Stagg and Shuttleworth, 1982[a], Laurén and McDonald, 1985, 1987), and Ca (Sayer *et al* , 1991) The biochemical basis of the recovery of the branchial ion regulatory function during acclimation likely concerns the chloride cells, the cells involved in the ion transport in the gills (Mayer-Gostan *et al* , 1987)

Relatively little is known about the mechanisms underlying the recovery of Cu-induced branchial impairment Generally, one or two aspects of Cu-induced impairment have been studied in fish after a certain exposure regime As a result, there is no complete picture of acclimation processes during Cu exposure in one species of fish In the present study we report on the relation between Cu accumulation in gills and blood and its effects on ion regulatory mechanisms The cichlid fish tilapia was chosen in this study because previous work on this species has characterized gill Ca transport mechanism (Flik *et al* , 1985) In addition to the studies with adult fish, also experiments with juvenile fish could be performed, which allowed us to minimize the stress associated with flux measurement To obtain an integrated picture of the processes taking place in the gills of tilapia during acclimation to Cu, effects on ionic regulation were determined using several parameters chloride cell -number and -diameter, Na and Ca fluxes between fish and the ambient water, gill Na^+/K^+-ATPase activity, gill Ca^{2+}-transport, and plasma ion concentrations Plasma ceruloplasmin levels were determined in order to study the involvement of the Cu-transporting protein during Cu exposure in fish

MATERIALS AND METHODS

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from our laboratory stock Fish were grown and held under artificial freshwater conditions with undetectable Cu concentrations (detection level below 0 1 $\mu g\ l^{-1}$) Artificial freshwater consisted of demineralized water supplemented with 1 3 mM $NaHCO_3$, 0 5 mM $CaCl_2$, 0 06 mM KCl and 0 2 mM $MgCl_2$, at pH 7 8 Composition and preparation of the water was based on the EEC instructions for artificial water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1 Acute toxicity for fish). Water was continuously aerated, filtered and refreshed by means of flow-through The light/dark regime was 12/12 h and the water temperature 26°C Fish were fed commercial tropical fishfood Tetramin™, 2% ($^{dw}/_{ww}$) of their body weight per day The Cu content of the food was 9 86 ± 0 16 μg Cu g^{-1} (means ± SE, n=10)

Whole body Na and Ca fluxes

Pilot experiments were performed to determine optimal experimental conditions Cu exposure regime, conditions of the fish (density, size, acclimation period), water quality during the flux periods (pH, temperature, nitrate- and ammonium- concentrations), anesthetic concentration, tracer injection volume, interaction between $CaCl_2$ and Na_2CO_3, peroxide digestion and rinsing of the fish after radio tracer exposure (data not shown)

Three days before the start of the experiment, 12 groups of 9 tilapia (weighing 1-2 g , about 2 months old) were placed randomly in 3 2 l flux chambers filled with artificial freshwater Fish were fed daily (2% $^{dw}/_{ww}$ Tetramin™) The food was eaten within 1 minute During the acclimation period, the water in the flux chambers was continuously aerated and refreshed by means of a flow-through-system (flow rate 0 24 $l\ h^{-1}$, 16-channel peristaltic pump, Watson Marlow) The exposure period started with the connection of each flux chamber to reservoirs filled with artificial freshwater each with a well defined Cu concentration (added as nitrate, Spectrosol, BDH, England) During the first 4 h of the exposure, the flow rate was 0 90 $l\ h^{-1}$, followed by a flow rate of 0 24 $l\ h^{-1}$ during the rest of the exposure period Cu concentrations in the reservoirs were monitored daily The Cu concentrations in the flux chambers were monitored every hour during the first 6 h of exposure, and at least once a day during the rest of the exposure period The Cu concentrations in the flux chambers were 0 (control), 50 (50Cu) and 200 (200Cu) μg Cu l^{-1} The actual Cu concentrations measured did not deviate more than 5% from the nominal Cu concentrations After 6 days of Cu exposure, Na^+ and Ca^{2+} influx and efflux were determined by means of radiotracers

For measurement of Na^+ and Ca^{2+} influx, 1 0 MBq l^{-1} $^{24}Na_2CO_3$ (IRI, Delft, The

Netherlands) and 0.75 MBq.l^{1} $^{45}CaCl_2$ (Amersham, England) were added to the flux chambers. $^{24}NaCO_3$ was neutralized to pH 7.5 with equimolar concentrations of hydrochloric acid After 5, 20 and 45 minutes, water samples for tracer measurement were taken After 45 minutes of tracer exposure, fish were quickly (within 1 minute) anaesthetized with phenoxy-ethanol in a final dilution of 1:400. Of each flux chamber, all 9 fish were briefly (2 seconds) rinsed in artificial freshwater containing 5mM Ca and 10 mM Na, followed by a rinse in artificial freshwater Subsequently, 4 fish were immediately killed in dry ice/acetone for determination of whole body Na^+ and Ca^{2+} influx.

To investigate Na^+ and Ca^{2+} efflux, the remaining 5 fish of each flux chamber were injected (i.p) with 0.17 MBq $^{24}Na_2CO_3$ (neutralized with HCl to pH 7.5) and 0 12 MBq $^{45}CaCl_2$. Fish were allowed to recover from anesthesia in freshwater with the same Cu concentrations as they were exposed to, and put back in their flux chambers containing radiotracer-free artificial freshwater with Cu Fish recovered from anesthesia within 1 minute after injection as indicated by a feeding response. The whole procedure took less than 5 minutes per flux chamber. Overnight, the flux chambers were continuously refreshed (0 24 l h^{1}). During efflux measurement, the water-flow was stopped, and tracer appearance in the water was monitored during 4 h. After this period, fish were anaesthetized (phenoxy-ethanol 1 400) and rinsed in artificial freshwater. Blood from the caudal vessels was taken by means of heparinized mini-capillaries (Hirschmann). After centrifugation (3 minutes 18000 g), plasma radiotracer concentration was determined in triplicate for each fish

^{24}Na in whole fish, plasma and water was immediately determined in a γ-counter (LKB) After one week (11 times the half-life of ^{24}Na), no ^{24}Na could be detected in the samples. Then, scintillation fluid was added to the water and blood samples for ^{45}Ca determination The fish were digested with peroxide (35%, 4-times 100 μl) at 40 °C during 3 days, and 1 day at 60°C, and the digests were dissolved in scintillation fluid ^{45}Ca was determined in water, blood and fish samples by means of a liquid scintillation counter (Pharmacia Wallac 1410).

Influx of Ca^{2+} and Na^+ was calculated on the basis of the total body radioactivity after 1 h of exposure to ^{24}Na and ^{45}Ca, and the respective mean tracer specific activities in the water For this calculation we assumed that during the influx period no significant backflux from the fish to the water occurred Efflux of Ca^{2+} and Na^+ was calculated from the tracer activities in the water and the specific Ca and Na activities in the plasma. In the exposed fish, the plasma concentrations of Ca and Na were determined to calculate the specific Ca and Na activities in the plasma The net flux is given as the difference between average influx and efflux in each flux chamber

Experimental design of experiments with mature fish

Six weeks before the start of the experiment four groups of 14 mature (mean weight 20 g) female tilapia were were kept in 80 l aquaria with continuously filtered and refreshed artificial

freshwater. The experiment started by connecting (by means of a 16 channel peristaltic pump; Watson Marlow) each aquaria to its own reservoir filled with artificial freshwater with or without (controls) a well-defined Cu concentration (added as nitrate; spectrosol, BDH, England). During the first 6 h the flow rate was 4.5 $l.h^{-1}$, followed by a flow rate of 1.5 $l.h^{-1}$. In this way, the Cu concentrations in the aquaria were gradually raised, reaching a plateau after 18 h. Cu concentrations in both stock solutions and aquaria were monitored every hour during the first 6 h, and at least once a day during the rest of the exposure period. The nominal Cu concentrations in the aquaria were 0, 50, 100 and 200 $\mu g.l^{-1}$, with the actual concentrations deviating maximally 5% from nominal concentrations. The exposure period lasted 6 d, and feeding was ended one day before sacrifice. At the end of the exposure period, blood samples were taken from the caudal blood vessels by means of heparinized capillaries, and fish were killed by spinal dissection. Blood cells and plasma were separated by centrifugation (3 minutes 18000 g). The left opercula were prepared for chloride cell counting with DASPEI vital staining (Wendelaar Bonga *et al.*, 1990), and the gill arches were prepared for either Cu measurement or plasma membrane isolation. Dissection instruments were systematically cleaned with 0.1% HNO_3 and alcohol to prevent contamination.

Cu measurement

After determination of wet and dry weights, gills, blood cells and plasma were destructed with nitric acid (65% HNO_3 ultrapur, Merck), and stored in 0.2% HNO_3 at 4°C until analysis. Water samples were acidified with HNO_3 to a final concentration of 0.2%. Cu concentrations were determined with a flameless Atomic Absorption Spectrometer (AAS, Philips PU 9200) connected to an electrothermal atomiser (Philips PU 9390X).

Isolation of plasma membranes

Plasma membranes of the branchial epithelia were isolated at 4°C as described by Flik *et al.* (1985), with some adjustments. Briefly, the soft tissue of the gills was scraped off with a glass microscope slide, and carefully homogenized with a glass-to-glass Dounce homogenizer (10 strokes) in an isotonic buffer containing 250 mM sucrose, 12.5 mM NaCl, 5 mM HEPES/TRIS pH 7.5, 0.1 mM EDTA, 100 $U.ml^{-1}$ aprotinin (Sigma) and 50 $U.ml^{-1}$ heparin. Nuclei and cellular debris (pellet P_0) were separated from membrane fractions (supernatant H_0) by centrifugation during 10 minutes at 550 g (Hereus). After centrifugation of the supernatant H_0 (50,000 rpm, 30 minutes; Beckmann Ultracentrifuge, Ti 70 rotor), membranes were collected in a fluffy pellet (P_1). This pellet was resuspended with a glass-to-glass Dounce homogenizer (100 strokes) in an isotonic sucrose buffer containing 250 mM sucrose, 5 mM HEPES/TRIS pH 7.5 and 5 mM $MgCl_2$. The membrane suspension was centrifuged differentially: 10 minutes at 1,000 g followed by 10 minutes at 9,500 g (Sorval RC-5B). Finally, the supernatant was centrifuged during 15

minutes at 20,000 g, resulting in the final membrane fraction, pellet P_3. These pellets P_3 were resuspended by passage through a 23-G needle (10 times) in a buffer containing 20 mM HEPES/TRIS pH 7.4, 1.5 mM $MgCl_2$ and 150 mM KCl (for Ca^{2+} transport studies) or 150 mM NaCl (for Na^+/K^+-ATPase studies). Membrane preparations P_3 and crude membrane homogenates H_0 were quickly frozen in cold CO_2/acetone, and used the next day for determination of protein content, enzyme- and transport-activity.

$Na/^+K^+$-ATPase activity

Na^+/K^+-ATPase activity in the H_0 and P_3 gill membrane fractions was determined by the method described by Flik *et al.* (1985). Routinely, 0.20 $mg.ml^{-1}$ saponin was added to optimize substrate accessibility. Membrane protein content was determined with a reagent kit (Biorad), using Bovine Serum Albumin (BSA, Sigma) as reference. Vesicles were incubated during 10 minutes at 37° C with medium containing 100 mM NaCl, 30 mM Imidazole, 0.1 mM EDTA, 5 mM $MgCl_2$ and either 15 mM KCl or 1 mM ouabain. Na_2ATP was added in a final concentration of 3 mM. The reaction was stopped by adding ice-cold TCA-solution. Inorganic phosphate (P_i) production was measured by the colorimetric Fiske-Subbarow technique using a commercially (Sigma) phosphate standard (Flik *et al.*, 1985).

Ca^{2+}-transport

ATP-dependent Ca^{2+}-transport was determined by means of a rapid filtration technique as described by van Heeswijk *et al.* (1984). Ca^{2+} and Mg^{2+} concentrations were calculated according to Schoenmakers *et al.* (1992) using the computer program CHELATOR. Ca^{2+}-transport was measured at a Ca^{2+} concentration of 10^{-6}M (V_{max}) Uptake of ^{45}Ca into membrane vesicles (P_3-fraction) was determined during 1-minute incubations without or in the presence of 3 mM ATP (Tris-ATP). The reaction was stopped in ice-cold isotonic medium containing 0.1 mM $LaCl_3$, and the suspension was filtered (Schleicher & Schüll ME 25, pore size 0.45 μm). Filters were rinsed twice with ice-cold medium and transferred to counting vials, dissolved in Aqualuma©. ^{45}Ca was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

Plasma

Plasma protein concentrations were determined by means of a reagent kit (Biorad) with BSA as reference. Plasma glucose was determined spectrophotometrically using a D-glucose kit (Boehringer Mannheim, UV-method). Concentrations of plasma Na and K were measured with a flame-photometric Auto Analyzer (Model IV, Technicon), while the Cl concentration was determined spectrophotometrically by the forming of ferrothiocyanate. The cresolphtalein complexone method (Sigma Diagnostics) was used for the determination of total plasma Ca concentration. Ca^{2+} and pH were measured by means of an Ionic Calcium analyzer (Radiometer)

as described by Fogh-Anderson (1981)

Plasma ceruloplasmin concentration was measured as *p*-phenylenediamine (PPD) oxidase activity, an assay based on the methods described by Houchin (1958) and Rice (1961) To validate the method of ceruloplasmin detection in plasma of tilapia, several parameters of the assay were tested a) substrates PPD and N-N dimethyl-PPD, b) incubation time (between 0 and 75 minutes), c) the pH of the buffer (between pH 4 and 10), d) incubation temperatures (4°, 20°, 26°, 37° C), e) the plasma volume (between 0 and 100 μl)

From the results of these tests, ceruloplasmin concentrations in plasma of tilapia was measured by the following method Plasma (15 μl) was mixed with 1 ml 1 2 M acetate/acetic acid buffer (pH 6 4) containing 0 1% PPD (Sigma) as substrate To avoid non-specific substrate oxidation, incubation was carried out in the presence of 0 02 mM EDTA Each plasma sample was incubated in duplicate Concommitantly, each plasma sample was incubated in the presence of 1 ml 0 5% NaN_3 (azide blank) The mixtures were incubated during 30 minutes at 37° C The reaction was stopped by the addition of 1 ml 0 5% NaN_3 Within one hour, the absorption was measured at 550 nm (LKB spectrophotometer). Ceruloplasmin concentration was expressed as the difference in absorbance between the sample and its azide blank

Statistics

Data are presented as means ± SE The Student's *t*-test was applied for statistical evaluation Significant differences between control and Cu exposed groups are indicated by asterisks with * $P < 0\ 05$, ** $P < 0\ 02$, *** $P < 0\ 01$ and **** $P < 0\ 001$

RESULTS

Exposure during 6 days to 50, 100 and 200 μgCu l^{-1} resulted in significantly increased Cu concentrations in the gills (Fig 1A) The increase in the Cu content was most prominent in the fish exposed to 200 μgCu l^{-1} Only in this group, the ceruloplasmin concentration in the plasma was increased over controls (Fig 1B) Cu exposure also resulted in an increased Cu concentration in the plasma (Fig 1D), but had no effect on the Cu content of blood cells (Fig 1C)

The number of opercular chloride cells increased in a dose-dependent way, resulting in significantly more chloride cells in the fish exposed to 100 and 200 μgCu l^{-1} (Fig 2A) In these groups, also the diameter of the chloride cells increased (Fig 2B)

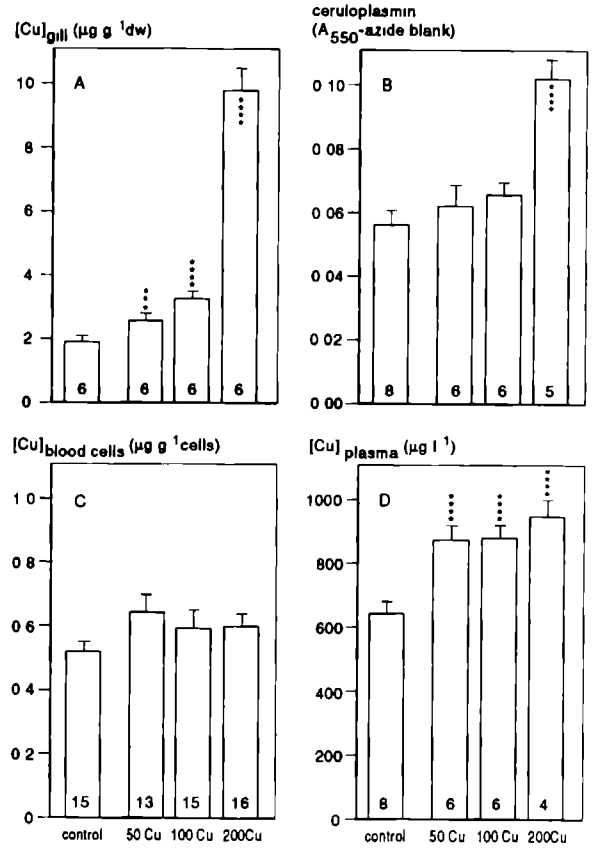

Figure 1. Cu concentrations in the gills (A), plasma ceruloplasmin levels (B) and Cu concentrations in the blood cells (C) and in the plasma (D) of fish exposed during 6 days to 0 50, 100 or 200 µg l⁻¹ Cu The number of fish per group is indicated in the bars Significant differences are indicated by asterisks

Whole body flux measurements showed that exposure to 200 µgCu l⁻¹ inhibited the Na influx, while the Na efflux remained unaffected The net flux was significantly lower, although still positive, in fish exposed to 200 µgCu l⁻¹ compared to control fish We did not observe an effect of waterborne Cu on Ca influx or efflux (Fig 3)

In the crude branchial homogenate (H_o) of fish exposed to 200 µgCu l⁻¹, both total and specific Na^+/K^+-ATPase activities were inhibited These observations were not reflected in the

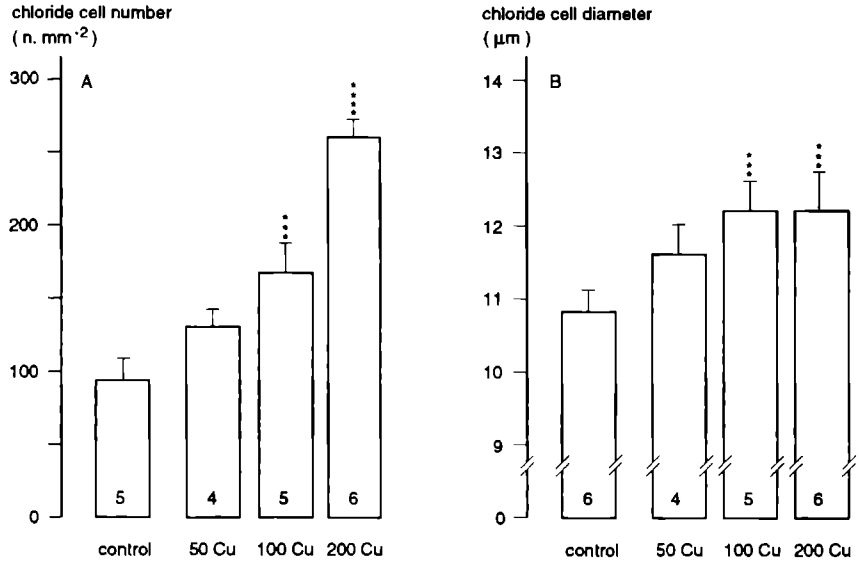

Figure 2. *Chloride cell number in the opercula (left panel) and diameter of the chloride cells (right panel) of fish exposed during 6 days to 0, 50, 100 or 200 µg.l^{-1} Cu. The number of fish per group is indicated in the bars. Significant differences are indicated by asterisks.*

total and specific Na^+/K^+-ATPase activities of the purified membrane fraction P_3. Cu exposure had no effect on the protein recovery during the process of membrane purification from H_o to P_3, while the enzyme purification was doubled in fish from the highest Cu concentration when compared to controls (Fig. 4).

Cu exposure had no effect on the Ca^{2+}-transport in the purified vesicle preparation of gill basolateral membranes (Table I). Plasma total Ca concentration decreased only in the fish exposed to 100 µgCu.l^{-1}. Plasma ionic Ca was not changed (Table I).

Exposure to 200 µgCu.l^{-1} resulted in decreased plasma Na and Cl concentrations (Table 2). The Na:Cl ratio was unchanged by Cu exposure. Compared to controls, the pH of the plasma of the 200 µgCu.l^{-1} exposed fish decreased. In this Cu exposed group, also the plasma potassium concentrations increased. Plasma protein concentrations did not change after Cu exposure. Only the glucose concentration in the plasma of the fish exposed to 200 µgCu.l^{-1} increased significantly as compared to controls.

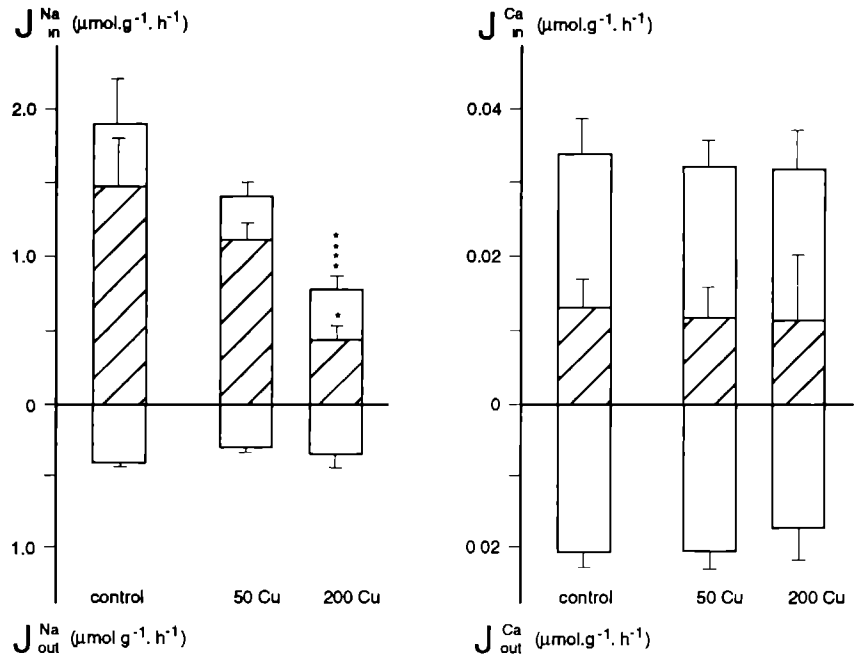

Figure 3. Na fluxes (left panel) and calcium fluxes (right panel) of fish exposed during 6 days to 0, 50 or 200 µg.l^{-1} Cu. Net fluxes are indicated by shaded bars Significant differences are indicated by asterisks

DISCUSSSION

The results of this study demonstrate that sublethal Cu exposure of fish affects several physiological parameters, such as the number of opercular chloride cells, Na^+ influx, and blood electrolyte composition and ceruloplasmin concentration.

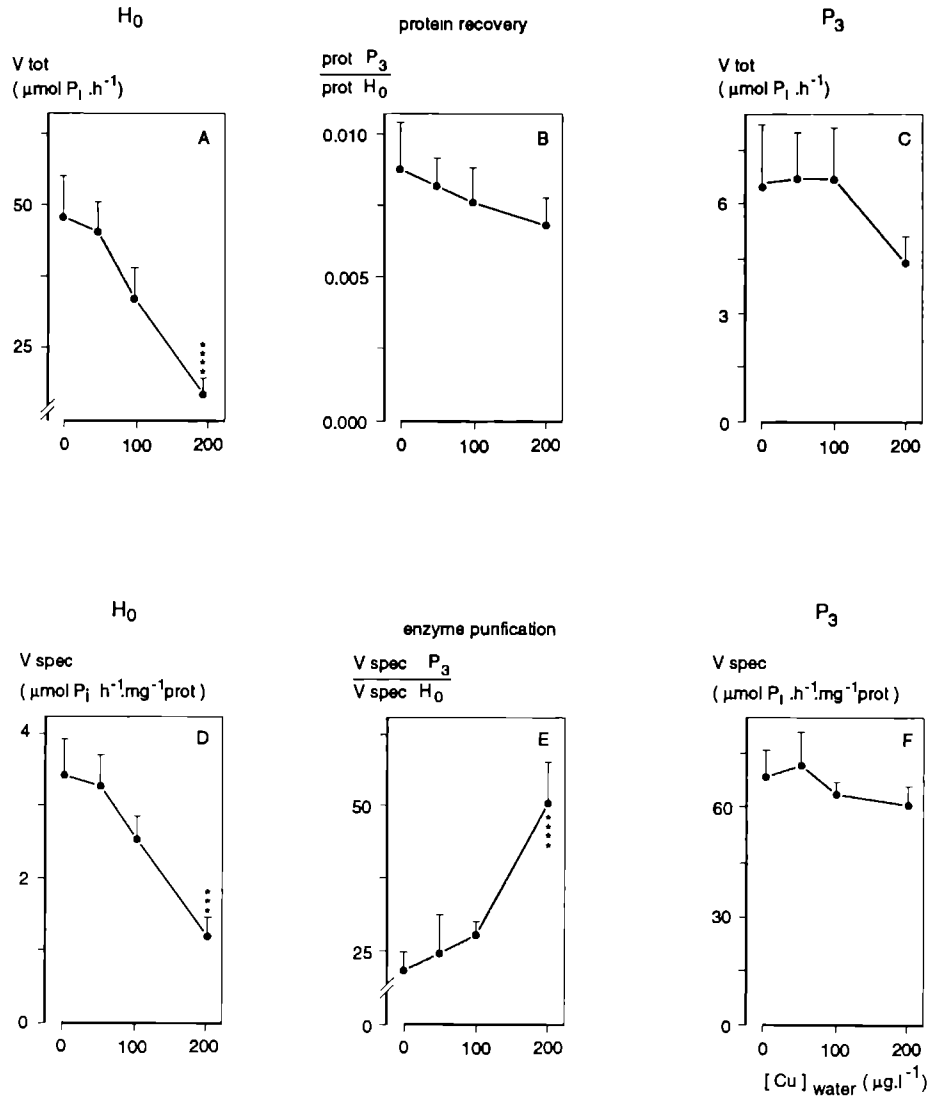

Figure 4. Total (upper panels) and specific (lower panels) Na^+/K^+-ATPase activity in crude branchial homogenate (H_0) and in purified branchial membrane preparation (P_3). Protein recovery and enzyme purification are also indicated of fish exposed during 6 days to 0, 50, 100 or 200 µg.l^{-1} Cu Significant differences are indicated by asterisks.

	Ca^{2+}- transport (nmol Ca. min^{-1}. mg^{-1}prot)	plasma [Ca] $_{total}$ (mM)	plasma [Ca^{2+}] (mM)
control	4.47 ± 0.40	6.96 ± 0.50	1.61 ± 0.05
50 Cu	3.94 ± 0.52	7.93 ± 1.27	1.65 ± 0.04
100 Cu	4.38 ± 0.59	4.78 ± 0.35***	1.71 ± 0.04
200 Cu	3.91 ± 0.51	6.78 ± 0.78	1.70 ± 0.05

Table 1. *Ca^{2+}-transport in purified branchial membrane preparation (P_i) and total and ionic Ca concentration in the plasma of fish exposed during 6 days to 0, 50, 100 or 200 $\mu g.l^{-1}$ Cu.*

Plasma	[Na^+] (mM)	[Cl^-] (mM)	[Na] · [Cl]	pH	[K^+] (mM)	protein (mg ml^{-1})	glucose (mg %)
control (n= 7)	154 ± 3	150 ± 1	1 03 ± 0 02	7 72 ± 0 03	2.73 ± 0 22	52 6 ± 2 7	66 4 ± 5 8
50Cu (n= 6)	145 ± 4	144 ± 2	1 01 ± 0.02	7 76 ± 0 06	2 47 ± 0 20	52 3 ± 4 7	66 4 ± 15 8
100Cu (n= 7)	150 ± 2	147 ± 1	1.02 ± 0 01	7 73 ± 0 03	3 37 ± 0 34	47 5 ± 2 7	50 8 ± 11 2
200Cu (n= 7)	142 ± 2****	143 ± 2****	1 00 ± 0.01	7 61 ± 0 03*	4 34 ± 0 39****	54.9 ± 1 5	109 4 ± 7 2****

Table 2. *Plasma Na, Cl, K, protein and glucose concentration, Na:Cl ratio and pH in the plasma of fish exposed during 6 days to 0, 50, 100 or 200 $\mu g.l^{-1}$ Cu Significant differences are indicated by asterisks*

Cu uptake and transport

After 6 days of exposure to Cu, the accumulation of this metal in the gill tissue was most prominent in the 200 $\mu g.l^{-1}$ group. In a previous study, we observed no difference in Cu accumulation in the gills of fish exposed for either 6 or 11 days. Therefore, in this study we

decided to expose the fish for 6 days In control fish, the Cu concentrations in blood plasma and blood cells are of the same magnitude, and comparable to concentrations reported for other fish species (600-1300 $\mu g\ l^{-1}$, Stagg and Shuttleworth, 1982, Bettger *et al* , 1987) Our results show that during exposure to waterborne Cu only the blood plasma and not the blood cells display an elevated Cu concentration This indicates that most of the Cu that enters the gills is transported via the blood plasma Our results are in line with observations in mammals (Task Group On Metal Accumulation, 1973, Frieden, 1979, Cousins, 1985) Cu-treatment of fish blood *in vitro* also resulted in association of Cu with plasma rather than with the blood cell fraction (Buckley *et al* , 1984) In the blood of mammals, Cu immediately binds to albumin and transcuprein, and is transported to the liver, where it is bound to ceruloplasmin, released into the blood, and distributed to other tissues (Weiss and Linder, 1985, Cousins, 1985) Cu bound to ceruloplasmin constitutes the larger part (90-95%) of plasma Cu, which makes ceruloplasmin the principal Cu transport protein in mammals (Frieden, 1979, Nederbragt *et al* , 1984) In a variety of vertebrate sera, the presence of this protein, as reflected by its *p*-phenylenediamine oxidase activity, has been reported (Frieden, 1979) In fish, ceruloplasmin has been demonstrated in plasma of carp (Yamamoto *et al* , 1977) Our results indicate that this protein is also present in the plasma of tilapia However, we did not observe a direct relationship between the plasma ceruloplasmin concentration and the water and/or plasma Cu concentration A direct relationship between plasma Cu concentration and ceruloplasmin level was observed in mammals after parenteral Cu administration In fish, however, waterborne Cu enters the general circulation primarily via the gills Probably, only during exposure to high levels of Cu, 200 $\mu g Cu\ l^{-1}$, ceruloplasmin synthesis is induced Cu exposure had no effect on the protein concentration in the plasma Comparable results are described in studies with brown bullhead and brook trout (McKim *et al* , 1970, Christensen *et al* , 1972)

Whole body ion-fluxes

Exposure to 200 $\mu g\ l^{-1}$ Cu reduced the Na^+ influx, whithout affecting the Na^+ efflux Consequently, the net Na^+ uptake decreased, although the Na balance was still positive It should be noted that the measured whole body fluxes almost completely represent gill fluxes (Flik *et al* , 1985) The flux experiments were performed under conditions which causes minimal additional disturbance, characterized by using an acclimation period, gradual Cu exposure, normal feeding regime and housing the fish in groups The advantage of our approach of ion-transport measurement is indicated by the high control net fluxes, as is demonstrated by a study of Dharmamba and Maetz, (1972) In contrast, no positive net fluxes were observed in control rainbow trout and brown trout (Lauren and McDonald, 1986, Reader and Morris, 1988, Reid and McDonald, 1988) Generally, only influx and net flux are measured, with net fluxes determined from changes in the ion concentration in the water, whereas efflux is calculated from the

difference between net flux and influx (Spry and Wood, 1985; Laurén and McDonald, 1986, 1987; Reader and Morris, 1988; Reid and McDonald, 1988). Branchial Na uptake is the result of Na^+/K^+-ATPase dependent Na^+ influx and Na^+ efflux via passive diffusional losses (Mayer-Gostan *et al.*, 1987; McDonald *et al.*, 1989, 1991; Wood, 1992). The inhibitory effect of Cu on the Na^+ influx may be due to the high affinity of Cu for -SH groups of transport enzymes such as Na^+/K^+-ATPase (Stagg and Shuttleworth, 1982; Beckman and Zaugg, 1988). Cu-induced disturbances of the Na^+ influx has also been observed in rainbow trout (Laurén and McDonald, 1986; McDonald *et al.*, 1989). Exposure to high concentrations of Cu has been shown to cause histological alterations in the gills (Baker, 1969). Structural damage can explain increase in ionic permeability and reduction in transport function commonly seen during the early phase of metal exposure. The absence of an effect of Cu on Na^+ efflux observed in the present study might therefore indicate that the structural integrity of the gills, which determines the permeability to Na, is not affected by the Cu concentrations used. Interestingly, only in the fish exposed to the highest Cu concentration the Na^+ influx were affected, although increased Cu concentrations in the gills were observed after exposure to all Cu concentrations used. This suggests that fish can cope with a certain increased Cu concentration in the gills before dysfunction becomes apparent. The Cu-induced inhibition of Na^+ influx in these fish exposed to the highest Cu concentration was reflected in a reduction in plasma Na concentration in mature fish. The Na:Cl ratio was unchanged, as a result of the concurrent decrease in plasma Cl concentration. Observations in the present study are in line with other reports on Cu exposed fish. In studies with flounder and rainbow trout, Na and Cl concentrations were affected similarly by Cu exposure (Stagg and Shuttleworth, 1982[a]; Laurén and McDonald, 1985; McDonald *et al.*, 1989).

In this study, Cu exposure had no effect on whole body Ca exchange with the water. This is in line with the unchanged plasma Ca concentration in mature fish observed in this study. Similar results have been reported for rainbow trout by Reid and McDonald (1988). A small and transient decrease in net Ca uptake was found in brown trout by Sayer *et al.* (1991). Influx rates of Ca^{2+} are usually substantially lower than those of Na^+ (Reid and McDonald, 1988). This was also observed in the present study with young fish. The branchial mechanisms regulating Na^+ and Ca^{2+} influxes are distinctly different from one another in hormonal control, ion-specific channels or carriers in the apical membrane and transport ATPases in the basolateral membrane of the chloride cells (Flik *et al.*, 1985; McDonald *et al.*, 1989). This is also reflected by the present observation that active branchial Ca^{2+}-uptake was, unlike Na^+/K^+-ATPase, not inactivated by Cu.

Chloride cells

In response to waterborne Cu we observed an increase in the number of chloride cells in the opercula which is known to reflect the chloride cell density in the gills in tilapia (Wendelaar Bonga *et al.*, 1990). Proliferation of chloride cells is a physiological response to agents affecting branchial ion uptake, such as Cd (Oronsaye and Brafield, 1984; Pratap and Wendelaar Bonga, 1993), and Cu (Baker, 1969). The increase in the number of chloride cells may be a compensatory response, playing a role in recovery from and acclimation to, heavy metals (Mallat, 1985; McDonald and Wood, 1993; Perry and Laurent, 1993). The ion transport capacity is related to the fraction of the chloride cells in contact with the external environment only (mature chloride cells) rather than to the total number of epithelial chloride cells, which includes young or degenerating cell stages (Wendelaar Bonga *et al.*, 1990). In a study on fish exposed to acid water (Wendelaar Bonga *et al.*, 1990) the rapid increase in chloride cell numbers reflected a higher turnover rate of these cells. Most of the cells were degenerating or immature, as evidenced by smaller cell diameters, and therefore unlikely to contribute to ion transport. In our study however, the increase in the number of chloride cells was unlikely to be the result of more small and immature cells, given the increased diameter after Cu exposure. These results therefore are more reminiscent of observations of chloride cell proliferation after exposure of trout to ion-deficient water (McDonald and Rogano, 1986; Perry and Laurent, 1989, 1993). In these studies, hyperplasia and hypertrophy of chloride cells resulted in an extension of the mean chloride cell area exposed to the water which coincided with an increased ion-transport activity of the gills (McDonald and Rogano, 1986; Perry and Laurent, 1989, 1993). However, our results on the effects of Cu demonstrate that an increase in the chloride cell number does not automatically imply an increase of the ion-transport capacity. Therefore, the increase in cell size and number observed in our experiment does not warrant restoration of the Na-transport activity.

Na- and Ca-transport mechanisms

The observed Cu-induced inhibition of Na^+ influx was not reflected in the specific Na^+/K^+-ATPase activity in membrane preparations of *in vivo* Cu-exposed fish. *In vitro* exposure to metals may cause a decrease in ATPase activity in membrane preparations (Stagg and Shuttleworth, 1982). The mechanisms underlying an inhibition of ATPase-dependent ion transport *in vivo* will however be more complicated. Several mechanisms may interfere with the process of membrane purification and *in vitro* determination of active transport mechanisms. Firstly, the Na^+/K^+-ATPase activity in the gills may be regulated by changing the number of active enzyme units present, as suggested by Stagg and Shuttleworth (1982). In the present study, the number of chloride cells was increased in Cu-exposed fish and this could account for the increase in Na^+/K^+-ATPase enrichment of the P_3-fraction in fish exposed to the highest Cu concentration. The increase in the number of chloride cells, however, is not reflected in the total Na^+/K^+-

ATPase activity. In the crude membrane homogenate H_o, the total enzyme activity was even significantly decreased in these Cu exposed fish. The second branchial mechanism in the process of compensation for Cu-induced ion losses might be a change the activity per enzyme unit (Stagg and Shuttleworth, 1982[b]). During exposure, the Cu concentration in the gills increased significantly, which may also occur in the chloride cells, which may inactivate the Na^+/K^+-ATPase activity in these cells. Such an effect seems to be reflected only in the specific Na^+/K^+-ATPase activity in the crude homogenate (H_0), and not in the P_3 fraction. One might argue that this might indicate reactivation of *in vivo* Cu-inhibited ATPase activity during the isolation procedure, assuming that most of the accumulated Cu is lost in this process. However, the amount of Cu in the P_3 fraction relative to the amount of Cu in the H_0 fraction is the same in controls and fish exposed to 100 $\mu g.l^{-1}$ Cu (39% and 42% respectively; Chapter 6). Therefore, differences in total Cu concentration between gills of controls and Cu exposed fish should equally influence the enzyme in H_0 and P_3 fractions. Thus the difference in Na^+/K^+-ATPase activity between H_0 and P_3 fractions cannot be attributed to reactivation due to loss of Cu from the membranes of the Cu exposed fish during the *in vitro* isolation procedure.

Both mechanisms may be involved during *in vitro* determination of Na^+/K^+-ATPase activity after *in vivo* Cu-exposure. In addition, *in vitro* ion-transport mechanism are determined in the absence of hormonal factors, which likely differ between controls and fish exposed to Cu (Pelgrom *et al.*, Chapters 4, 7), and at optimal Na concentrations. It should also be noted, that in most vertebrate tissues Na^+/K^+-ATPase is a heterogenous population of enzyme units. In a recent study of Middleton *et al.*, (1993) it was demonstrated, that not all forms of Na^+/K^+-ATPase in kidney cells were regulated by PKC (protein kinase C) phosphorylation, a mechanism known to be affected by Cu. Therefore, enzyme heterogeneity may contribute to the response diversity.

Few data are available of Cu-induced effects on branchial Na^+/K^+-ATPase activity after *in vivo* exposure. In flounder, Stagg and Shuttleworth (1982) observed no effect after *in vivo* Cu-exposure, whereas Laurén and McDonald (1987) observed in trout an inhibition of Na^+/K^+-ATPase specific activity, which was compensated by an increase in the microsomal protein concentration. In the latter study, however, no Cu-accumulation in the gills was measured.

To our knowledge, no data are available on the effects of *in vivo* Cu-exposure on Ca^{2+} transport in gill membrane vesicles. In this study, Ca^{2+}-transport was not affected by Cu exposure and the increased Cu concentration in the gills. These observations are in line with the flux data, and confirm the specific action of Cu on Na.

Plasma ions and glucose

Our results demonstrate that the effects of Cu exposure on plasma is not limited to disturbed Na and Cl levels, confirming the complexity of ambient Cu on these fish. In the fish exposed to 200 $\mu g.l^{-1}$ Cu, acid/base regulation was also disturbed, as indicated by a pH decrease. It has been

suggested by Laurén and McDonald (1985) that Cu exposure leads to a general increase in the permeability of cell membranes, which partly explain the increase in the plasma concentrations of potassium.

Hyperglycemia is a common response to stressors in freshwater fish, and has been considered as an indicator of sublethal environmental pollutions (Hatting, 1976). In the present study, an increased concentration of glucose in the plasma was observed in the highest Cu exposed fish only. This observation is in line with results from Cu exposed brown bullhead (Christensen *et al.*, 1972) and rainbow trout (Laurén and McDonald, 1985). A rise in the plasma glucose concentration indicates an activated carbohydrate metabolism in this species under the control of cortisol (Christensen *et al.*, 1972; Balm, 1986).

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CHAPTER 4

EFFECTS OF COMBINED WATERBORNE Cd AND Cu EXPOSURES ON IONIC COMPOSITION AND PLASMA CORTISOL IN TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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ABSTRACT

Plasma ions and cortisol levels were studied in immature tilapia exposed for 6 days to a range of sublethal concentrations of Cu (50, 100 and 200 μg Cu l^{-1}), Cd (20, 35 and 70 μg Cd.l^{-1}) and to combinations of these metals (50 μg Cu.l^{-1} + 20 μg Cd l^{-1}, 100 μg Cu.l^{-1} + 35 μg Cd.l^{-1} and 200 μg Cu l^{-1} + 70 μg Cd.l^{-1}). Our data show, that Na and Ca were markedly, although not exclusively, affected by Cu and Cd respectively. Plasma Na concentration were most prominently decreased in Cu-exposed fish, with less pronounced effects in Cd-exposed fish. In fish exposed to 70 μg Cd l^{-1} the plasma Ca concentration was half of the control value Cu-induced changes of plasma Ca concentrations were less strongly marked

In combined Cu/Cd exposed fish, Na, Ca and Cl concentrations were significantly changed. Important in the present study was the notion that, in combined Cu/Cd exposed fish, the changes in Na and Ca levels could not be explained by synergism or addition of the effects observed in single metal exposed fish

Plasma cortisol levels were increased in Cu-exposed fish, but an increase was not observed in the Cu/Cd co-exposed fish It is argued that the absence of this cortisol response contributes to an inadequate recovery of ionic disturbances in the Cu/Cd co-exposed fish

INTRODUCTION

Freshwater fish take up most of the ions necessary for growth and ionic homeostasis from the water via the gills (Eddy, 1982) Branchial function is very sensitive to environmental stressors.

During exposure to waterborne heavy metals, the active uptake of ions from the water is initially impaired (Laurén and McDonald, 1987, Verbost *et al* , 1989), leading to disturbances of ionic homeostasis (McDonald *et al* , 1989, Pratap *et al* , 1989, McDonald and Wood, 1993) The physiological disturbances caused by Cu and Cd appear to be metal-specific In several fish species, sublethal exposure to Cu, an essential metal, primarily affects plasma sodium and chloride concentrations (Stagg and Shuttleworth, 1982, Laurén and McDonald, 1985, Reid and McDonald, 1988, McDonald *et al* , 1989, Muñoz *et al* , 1991) Impaired ionic regulation was also reflected in whole body ion-composition (Laurén and McDonald, 1987, Sayer *et al* , 1991) Exposure to Cd however, a non-essential metal, in most cases affects Ca metabolism (Reid and McDonald, 1988, Fu *et al* , 1989), but has occasionally been reported to affect the sodium balance (McCarty and Houston, 1976, Giles, 1984)

After some time, fish demonstrate the ability to restore whole body and plasma ion concentrations after ionic disturbances due to sublethal metal exposure (Fu *et al* , 1989, McDonald *et al* , 1989) This process of restoring normal ionic regulation, which is interpreted to indicate acclimation, requires energy Cortisol regulates both energy mobilization and ion homeostasis in fish, and these functions are of particular importance during adaptation to stressors (Mazeaud *et al* , 1977) Exposure to sublethal concentrations of Cu or Cd has been shown to result in a rise of plasma cortisol levels in several species (Donaldson and Dye, 1975, Fu *et al* , 1989, Muñoz *et al* , 1991), which has been interpreted as an adaptive response intended to correct the ionoregulatory and metabolic imbalance (Mazeaud *et al* , 1977, Haux and Larsson, 1984, Laurén and McDonald, 1986, Muñoz *et al* , 1991, Perry *et al* , 1992)

Because of adaptive regulation mechanisms, enabling fish to respond to changed conditions, fish can usually tolerate moderately increased concentrations of one heavy metal (McDonald and Wood, 1993) However, environmental pollution is not limited to one heavy metal, and generally fish are exposed to mixtures of heavy metals Yet, in spite of the amount of data published on the effects of waterborne exposure of Cu and Cd singly, information on the effects of Cu/Cd mixtures on aquatic organisms is limited and not uniform In seawater herring, combined Cu/Cd exposure had an additive effect on embryonic survival and hatching succes (Westernhagen *et al* , 1979), and a more-than-additive effect of acutely lethal Cu/Cd concentrations was observed in a study with the freshwater amphipod *Gammarus lacustris* of deMarch (1988) In a previous study on metal accumulation (Pelgrom *et al* , 1994), we observed significant differences in whole body Cd burden in Cu/Cd co-exposed fish compared with fish exposed to Cd only The question therefore arises, whether fish are able to cope with the effects of combined Cu/Cd exposure In this study, juvenile tilapia, *Oreochromis mossambicus*, were exposed for 6 days to sublethal concentrations of Cu or Cd and combinations of these metals To examine the adaptive performance of fish in response to single and combined metal exposures, plasma cortisol levels were related to whole body and plasma ion concentrations of the experimental fish

MATERIALS AND METHODS

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from our laboratory stock Fish were held, from 9 days after hatching, under artificial freshwater conditions with undetectable Cu and Cd concentrations (detection levels below 0 1 and 0 01 $\mu g\ l^{-1}$ respectively) The artificial freshwater consisted of demineralized water supplemented with 1 3 mM $NaHCO_3$, 0 5 mM $CaCl_2$, 0 06 mM KCl and 0 2 mM $MgCl_2$, at pH 7 8 Composition and preparation of the water was based on the european community (EEC) instructions for artificial water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1 Acute toxicity for fish) Water was continuously aerated, filtered and refreshed by means of flow through The light/dark regime was 12/12 h and the water temperature 26°C Fish were fed commercial tropical fish food Tetramin™, 2% $^{dw}/_{ww}$ per day The Cu and Cd contents of the food were 9 86 ± 0 16 μg Cu g^{-1} and 0 22 ± 0 01 μg Cd g^{-1} dry food (means ± SE, n=10) (Pelgrom *et al* , 1994)

Experimental design

Three days before the start of the experiment, 11 groups of 15 immature fish (weighing 1-2 grams, 2 months old) were placed randomly in 3 2 l small aquaria (flux chambers) filled with artificial freshwater During the acclimation period and the metal exposure, fish were fed 2% $^{dw}/_{ww}$ Tetramin™ per day The experimental design was comparable with that of the whole body flux experiments performed by Pelgrom *et al* (1995) Briefly, the exposure period started with the connection of each aquarium to reservoirs filled with artificial freshwater with well-defined Cu and Cd concentrations (added as nitrate, Spectrosol, BDH, U K) The metal concentrations, randomly distributed over the aquaria, were raised gradually to 50, 100 or 200 $\mu g\ l^{-1}$ Cu, 20, 35 or 70 $\mu g\ l^{-1}$ Cd or 50+20, 100+35, 200+70 $\mu g\ l^{-1}$ Cu+Cd Two groups served as controls The metal concentrations in the water (monitored at least once daily by means of a flameless atomic absorption spectrometer [AAS, Philips PU 9200] connected with an electrothermal atomizer [Philips PU 9390X]), deviated not more than 5% of the nominal metal concentrations After 6 days of metal exposure, fish were anaesthetized with phenoxy-ethanol (1 400) The metal exposures had no effect on the time necessary for the fish to become anaesthetized Seven anaesthetized fish were killed in dry ice/acetone, and whole body Na, Ca and total phosphate (P) concentrations were determined (Pelgrom *et al* , 1994) After recovery from anaesthesia, the remaining fish were returned to their own aquarium On the next day, the fish were anaesthetized (phenoxy-ethanol), and blood from the caudal vessels was taken by means of heparinized mini-capillaries (Hirschmann) After centrifugation, Na, Cl, Ca and cortisol concentrations were determined in the plasma Also whole body Na, Ca and P concentrations were determined

Blood plasma measurements

Na concentrations in the plasma were determined with a flame-photometric Auto Analyzer (Model IV, Technicon). The Cl concentrations were determined spectrophotometrically via the formation of ferrothiocyanate (O'Brien, 1962). Total plasma Ca concentrations were measured by means of the cresolphtalein complexone method (Sigma Diagnostics). Cortisol levels were determined by Radio Immuno Assay (RIA) as described by Balm *et al.* (1994).

Whole body measurements

Fish were weighed and lyophilized. After determination of the dry weight, the fish were completely destructed by the following procedure: 1h at 40°C after addition of 150 μl 65% HNO_3 (Merck, ultrapur), 1h at 75°C after addition of 200 μl HNO_3 and subsequently dried overnight at 110 °C. The samples were dissolved in 4 ml 0.1% HNO_3 (final concentration) and stored until ion analyses. Whole body Cl concentrations could not be determined in HNO_3-destructed fish. Whole body Na, Ca and P contents were determined by using an Inductive Coupled Plasma (ICP) atomic emission spectrometer (Plasma IL 200, Thermo Electron U.S.A.).

Statistics

Data are presented as means $\pm$ SE. Differences between groups were tested for significance by Student's *t*-test for unpaired observations. The two control groups did not differ significantly for all parameters tested, and were therefore pooled for statistical comparisons. Significant differences between metal-exposed and control fish are indicated by asterisks, whereas significant differences between single metal exposed and Cu/Cd co-exposed groups are indicated by circles, with: * or ● : $P < 0.05$; ** or ●● : $P < 0.02$; *** or ●●● : $P < 0.01$ and **** or ●●●● : $P < 0.001$.

RESULTS

Sodium and chloride

The concentrations and ratios of Na and Cl in the plasma of fish exposed for 6 days to sublethal concentrations of Cu, Cd or Cu+Cd are shown in Fig. 1. Single and combined metal exposure to the highest metal concentrations tested, significantly decreased the plasma Na concentration (Fig. 1A). In fish exposed to 200 μg $Cu.l^{-1}$ + 70 μg $Cd.l^{-1}$, the decrease in plasma Na was similar to the effect observed in fish exposed to 200 μg $Cu.l^{-1}$ singly. Compared with controls, plasma Cl concentrations were significantly lower in all Cu-exposed groups (Fig. 1B). Decreased plasma Cl

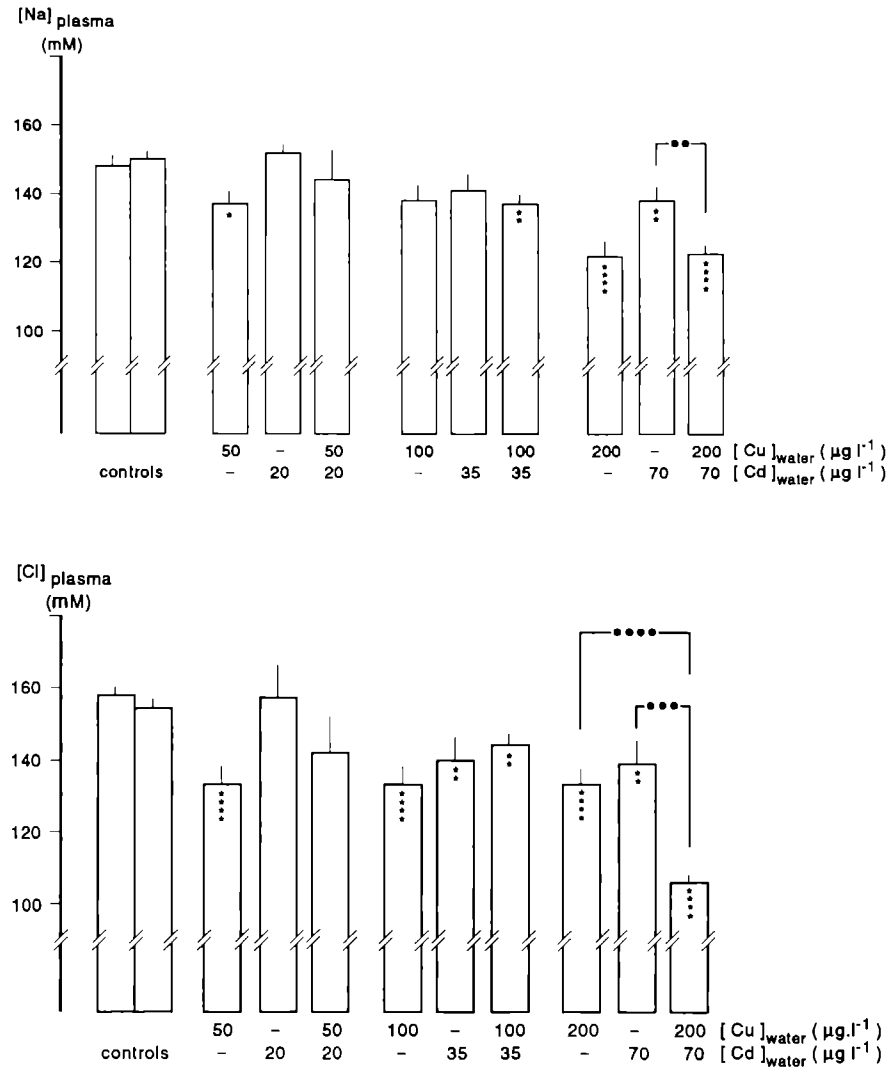

Figure 1A, B. *Concentrations of Na (A, upper panel) and Cl (B, lower panel) (in mM) in plasma of immature tilapia exposed for 6 days to sublethal concentrations of Cu and Cd, singly and in combination. Data are means ± SE (n=5) Significant differences between control and experimental groups of fish are indicated by asterisks in the bars, whereas significant differences between single and combined metal exposed fish are indicated by closed circles between the bars.*

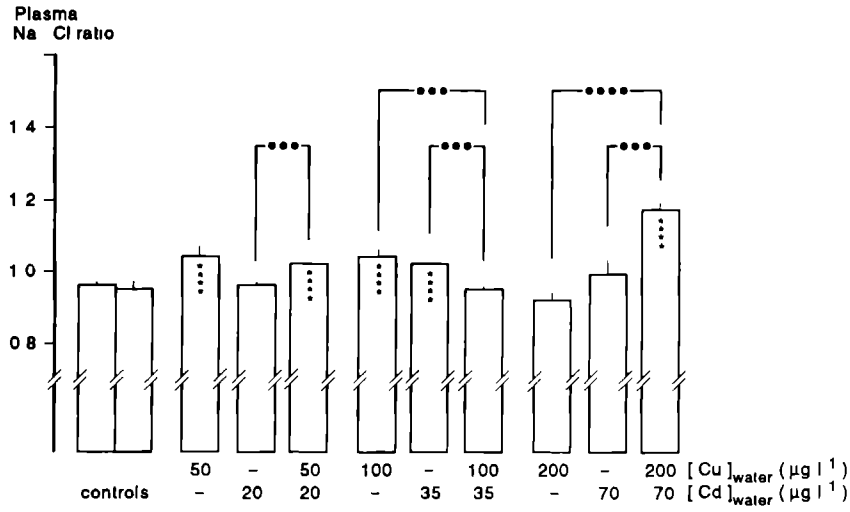

Figure 1C. Ratios of Na/Cl in plasma of immature tilapia exposed for 6 days to sublethal concentrations of Cu and Cd, singly and in combination Data are means ± SE (n=5) Significant differences are indicated as described in Fig 1A,B

concenterations were also observed in fish exposed to 35 and 100 µg Cd l^{-1}, singly and in combination with Cu. In fish exposed to the highest Cu/Cd combination, the plasma Cl concentration was significantly lower than in single Cu or Cd exposed fish

Na/Cl ratios were increased in fish exposed to 50 and 100, but not 200, µg Cu l^{-1} (Fig 1C) In Cd-exposed fish, an increase in the Na/Cl ratio was only observed in the 35 µg Cd l^{-1} group The Na/Cl ratios observed in combined Cu/Cd exposed fish were significantly different from the effects observed in the single metal exposed fish, particularly in fish co exposed to the highest metal concentrations In these combined Cu/Cd exposed fish, the Na/Cl ratio was significantly increased, whereas the single metal exposed fish showed no change in the Na/Cl ratio

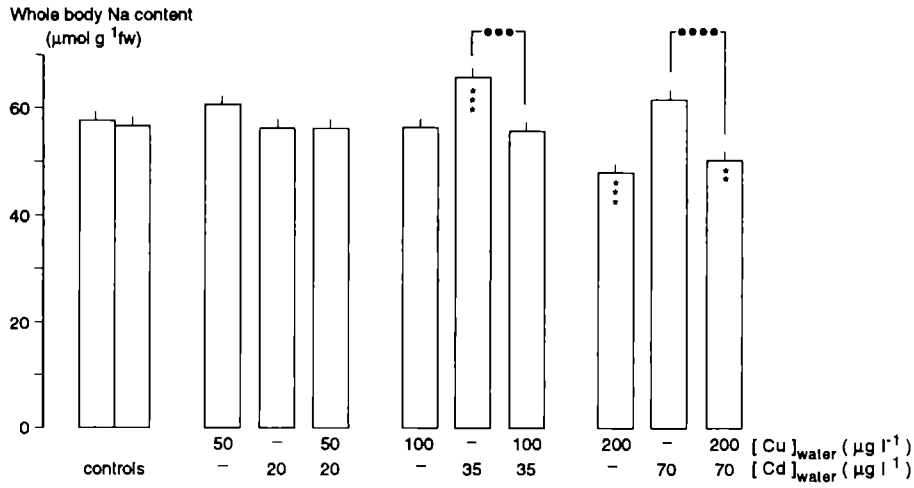

Figure 2. Whole body Na contents (in µmol per gram fresh weight) of immature tilapia exposed for 6 days to sublethal concentrations of Cu and Cd, singly and in combination Data are means ± SE of at least 9 fish Significant differences are indicated as described in Fig 1

Metal induced differences in whole body Na concentrations were few (Fig. 2). Only in fish exposed to 200 µg Cu l^{-1} and to 200 µg Cu.l^{-1} + 70 µg Cd.l^{-1} whole body Na was significantly decreased, whereas in fish exposed to 35 µg Cd l^{-1} the Na concentration was increased. At the two higher metal concentrations tested, whole body Na content in combined Cu/Cd exposed fish was comparable with those in fish exposed to Cu, but not to Cd

Calcium

Changes in plasma Ca concentrations caused by Cu and/or Cd are shown in Fig 3 Exposure to 50 and 100 µg Cu l^{-1} resulted in a slight, although significant, increase and decrease respectively in plasma Ca levels Cd exposure to 20, 35 and 70 µg l^{-1} Cd resulted in an increase, no effect and decrease of the plasma Ca concentrations respectively. In fish exposed to 70 µg Cd.l^{-1}, plasma Ca levels were even decreased to half of the concentration observed in control fish. Compared with controls, in Cu/Cd co-exposed fish the plasma Ca concentrations were significantly decreased in all three combinations tested Plasma Ca levels in co-exposed fish were also significantly different from the levels in Cu (except 200 Cu) or Cd exposed fish, although apparently not in an additive or synergistic way

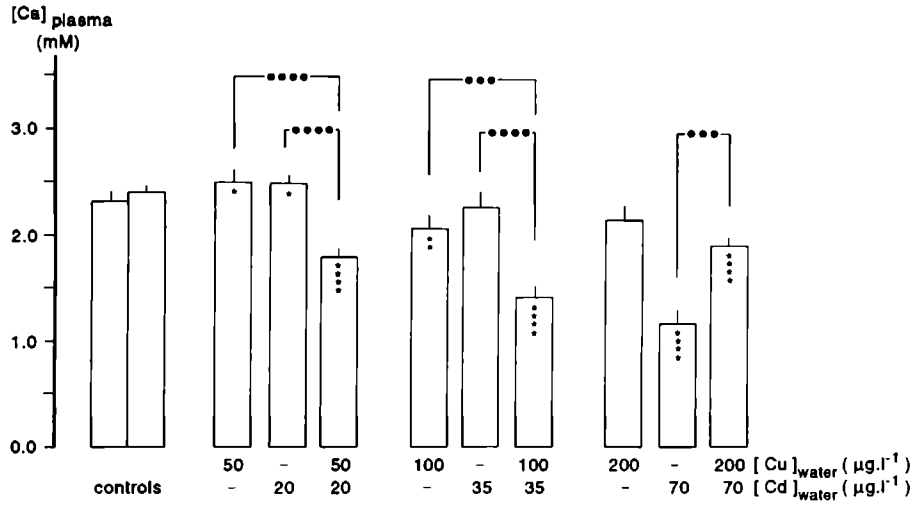

Figure 3. Total Ca concentrations (in mM) in plasma of fish exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination. Data are means ± SE (n=5). Significant differences are indicated as described in Fig.1.

The whole body Ca content (Fig.4) was only affected in fish singly exposed to the highest Cu or Cd concentrations, whereas in combined Cu/Cd exposed fish no effect was observed. Compared with fish exposed to 20 or 35 μg $Cd.l^{-1}$, whole body Ca levels in the combined Cu/Cd exposed fish were significantly lower and higher respectively. Metal exposure had no effect on whole body P content (Table 1), except in fish exposed to 35 μg $Cd.l^{-1}$. Metal-induced changes in the whole body Ca/P ratio (Table 1) were solely observed in fish exposed to 35 μg $Cd.l^{-1}$ or 200 μg $Cu.l^{-1}$ singly (Table 1).

Cortisol

Plasma cortisol concentrations are shown in Fig. 5. In Cu-exposed fish, but not in Cd-exposed fish, the plasma cortisol levels were increased in relation to the Cu concentration in the water. The Cu-induced increase in plasma cortisol concentration was absent in the corresponding Cu/Cd co-exposed groups of fish.

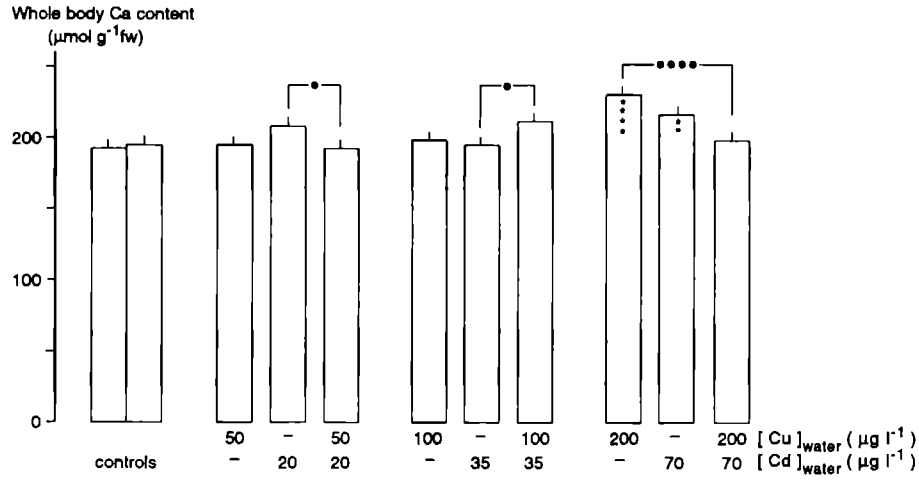

Figure 4. Whole body Ca contents (in µmol per gram fresh weight) of immature tilapia exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination Data are means ± SE of at least 9 fish Significant differences are indicated as described in Fig 1

DISCUSSION

From the results presented in this study, two major conclusions are drawn. Firstly, in many instances the effects observed on ion composition in combined Cu/Cd exposed fish were significantly different from those observed in single metal exposed fish Our results indicate that the effects observed during combined Cu/Cd exposure cannot be predicted from the effects observed in single metal exposed fish Secondly, combined Cu/Cd exposure abolished the Cu-induced increase in plasma cortisol levels.

Sodium and chloride

The observed effects of single metal exposure on ionic composition are in line with our previous results and those of other investigators, who reported that the Na balance was most prominently affected by Cu, while Cd had a less pronounced effect (McDonald *et al* , 1989; Pelgrom *et al.*,

	Whole body P content (µmol g^{-1} fw)	Whole body Ca:P ratio
controls	222 ± 8	0 92 ± 0 05
	219 ± 7	0 84 ± 0 03
50 Cu	230 ± 5	0 84 ± 0 03
20 Cd	225 ± 5	0 94 ± 0 04
50 Cu + 20 Cd	225 ± 7	0 87 ± 0 03
100 Cu	222 ± 5	0 91 ± 0 04
35 Cd	252 ± 8***	0 81 ± 0 04**
100 Cu + 35 Cd	214 ± 5	0 99 ± 0 03
200 Cu	210 ± 5	1 10 ± 0 02****
70 Cd	231 ± 6	0 93 ± 0 03
200 Cu + 70 Cd	220 ± 7	0 94 ± 0 03

Table 1. *Whole body P contents (in µmol per gram fresh weight) and whole body Ca/P ratios of immature tilapia exposed for 6 days to sublethal Cu and Cd concentrations, singly and in combination. Data are means ± SE of at least 9 fish. Significant differences are indicated as described in Fig. 1.*

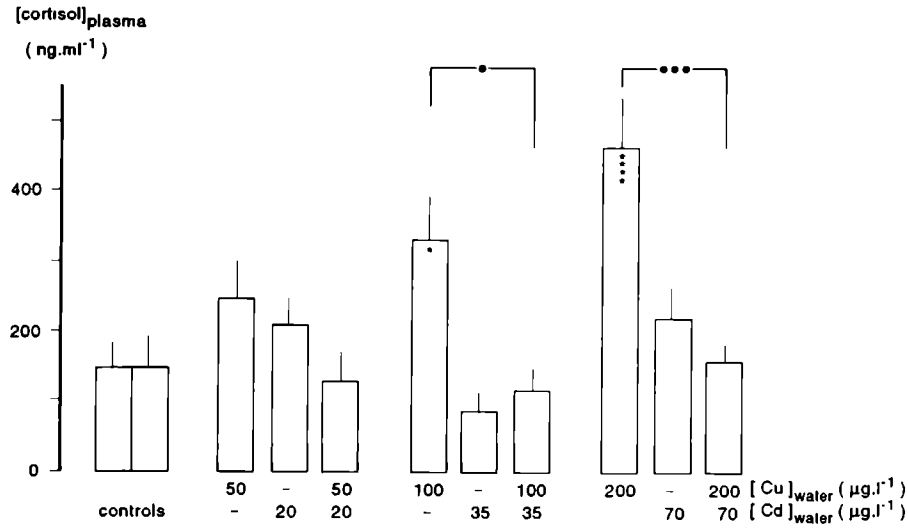

Figure 5. *Plasma cortisol levels (in nanogram per ml) of immature tilapia exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination. Data are means ± SE (n=5) Significant differences are indicated as described in Fig. 1.*

1995) A Cu-induced decrease in plasma Na concentration has also been reported for flounder (Stagg and Shuttleworth, 1982) and rainbow trout (Laurén and McDonald, 1985, Reid and McDonald, 1988, Muñoz *et al* , 1991) In a previous study with mature tilapia, we observed a decreased plasma Na concentration after exposure to 200 μgCu l^{-1} (Pelgrom *et al* , 1995). Upon Cd exposure, either no effect (Smith *et al* , 1976; Christensen *et al* , 1972) or, compared with Cu exposure, less pronounced decreases of plasma Na concentrations were observed (Giles, 1984, Reid and McDonald, 1988, Fu *et al* , 1989) In our study, not only single exposure to 200 μgCu l^{-1}, but also co-exposure to 200 μgCu l^{-1} and 70 μgCd l^{-1} resulted in a decreased plasma Na concentration No other data are available concerning the effects of Cu/Cd co-exposure on plasma ion composition in fish, which illustrate the necessity for further study.

The observed decreases of plasma Na levels in fish exposed to 200 μg Cu l^{-1} and 200 μg Cu l^{-1} + 70 μg Cd l^{-1} were also reflected in the whole body Na concentration Laurén and McDonald (1987) also observed a Cu-induced decrease in whole body Na concentration after 24h The Cu induced disruption of the Na balance in their fish was reflected in plasma concentrations and in the whole body level Laurén and McDonald (1986) previously concluded that about 75% of the total body Na content may be considered as exchangeable Consequently, major Cu-induced changes in the plasma Na concentration will be readily reflected in the whole body Na concentration

In general, plasma Na and Cl tend to be similarly affected by waterborne toxicants (McDonald *et al* , 1989) In our study, exposure to Cu as well as to Cd also resulted in a decreased plasma Cl concentration Both Cu-induced (McKim *et al* , 1970, Stagg and Shuttleworth, 1982, Laurén and McDonald, 1985) as well as Cd-induced (Christensen *et al* , 1972, Giles, 1984) reduction of plasma Cl concentrations have been described In fish exposed to the two highest combinations of Cu and Cd, plasma Cl levels were also decreased In fish exposed to a combination of 200 μg Cu l^{-1} + 70 μg Cd l^{-1}, the plasma Cl concentration was even significantly lower than in Cu or Cd exposed fish, resulting in more than-addition of the effects of the metals separately

As a consequence of their electroneutral coupling (Perry and Laurent, 1993), reduction of Cl uptake is believed to reduce HCO_3 excretion, while reduced Na uptake will affect Na^+/H^+ exchange Therefore, an imbalance in the plasma Na/Cl ratio has been interpreted to reflect a disturbed acid-base regulation (Perry and Laurent, 1993) In the present study, metal-induced changes of the Na/Cl ratios were observed In fish exposed to combinations of Cu and Cd, plasma Na/Cl ratios were significantly different from the ratios in single metal exposed fish Disturbance in the Na/Cl ratio was most obvious in fish co-exposed to 200 μg Cu l^{-1} + 70 μg Cd l^{-1}, whereas in Cu or Cd exposed fish Na/Cl ratios were not affected Therefore, the change in Na/Cl ratio in combined Cu/Cd exposed fish cannot be described only by additive or synergistic action of the metals

Calcium

In addition to changed plasma Na and Cl concentrations, waterborne metal exposure also affected Ca-homeostasis In fish exposed to 70 μg Cd l^{-1} plasma Ca concentrations were decreased most prominently to half of the control plasma Ca values It has been demonstrated in rainbow trout, that Cd-induced effects on Ca apparently result from a specific reduction of Ca^{2+} uptake via the gills (Verbost *et al* , 1989, Reid and McDonald, 1988) Cu or Cd induced impairment of the Ca balance has also been reported for rainbow trout (Giles, 1984, Reid and McDonald, 1988), carp (Koyama and Itazawa, 1977) and tilapia (Fu *et al* , 1989, Pelgrom *et al* , 1995) Effects of Cu/Cd co-exposure on the ion-homeostasis have not been studied before In all Cu/Cd co-exposed groups of fish we observed significantly lower plasma Ca concentrations than in controls Even more striking was the observation that the decrease of plasma Ca in Cu/Cd co-exposed fish differed from that observed in single metal exposed fish In the presence of Cu, even low concentrations of Cd reduced plasma Ca levels, whereas this effect was absent in fish exposed to Cd alone It should be noticed that, unlike other studies on Cd exposure (Giles, 1984, Fu *et al* , 1989), these effects on plasma Ca concentrations were observed in immature fish

Changes in the Ca concentration of the plasma were not reflected in the whole body Ca concentration Reid and McDonald (1988) have demonstrated for rainbow trout, that the Ca turnover, as a percentage of the whole body Ca concentration, is about 100 fold lower than the Na turnover In teleosts, plasma Ca represents less than 3-6% of the total body Ca (Flemming, 1974) Therefore, even a decrease to half of the control plasma Ca concentration, as observed in the present study, will not be readily reflected in whole body Ca levels In line with this assumption and with our results, Fu *et al* (1989) observed no Ca mobilization from bone in hypocalcemic tilapia exposed to waterborne Cd In a previous study on Cu, we observed in fish exposed to 50 or 200 μg Cu l^{-1} no changes in whole body Ca fluxes (Pelgrom *et al* , 1995) Together with the observed effects on plasma and whole body Ca concentrations, this illustrates that conclusions on metal-induced effects on Ca should preferably not be based on one of the three parameters solely (whole body flux, plasma Ca or whole body Ca)

Metal exposure had also no effect on whole body P concentration As a result, in the present study the Ca/P ratios are not different in metal exposed fish For Cd exposed fish, comparable results have been reported by Fu *et al* (1990)

Cortisol

In the present study, plasma cortisol levels were determined to gain insight in their adaptive performance to counteract the metal induced disturbance of ion homeostasis Plasma cortisol levels in the control fish were relatively high, although within the range of plasma cortisol levels observed in mature control fish Data on plasma cortisol levels in young fish are scarce (Pottinger

and Mosuwe, 1994), and we cannot therefore exclude the possibility that the control cortisol values measured may be characteristic for the developmental stage of the species used presently We do not assume that they indicate stressful experimental conditions, because the high net sodium fluxes observed in fish kept under these conditions (Pelgrom *et al* , 1995) are reliable indications of well being in this species (Dharmamba and Maetz, 1972)

Plasma cortisol levels were elevated in fish exposed to Cu, but not to Cd, which confirm observations by Donaldson and Dye (1975) on sockeye salmon and Muñoz *et al* (1991) on rainbow trout More important, however, is the absence of a Cu-induced cortisol response in Cu/Cd co-exposed fish, in which Cd co-accumulated with Cu (Pelgrom *et al* , 1994) Obviously, the cortisol response induced by Cu alone was restrained in the presence of Cd, although Cd itself did not affect plasma cortisol levels It is unlikely that the whole body Cu concentration is of sole importance for the rise in cortisol, because we previously demonstrated that whole body Cu accumulation did not differ between Cu and Cu/Cd exposed fish (Pelgrom *et al* , 1994) Cortisol is known to affect osmotic and ionic regulation, and is also released in the circulation as part of the stress response of fish (Mazeaud *et al* , 1977) The absence of a cortisol response might be interpreted to indicate either succesful adaptation or a defect in the stress response This latter could result from a direct action of the metals (e g metal effects in the central nervous system) or alternatively could be associated with disturbed homeostasis Obviously, the absence of a cortisol response in our Cu/Cd co-exposed fish cannot be related to succesful adaptation given the present observations and our previous results showing altered whole body water content in both Cu- as well as Cu/Cd co-exposed fish (Pelgrom *et al* , 1994) In the present study, the plasma ion balance of Cu/Cd co-exposed fish was significantly disturbed after 6 days of metal exposure, even more than in fish exposed to Cu singly Therefore, we interpret the ionic disturbances in Cu/Cd co exposed fish as major consequences of the absence of a cortisol response, leading to impairment of the cortisol mediated adaptive responses in these fish Cortisol is known to induce proliferation of chloride cells (Perry *et al* , 1992) in the branchial and opercular epithelia of fish This has been interpreted as a reaction of the fish to compensate for ion-losses (Fu *et al* , 1989, Muñoz *et al* , 1991, Perry *et al* , 1992, Wood, 1992) In addition, cortisol also regulates carbohydrate and protein metabolism in fish (Mazeaud *et al* , 1977) In a previous study, we observed an increase in the plasma glucose concentration and the number of opercular chloride cell numbers in Cu exposed fish (Pelgrom *et al* , 1995) This increase in plasma glucose concentration and chloride cell numbers was probably mediated by an increase in cortisol release of the head kidney Pickering and Pottinger (1987) and McMaster *et al* (1994) observed a suppressed cortisol response in salmonids and white sucker experiencing more than one form of stress or exposed to a complex mixture of contaminants Our results show that a suppression of a cortisol response during exposure to combinations of environmental stressors may be a more general phenomenon than recognized previously This demonstrates that plasma

cortisol levels are not always a reliable index of environmental stress

In conclusion, this study shows that effects on ionic composition and plasma cortisol concentrations in combined Cu/Cd exposed fish are not predictable from observations of single metal exposed fish Since heavy metal contamination of freshwaters usually involves mixtures, our results demonstrate the need for further study on the the adverse effects of Cu/Cd combinations on fish

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CHAPTER 5

CALCIUM FLUXES IN JUVENILE TILAPIA, *OREOCHROMIS MOSSAMBICUS*, EXPOSED TO SUBLETHAL WATERBORNE Cd, Cu OR MIXTURES OF THESE METALS.

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ABSTRACT

In juvenile tilapia the effects of waterborne Cu, Cd and combinations of these metals on whole body Ca fluxes were studied, using ^{45}Ca as tracer. The maintenance of Ca-homeostasis in fish is crucial throughout life, and in young fish, Ca is also critical for growth. Single metal exposure had no effect on Ca fluxes after 6 days of exposure In fish co-exposed to 200 μg Cu.l^{-1} + 70 μg Cd l^{-1} however, Ca influx was significantly decreased, whereas Ca efflux was not affected. As a result, the net flux was decreased. Since the effect on Ca fluxes observed in Cu/Cd co-exposed fish could not have been predicted from the effects of single metal exposures, this study underscores the impact of interactions between toxicants Since natural freshwaters are commonly polluted by mixtures of metals, interactions between toxicants are important in risk assessment of heavy metals

INTRODUCTION

The gills of freshwater fish represent the largest part of the total body surface area At the branchial epithelium the distance between the water and the blood is only a few microns (Hughes, 1984) This, together with their crucial role in physiological homeostasis and energy metabolism (Lyndon, 1994) and their delicate structure, make the gills a sensitive target organ for waterborne pollutants (Perry and Laurent, 1993). Many toxicants, such as waterborne heavy metals, not only enter the organism through the gills (Playle *et al* , 1992), but also exert their primary toxic

effects on the branchial epithelium by interfering with one or more of the essential physiological processes, such as calcium (Ca) transport (Wood, 1992) It appears that dysfunctions in branchial ionoregulatory mechanisms underlay most of the physiological effects of sublethal heavy metal exposure (Laurén and McDonald, 1985, 1986, McDonald and Wood, 1993) Exposure to waterborne copper (Cu) or cadmium (Cd), metals which are commonly present in polluted waters, has been shown to affect ion composition of the blood (Larsson *et al* , 1981, Giles, 1984, Laurén and McDonald, 1987, Reid and McDonald, 1988, Fu *et al* , 1989, Pelgrom *et al* , 1995[a], 1995[b]) However, large differences exist in literature concerning exposure conditions such as water quality, waterborne metal concentrations and species studied (Laurén and McDonald, 1986, 1987, Reid and McDonald, 1988) Only for trout, a model for a Cd-induced inhibition of Ca^{2+} uptake has been presented (Verbost *et al* , 1987, 1989)

Metal contamination of aquatic environments usually involves mixtures of heavy metals and therefore, it is imperative to study metal interactions in fish to get more insight in the effects of mixtures of contaminants Most studies on metal interactions, including Cu/Cd interactions, are *in vitro* studies with cell cultures or deal with tissue studies in mammals (Ashby *et al* , 1980, Meshitsuka *et al* , 1982, Sharma *et al* , 1985) In the present study, we investigated metal interactions *in vivo*, to further substantiate the relevance of these interactions for physiological processes in organisms As a first step towards a more realistic approach to study the impact of heavy metal contamination (Lourdes *et al* , 1993), we previously investigated interactions between the two metals Cu and Cd To the best of our knowledge, these were the first investigations specifically designed to study the effects of sublethal Cu/Cd co-exposure on freshwater fish ionoregulatory mechanisms In these studies, we observed that metal interactions take place during accumulation in the gills, and disturbances in the plasma ion composition were most prominent in Cu/Cd co-exposed fish (Pelgrom *et al* , 1995[b], 1995[c]) The present study addresses Ca regulation by this tissue, because the maintenance of Ca-homeostasis in fish is crucial throughout life In young fish, Ca is also critical for growth (Reader *et al* , 1988) Ca is mainly taken up via the gills (Flik *et al* , 1993) Previous studies (Verbost *et al* , 1987, Reid and McDonald, 1988, Fu *et al* , 1989) have shown that Cd mainly impairs Ca-homeostasis, whereas Cu negatively affects sodium (Na) balance in fish, and has only minor effect on Ca-balance It is not known what the effects of combined Cu/Cd exposures are on Ca homeostasis in fish

Recently, Wood (1992) has reviewed the advantages of flux studies to investigate the effects of waterborne metal exposure By measuring ion fluxes the primary toxic events are quantified which is substantiated by the fact that these branchial toxic insults of ionic uptake not necessarily result in altered blood ion composition, another parameter frequently used as an index of metal toxicity However, most flux studies concern Na and Cl fluxes, and information on Ca is limited We therefore determined whole body Ca influx, efflux and net flux in fish exposed during 6 days to sublethal concentrations of Cu, Cd and Cu+Cd

MATERIALS AND METHODS

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from our laboratory stock. Fish were held, from 9 days after hatching, under artificial freshwater conditions with undetectable Cu and Cd concentrations (detection levels below 0.1 and 0.01 $\mu g.l^{-1}$ respectively). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM HNO_3, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8. Composition and preparation of the water was based on the European Community (EEC) instructions for artificial water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1· Acute toxicity for fish). Water was continuously aerated, filtered and refreshed by means of flow-through The light/dark regime was 12/12 h and the water temperature 26°C. Fish were fed commercial tropical fish food Tetramin™, 2% dw/ww per day. The Cu and Cd contents of the food were: 9.86 ± 0.16 $\mu g\ Cu.l^{-1}$ and 0.22 ± 0 01 $\mu g\ Cd.l^{-1}$ dry food (means ± SE; n=10) (Pelgrom *et al.*, 1994).

Whole body Ca fluxes

Three days before the start of the experiments, 12 groups of 9 juvenile fish (weighing 1-2 g., about 2 months old) were placed randomly in 3.2 l flux chambers filled with artificial freshwater. Throughout the experimental period, fish were fed daily (2% dw/ww Tertramin™), food was eaten within 1 minute. During the acclimation period, the water in the flux chambers was continuously aerated and refreshed by means of a flow-through-system (flow rate 0.24 $l.h^{-1}$; multi-channel peristaltic pump, Watson Marlow) The experimental conditions were the same as tested and described for the whole body flux experiments in Pelgrom *et al.* (1995[a]). Briefly: the exposure period started after connection of each flux chamber to reservoirs filled with artificial freshwater with well-defined Cu and Cd concentrations (added as nitrate, Ultrapur, Spectrosol, BDH, England). Metal concentrations, randomly distributed over the flux chambers (3 to 4 flux chambers per experimental group; 12 flux chambers simultaneously), were raised gradually (Pelgrom *et al.*, 1995[b]) to 50, 100 or 200 $\mu g.l^{-1}$ Cu, 20, 35 or 70 $\mu g.l^{-1}$ Cd or 50+20, 100+35, 200+70 $\mu g\ l^{-1}$ Cu+Cd by flow through (flow rate: 4 h 0 90 $l.h^{-1}$ followed by a flow rate of 0.24 $l\ h^{-1}$). Prior to the experiments with combined Cu/Cd exposures, we performed an experiment with 20 and 70 $\mu g.l^{-1}$ Cd Three groups served as controls (no Cu and Cd added) during each experiment. The metal concentrations in the water, monitored at least once a day by means of flameless atomic absorption spectrometer (AAS, Philips PU 9200), did not deviate more than 5% of the nominal metal concentrations. After 6 days of metal exposure, Ca^{2+} influx and efflux were determined by means of ^{45}Ca

For measurement of Ca^{2+} influx, 0.75 $MBq.l^{-1}$ $^{45}CaCl_2$ (Amersham, England) was added to

the flux chambers, and flow-through was stopped After 45 minutes of tracer exposure, water samples were taken, and fish were quickly anaesthetized with phenoxy-ethanol (1 400) After rinsing, 4 fish per group were immediately killed in dry ice/acetone for whole body Ca^{2+} influx determination

To investigate Ca^{2+} efflux, the remaining 5 fish of each flux chamber were intraperitoneally injected with 0 12 MBq $^{45}CaCl_2$ (Pelgrom *et al* , 1995[a]) After recovery from anaesthesia, fish were put back in their flux chambers which in the mean time had been rinsed and filled with radiotracer-free experimental water Subsequently, the flux chambers were continuously flowed-through overnight (0 24 $l.h^{-1}$). During efflux measurement, the water-flow was stopped, and tracer appearance in the water was monitored during 4 h to assess Ca^{2+}-efflux After this period, fish were anaesthetized, and blood was taken from the caudal vessels after severing the tail Plasma radiotracer concentrations were determined in triplicate for each fish

Whole body ^{45}Ca was determined after tissue digestion with hydrogen peroxide (30%) Scintillation fluid was added to the digested fish, water and blood samples, and $^{45}Ca^{2+}$ was determined by means of a liquid scintillation counter (Pharmacia Wallac 1410)

Ca^{2+}-influx was calculated on the basis of the total body radioactivity per hour of exposure to $^{45}Ca^{2+}$, and the respective mean tracer specific activities in the water (Pelgrom *et al* , 1995[a]) Ca^{2+}-efflux was calculated from the tracer activity in the water and the specific Ca activities in the plasma In the exposed fish, the plasma total Ca concentrations were determined to calculate the specific Ca activities in the plasma (cresolphtalein complexone method, Sigma Diagnostics) The net flux is given as the calculated difference between the average influx and the efflux for each flux chamber

Statistics

Data are presented as means ± SE The Mann Whitney *U* test was applied for statistical evaluation Significant differences between control and metal exposed groups are indicated by asterisks, with * $P < 0\ 05$, ** $P < 0\ 02$, *** $P < 0\ 01$ and **** $P < 0\ 001$

Significant differences between single Cu and Cu/Cd co exposed fish are indicated by **a** ($P < 0\ 02$), whereas significant differences between single Cd and Cu/Cd co-exposed fish are indicated by **b** ($P < 0\ 02$)

RESULTS

Cd

Exposure during 6 days to 20 or 70 $\mu g.l^{-1}$ Cd had no effect on Ca^{2+}-influx, -efflux and net-flux (Fig. 1). Concomitantly determined Na^+-fluxes in these Cd exposed fish also showed no effect on Na^+ fluxes (data not shown).

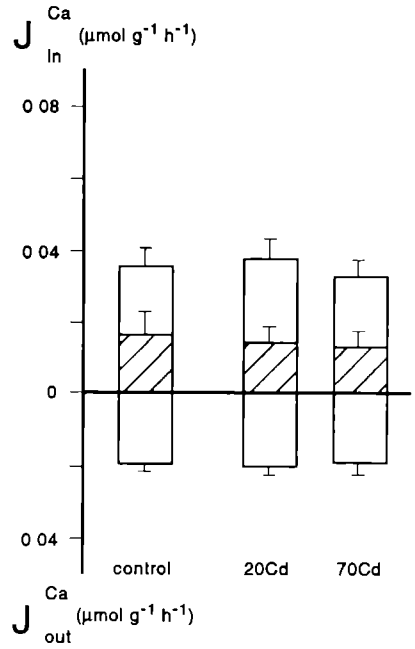

Figure 1. Ca^{2+}-influx, indicated by J_{in} (open bars above zero, n=16), Ca^{2+}-efflux, indicated by J_{out} (open bars below zero, n=4) and net Ca^{2+} flux (shaded bars, n=4) of fish exposed during 6 days to 20 or 70 µg Cd l^{-1} or no Cd (controls)

Cd and Cu

Figures 2 and 3 show Ca^{2+}-influx, -efflux and net-flux of fish after single and combined exposure to 50 $\mu g\ l^{-1}$ Cu and 20 $\mu g\ l^{-1}$ Cd (Fig 2) or 200 $\mu g.l^{-1}$ Cu and 70 $\mu g\ l^{-1}$ Cd (Fig.3). Single metal exposure had no effect on Ca-fluxes Exposure to the highest Cu/Cd combination however resulted in significantly reduced Ca-influx compared to controls and single metal exposed fish, whereas Ca efflux was not affected. As a result, the net Ca^{2+}-flux in Cu/Cd co-exposed fish was

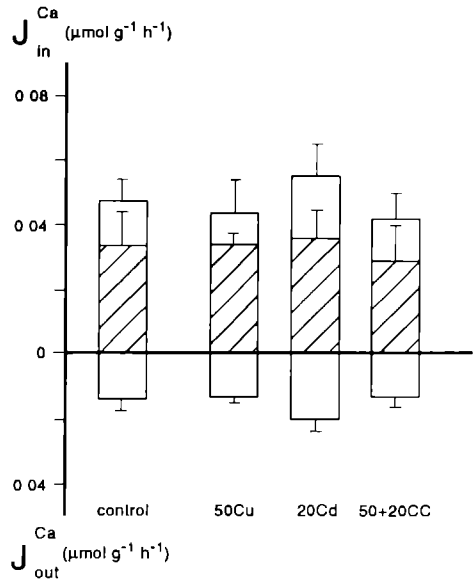

Figure 2. *Ca^{2+}-influx, indicated by J_{in} (open bars above zero, n=12), Ca^{2+}-efflux, indicated by J_{out} (open bars below zero, n=3) and net Ca^{2+} flux (shaded bars, n=3) of fish exposed during 6 days to 50 µg Cu l^{-1}, 20 µg Cd l^{-1}, or 50 µg Cu l^{-1} + 20 µg Cd l^{-1}*

significantly decreased compared to controls and single Cd exposed fish Moreover, the combined Cu/Cd exposed fish was the only experimental group in which influx did not exceed efflux In these fish therefore, Ca balance was not positive

DISCUSSION

The present results show that the combination of Cu and Cd significantly decreased Ca^{2+}-influx, whereas exposure to these concentrations Cu or Cd singly had no effect Ca^{2+}-efflux was not affected by Cu/Cd co-exposure. Consequently, the Cu/Cd co-exposed fish were not in positive Ca balance Maintenance of a positive Ca balance is important through most of fish life, but is crucial for young, growing fish when body Ca-levels increase several fold within a relatively

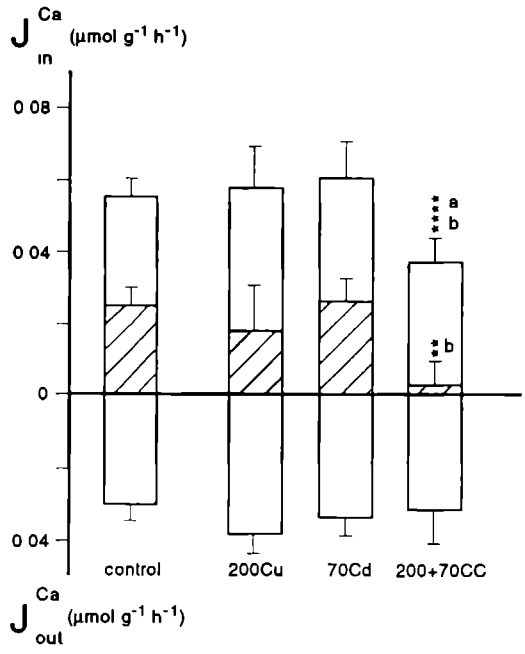

*Figure 3. Ca^{2+}-influx, indicated by J_{in} (open bars above zero, n=12), Ca^{2+}-efflux, indicated by J_{out} (open bars below zero, n=3) and net Ca^{2+} flux (shaded bars, n=3) of fish exposed during 6 days to 200 µg Cu l^{-1}, 70 µg Cd l^{-1}, or 200 µg Cu l^{-1} + 70 µg Cd l^{-1} Significant differences between controls and metal exposed fish are indicated by asterisks, with * P< 0 05, and *** P< 0 01 Significant differences between Cu-exposed and combined Cu/Cd exposed fish are indicated by a (P< 0 02), whereas b indicates significant differences between Cd-exposed and combined Cu/Cd exposed fish*

short period (Rombough and Garside, 1984; Reader and Morris, 1988). The observed inhibition of Ca uptake in young tilapia will have consequences for fish development in polluted areas.

Cd

In tilapia exposed during 6 days to both sublethal concentrations of waterborne Cd tested, Ca^{2+} and Na^{+} fluxes were not different from control values Previous studies however, reported that Cd exposure, at these concentrations, primarily affects Ca-homeostasis (Giles, 1984, Reid and McDonald, 1988; Fu *et al* , 1989; Pelgrom *et al* , 1995[b]) Recently, Reid and McDonald (1991) and Playle and Dixon (1993[a], 1993[b]) have shown that (apical) gill binding affinity for Cd^{2+} was similar to that for Ca^{2+}, which may underlay the specific effects of Cd on Ca-homeostasis From

previous studies, including flux experiments, with rainbow trout it furthermore appeared that Cd-induced hypocalcemia was mainly caused by inhibition of specific active Ca uptake mechanisms via the gills (Verbost *et al.*, 1987; Reid and McDonald, 1988). In comparing the present results with flux data from other studies, it should be noticed, that the experimental set up in the present study differed in several important aspects. Firstly, the species studied. Cd-induced inhibition of Ca^{2+}-uptake has so far been observed in rainbow trout only. It has become clear that some species of teleost fish, including rainbow trout, are especially sensitive to waterborne metals (Roberts *et al.*, 1979), whereas others such as roach, perch and tilapia can tolerate much higher concentrations (Solbé and Flock, 1975; this study). Secondly, a 3-day acclimation period was used prior to the metal exposure in the present study, and not by others (Reader and Morris, 1988; Reid and McDonald, 1988). It has been demonstrated by Dharmamba and Maetz (1972) that during the first 24 h after the introduction of fish into flux chambers, net ion fluxes were negative in control fish. After 24-48 h, net ion uptake was observed, indicating acclimation to the experimental conditions (Dharmamba and Maetz, 1972). Therefore, a long acclimation period seems a prerequisite for reducing the effects of stress on the flux measurements. The beneficial influence of the acclimation period is reflected by the highly positive Na and Ca uptake rates in our control fish, which were much higher than those in flux studies where fish were not preacclimated (Reader and Morris, 1988; Reid and McDonald, 1988). Thirdly, there is a difference in the exposure time and regime. The Cd-induced inhibition of Ca-uptake was observed in trout acutely exposed during 12-24 h (Verbost *et al.*, 1987; Reid and McDonald, 1988), whereas in the present study Ca^{2+} fluxes were determined after 6 days of exposure, which was initiated gradually rather than acutely. Fourthly, the present flux data were obtained with fed fish, whereas Reader and Morris (1988) and Reid and McDonald (1988) performed their flux experiments after a 5-days starvation period. Finally, the present flux studies were performed under artificial freshwater conditions. Generally, tapwater contains Cu. For example, we have measured that Nijmegen tapwater contains up to 50 $\mu g.l^{-1}$ Cu (personal observations). Therefore, results on flux studies on Cd exposure which are performed in tapwater are probably complicated by the presence of Cu in the water.

Cd and Cu

The absence of a Cd-induced inhibition of Ca-uptake after 6 days may be interpreted to indicate acclimation. However, acclimation to sublethal Cd concentration did not occur in the presence of Cu, since Ca-uptake was inhibited in Cu/Cd co-exposed fish. For trout, it has been shown that Cd interacts with Ca uptake mainly through effects on basolateral Ca^{2+}-ATPase activity, the Ca pump (Verbost *et al.*, 1989). The present study shows Cu/Cd interaction with Ca uptake mechanisms in the gills of tilapia. In a previous study with tilapia exposed during 6 and 11 days to combinations of Cu and Cd, we observed Cu/Cd interactions resulting in different

accumulation patterns in the gills, in particular enhanced Cd accumulation in Cu/Cd exposed fish (Pelgrom *et al* , 1995[c]) It may be suggested that those interactions may also exert effects intracellularly at the level of the Ca pump *In vitro* Cu/Cd exposure of cell cultures resulted in an increased toxicity of these metals, as concluded from the protein content of trout hepatocytes (Denizeau and Marion, 1990) and cultured (KB) cells (Meshitsuka *et al* , 1982, 1987) Meshitsuka and coworkers (1982, 1987) suggested that the toxicity of Cd did not solely depend on the amount of Cd absorbed by the cells but also on cofactors such as Cu, though Cu itself had no effect To get insight in the effects of combined Cu/Cd exposures on physiological processes in organisms, whole body studies are necessary, and the present study substantiates the importance of *in vivo* studies on Cu/Cd interactions Knowledge about interactions between toxicants during *in vivo* exposures of organisms will contribute to a better risk assessment of polluted environments

In previous studies on metal-exposed tilapia, we observed differences in plasma cortisol levels, in particular between Cu and Cu/Cd co-exposed fish (Pelgrom *et al* , 1995[b]) Regulation of the homeostasis of ions, including Ca, is partly controlled by cortisol, which is known to induce chloride cell proliferation and concomitantly stimulate ionic uptake (Laurent and Perry, 1990, Muñoz *et al* , 1991, Wood, 1992) In addition, whole body metal accumulation (Pelgrom *et al* , 1994) and distribution (Pelgrom *et al* , 1995[c]) between single metal and Cu/Cd co-exposed fish were different Those observations, together with the present results, illustrate the fundamentally different responses in Cu/Cd co-exposed fish compared to single metal exposed fish

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CHAPTER 6

STRUCTURAL AND PHYSIOLOGICAL CHANGES IN THE GILLS OF TILAPIA, *OREOCHROMIS MOSSAMBICUS*, IN RESPONSE TO SINGLE AND COMBINED COPPER AND CADMIUM EXPOSURE.

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submitted for publication

ABSTRACT

During waterborne metal exposure, gills are the primary target organ. Consequently, increased metal-tolerance will depend on specific structural and physiological adjustments of the branchial tissue. To study the response of fish to sublethal waterborne concentrations of Cu and Cd, we exposed mature tilapia (*Oreochromis mossambicus*) for 6 days to 100 μg $Cu.l^{-1}$ or 35 μg $Cd.l^{-1}$. To get more insight in the effects of combined Cu/Cd exposure, we also exposed tilapia to 100 μg $Cu.l^{-1}$ + 35 μg $Cd.l^{-1}$.

It is demonstrated, that Cu and Cd have metal-specific effects on the gills. Exposure to Cu resulted in an increased number of branchial chloride cells and an increase in the average diameter of these cells. In Cd-exposed fish, most obvious were the increased numbers of lysosomes and of apoptotic chloride cells, and an inhibition of the active branchial Ca^{2+}-transport activity. In fish exposed to combined Cu/Cd, the observed effects were complex. Although Cu and Cd accumulated both in the gill tissue, the effects observed were not a summation of the single metal-induced effects, as shown by plasma ceruloplasmin levels, Na^+/K^+-ATPase activity and chloride cell numbers. Therefore, there seems to be no apparent relationship between metal accumulation in the gills and the structural and physiological changes in this tissue.

INTRODUCTION

Aquatic pollution by heavy metals is a widespread problem. It has been shown that

sublethal concentrations of waterborne metals such as copper (Cu) and cadmium (Cd) seriously affect the physiological processes in fish (McDonald and Wood, 1993) The branchial epithelium is the site of the most intimate contact between the water and the internal milieu of the fish Cu and Cd not only enter the body via the gills (Segner, 1987, Battaglini *et al* , 1993), but also exert their primary toxic effects by interfering with one or more of physiological processes in this tissue (Wood, 1992, McDonald and Wood, 1993) The branchial epithelium is a sensitive and metabolically active tissue, and plays a crucial role in gas exchange, acid-base regulation, nitrogenous waste excretion and ionic regulation (Wood, 1992, Perry and Laurent, 1993) When Cu or Cd enters the cell, interference with enzymatic activities result in functional impairment, leading to ionoregulatory disturbances (Giles, 1984, Reid and McDonald, 1988, Pratap *et al* , 1989, Verbost, 1989, McDonald and Wood, 1993, Pelgrom *et al* , 1995[b]) Several investigators have argued that Cu mainly impairs sodium (Na) homeostasis, whereas Cd negatively affects the calcium (Ca) balance in fish (Reid and McDonald, 1988, 1991, McDonald and Wood, 1993) Since the gills are the initial target organ for waterborne metals, it follows that increased tolerance will depend on specific biochemical and physiological adaptation of the gill tissue (McDonald and Wood, 1993) In many instances, the compensatory adjustments of gill function concur with morphological adjustments For example, proliferation of chloride cells, cells involved in ionic uptake in freshwater teleosts (Perry and Laurent, 1993), has been observed after chronic exposure to sublethal concentrations of Cd (Parrott and Sprague, 1993, Pratap and Wendelaar Bonga, 1993) and Cu (Baker, 1969, Gupta and Rajbanshi, 1981) However, also maladaptive structural changes in the gills of metal-exposed fish have been observed (Gupta and Rajbanshi, 1981, Mallatt, 1985, Pratap and Wendelaar Bonga, 1993)

Despite the increasing knowledge about the toxic mechanisms of sublethal concentrations of either Cu or Cd for fish, little is known about the effects of combined Cu/Cd exposures The potential toxic effect of mixtures of heavy metals for fish has lately become a subject of growing interest (deMarch, 1988, Ma Lourdes *et al* , 1993) Although it has been shown that the toxic mechanisms of Cu and Cd are distinctly different (Reid and McDonald, 1988, 1991), metal-interactions during combined Cu/Cd exposure have been demonstrated in some studies Cu/Cd interactions have been observed in mammals (Irons and Smith, 1976) and in cultured cell systems (Sakamoto and Kozuka, 1992) In fish, we recently demonstrated Cu/Cd interactions during combined exposures concerning whole body and organ metal accumulation, ionic composition of plasma and tissue, and plasma cortisol levels (Pelgrom *et al* , 1994, 1995[b]) Cortisol is known to regulate the mobilization of energy substrates as well as ion homeostasis in fish experiencing unfavourable water conditions (Laurent and Perry, 1990)

The aim of the present study was twofold Firstly, gill ultrastructure at the electron microscope level as well as the number and diameter of chloride cells by light microscopic techniques were investigated in tilapia (*Oreochromis mossambicus*) after either single or combined exposure to Cu and Cd To study potential structural changes in relation to metal-induced effects on ionoregulatory function in the tissue, we determined Na^+/K^+-ATPase activity and Ca^{2+}-transport in the gills Secondly, the transport and subcellular distribution of the metals were studied after single and combined exposure. After uptake of Cu and Cd by the gills, part of it is cleared to the blood (Task Group on Metal Accumulation, 1973) From studies on mammals it is known, that the majority of circulating Cu is associated with the protein ceruloplasmin, which is synthesized in the liver (Cousins, 1985) From these studies it has become apparent that ceruloplasmin plays a role in the transport and distribution of Cu to the organs A similar mechanism for Cu transport and distribution after uptake by the gills may also apply to fish (Pelgrom *et al* , 1995[a]) In mammals it also has been demonstrated that serum ceruloplasmin levels are decreased after Cd treatment (Frieden, 1979), thereby creating the possibility that Cu/Cd interaction might also occur at this level As far as we know, metal interactions on ceruloplasmin levels have not been studied in fish, and we therefore measured plasma Cu and Cd concentrations and ceruloplasmin levels in the experimental groups

MATERIALS AND METHODS

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from own laboratory stock Fish were grown and held under artificial freshwater conditions with undetectable Cu and Cd concentrations (detection limit 0 1 and 0 01 $\mu g\ l^{-1}$ respectively) Artificial freshwater consisted of demineralized water supplemented with 1 3 mM $NaHCO_3$, 0 5 mM $CaCl_2$, 0 06 mM KCl and 0 2 mM $MgCl_2$, at pH 7 8 Composition and preparation of the water was based on the EEC instructions for artificial water for use in toxicity studies with fish (EEC Directives 84/449/EEC Annex 5 method c1 Acute toxicity for fish) Water was continuously aerated, filtered and refreshed by means of flow-through (Pelgrom *et al* , 1994) The light/dark regime was 12/12 h and the water temperature 26°C Fish were fed commercial tropical fish food Tetramin™, 2% ($^{dw}/_{ww}$) of their body weight per day Cu and Cd contents of the food were 9 86 ± 0 16 μg Cu g^{-1} dry food and 0 22 ± 0 01 μg Cd g^{-1} dry food (means ± SE, n=10)

Experimental design

Six weeks before the start of the experiment, sexually mature female fish (mean weight of 20 g) were divided into 4 groups of 14 fish each, and kept in 80 l aquaria with artificial freshwater, continuously filtered and refreshed by means of a 16-channel peristaltic pump (Watson Marlow) The experiment started by switching the water supply from each aquarium to its own reservoir filled with artificial freshwater with or without (controls) a well-defined Cu and Cd concentration (added as nitrate, Spectrosol, BDH, England) During the first 6 h the flow rate was 4 5 l h^{-1}, followed by a flow rate of 1 5 l h^{-1} In this way, the metal concentrations in the aquaria were gradually raised, reaching a plateau after 18 h (Pelgrom *et al* , 1994) The measured concentrations in the aquaria deviated maximally 5% from the nominal concentrations (100 μg Cu l^{-1}, 35 μg Cd l^{-1} or 100 μg Cu l^{-1}+ 35 μg Cd l^{-1}) The exposure period lasted 6 days, and feeding was ended the day before sacrifice At the end of the exposure period, blood samples were taken from the caudal vessels by means of capillaries, and fish were killed by spinal dissection Blood cells and plasma were separated by centrifugation, and plasma was stored at -20°C until measurement of Cu, Cd and ceruloplasmin contents

Plasma ceruloplasmin

Plasma ceruloplasmin content was measured as *p*-phenylenediamine (PPD) oxidase activity The method of ceruloplasmin detection has been validated for plasma of tilapia (Pelgrom *et al* , 1995[a]) A 15 μl plasma sample was mixed with 1 ml of 1 2 M acetate/acetic acid buffer (pH 6 4) containing 0 1% PPD (Sigma) as substrate To avoid non-specific substrate oxidation, incubation was carried out in the presence of 0 02 mM EDTA Each plasma sample was incubated in duplicate Concomitantly, each plasma sample was incubated in the presence of 1 ml 0 5% NaN_3 (azide blank) The mixtures were incubated during 30 minutes at 37°C The reaction was stopped by the addition of 1 ml 0 5% NaN_3 Within one hour, the absorption was measured at 550 nm (LKB Spectrophotometer) Plasma ceruloplasmin content was expressed as the difference in absorbance between the sample and its azide blank

Cu and Cd measurement

Plasma, gills, crude gill homogenates (H_0) and purified plasma membrane preparations (P_3) were digested with nitric acid (65% HNO_3 Ultrapur, Merck), and stored in 0 2% HNO_3 at 4°C until analysis (Pelgrom *et al* , 1995[a,c]) Water samples were acidified with HNO_3 to a final concentration of 0 2% Cu and Cd concentrations were determined with a flameless Atomic Absorption Spectrometer (AAS, Philips PU 9200) connected to an electrothermal atomizer (Philips PU 9390X)

Chloride cell measurement

The left opercula were prepared for numerical chloride cell densities (n/mm^2) and chloride cell diameter measurement after DASPEI (ICN Biochemicals Inc., Plainview, NY) vital staining (Wendelaar Bonga *et al.*, 1990). The opercular chloride cell numbers are considered to reflect the gill chloride cell numbers, since the opercular epithelium is a continuous extension of the epithelium covering the gills (Wendelaar Bonga *et al.*, 1990). The middle part of the first left gill arches were prepared for electron microscopy as described by Wendelaar Bonga and van der Meij (1989). The remaining gill arches were prepared for either Cu and Cd measurement or plasma membrane isolation.

Isolation of plasma membranes

Plasma membranes of the branchial epithelia were isolated at 4°C based on the method described by Flik *et al.* (1985), with some adjustments. This procedure leads to a good enrichment of the Na^+/K^+-ATPase and Ca^{2+}-ATPase: the vesicles are leaky and the degree of mitochondrial contamination is low. Briefly, the soft tissue of the gills was scraped off with a glass microscope slide, and carefully homogenized with a glass-to glass Dounce homogenizer (10 strokes) in an isotonic buffer containing 250 mM sucrose, 12.5 mM NaCl, 5 mM HEPES/TRIS pH 7.5, 0.1 mM EDTA, 100 U.ml^{-1} aprotinin (Sigma) and 50 U.ml^{-1} heparin. Nuclei and cellular debris (pellet P_0) were separated from membrane fractions (supernatant H_0) by centrifugation during 10 minutes at 550 g (Hereus). After centrifugation of the supernatant H_0 (50,000 rpm, 30 minutes; Beckmann Ultracentrifuge, Ti 70 rotor), membranes were collected in a fluffy pellet (P_1). This pellet was resuspended with a glass-to-glass Dounce homogenizer (100 strokes) in an isotonic sucrose buffer containing 250 mM sucrose, 5 mM HEPES/TRIS pH 7.5 and 5 mM $MgCl_2$. The membrane suspension was centrifuged differentially: 10 minutes at 1,000 g followed by 10 minutes at 9,500 g (Sorval RC-5B). Finally, the supernatant was centrifuged during 15 minutes at 20,000 g, resulting in the final membrane fraction, pellet P_3. These pellets P_3 were resuspended by passage through a 23-G needle (10 times) in a buffer containing 20 mM HEPES/TRIS pH 7.4, 1.5 mM $MgCl_2$ and 150 mM KCl (for Ca^{2+}-transport studies) or 150 mM NaCl (for Na^+/K^+-ATPase studies). Membrane preparations P_3 and crude homogenates H_0 were quickly frozen in cold CO_2/acetone, and used the next day for determination of protein content, enzyme- and transport-activity.

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity in the H_0 and P_3 gill membrane fractions were determined by the method described by Flik *et al.* (1985). Routinely, 0.20 mg.ml^{-1} saponin was added to optimize substrate accessibility. Membrane protein content was determined with a reagent

kit (Biorad), using Bovine Serum Albumin (BSA, Sigma) as reference. Vesicles were incubated during 10 minutes at 37°C with medium containing 100 mM NaCl, 30 mM Imidazole, 0.1 mM EDTA , 5 mM $MgCl_2$ and either 15 mM KCl or 1 mM ouabain. Na_2ATP was added in a final concentration of 3 mM. The reaction was stopped by adding ice-cold TCA-solution. Inorganic phosphate (P_i) production was measured by the colorimetric Fiske-Subbarow technique using a commercially (Sigma) phosphate standard (Flik *et al.*, 1985).

Ca^{2+}-transport

ATP-dependent Ca^{2+}-transport was determined by means of a rapid filtration technique as described by Van Heeswijk *et al.* (1984). Ca^{2+} and Mg^{2+} concentrations were calculated according to Schoenmakers *et al.* (1992) using the computer program CHELATOR. Ca^{2+}-transport was measured at a Ca^{2+} concentration of 10^{-6} M (V_{max}). Uptake of $^{45}Ca^{2+}$ into membrane vesicles (P_3-fraction) was determined during 1-minutes incubations without or in the presence of 3 mM ATP (Tris-ATP). The reaction was stopped in ice-cold isotonic medium containing 0.1 mM $LaCl_3$, and the suspension was filtered (Schleicher & Schüll ME 25, pore size 0.45μm). Filters were rinsed twice with ice-cold medium and transferred to counting vials and dissolved in Aqualuma©. ^{45}Ca was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

Statistics

Results are presented as means ± SE. Differences between groups were tested for significance by the Mann-Whitney *U*-test. Significant differences between metal-exposed and control fish are indicated by asterisks, whereas significant differences between Cu-exposed and Cu/Cd co-exposed fish are indicated by **a**, and differences between Cd-exposed and Cu/Cd co-exposed fish by **b**.

* (**a b**): $P<0.05$; ** (**aa bb**): $P<0.02$; *** (**aaa bbb**): $P<0.01$ and **** (**aaaa bbbb**): $P<0.001$.

RESULTS

Plasma

Plasma Cu and Cd concentrations and ceruloplasmin are presented in Table 1. The plasma Cu and Cd concentrations were increased in fish exposed to Cu or Cd respectively. The Cu and Cd concentrations in plasma of combined Cu/Cd exposed fish were increased to the same levels as observed in fish exposed to the metals singly. Plasma ceruloplasmin levels were decreased in Cd-exposed fish, but not in Cu/Cd exposed fish.

	[Cu] $_{plasma}$ ($\mu g.l^{-1}$)	[Cd] $_{plasma}$ ($\mu g.l^{-1}$)	Ceruloplasmin (A_{550}-azide blank; $x10^{-3}$)
control (n= 8)	679.8 ± 37.3	10.4 ± 1.2	18.0 ± 1.8
Cu (n= 7)	$880.0 \pm 38.0^{***}$	11.3 ± 1.2	19.1 ± 0.9
Cd (n= 8)	666.8± 27.7	$14.5 \pm 0.8^{***}$	$13.3 \pm 1.6^{*}$
Cu+Cd (n= 7)	$868.6 \pm 46.0^{***}_{bbb}$	$20.6 \pm 4.9^{****}_{aa}$	17.4 ± 0.6^{b}

Table 1. *Plasma Cu and Cd concentrations and ceruloplasmin in tilapia exposed for 6 days to 100 μg Cu.l^{-1} (Cu), 35 μg Cd.l^{-1} (Cd) or 100 μg Cu l^{-1} + 35 μg Cd.l^{-1} (Cu+Cd). Significant differences between controls and metal-exposed fish are indicated by asterisks, whereas significant differences between single Cu or Cd exposed fish and combined Cu+Cd exposed fish are indicated by* ***a*** *or* ***b*** *respectively.*

Chloride cells

Figure 1 shows the opercular chloride cell number (panel A) and diameter (panel B) of control and metal exposed tilapia. Chloride cell numbers of fish exposed to Cu and Cd, singly and in combination, are increased. However, the chloride cell numbers observed in combined Cu/Cd exposed fish were significantly lower than those in fish exposed to one of these metals. This effect was also observed in additional experiments with tilapia exposed singly and in combination to 50 μg Cu.l^{-1} and 20 μg Cd.l^{-1}, and to 20 μg Cu.l^{-1} and 5 μg Cd.l^{-1} (data not shown). The diameter of the chloride cells was increased in Cu-exposed fish, but not in Cd or Cu/Cd co-exposed fish.

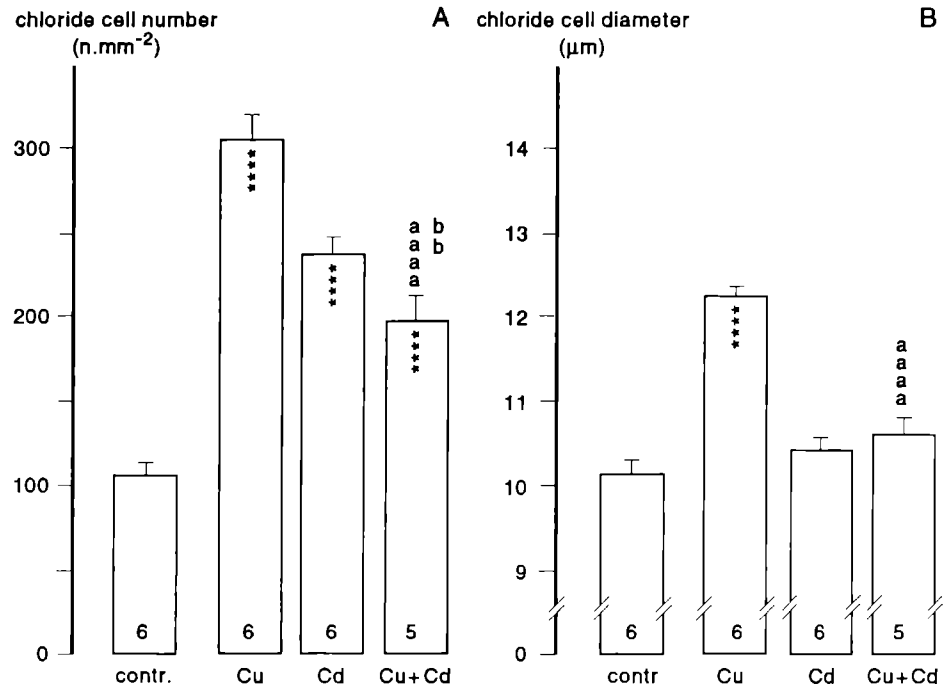
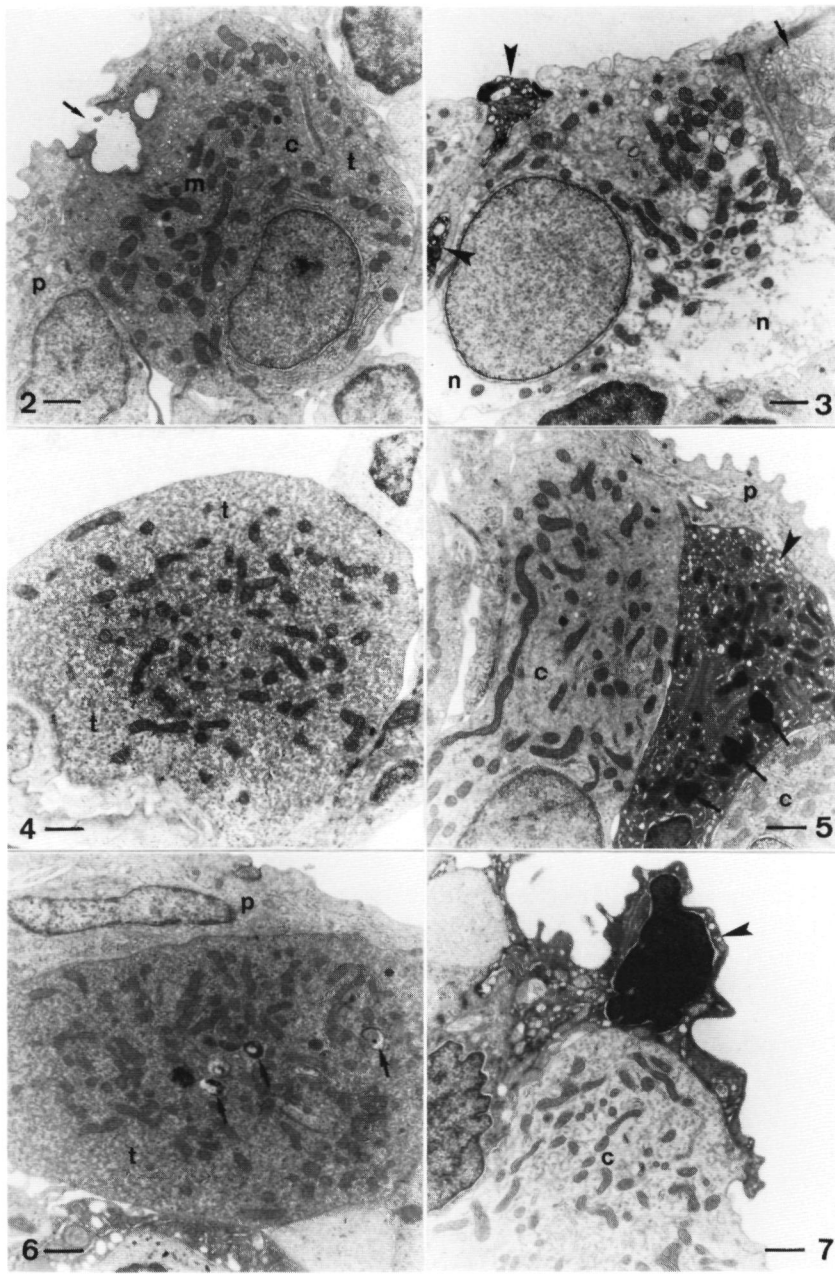


Figure 1. *Chloride cell number (panel A) and diameter (panel B) in operc ula of tilapia exposed for 6 days to 100 µg Cu l^{-1} (Cu), 35 µg Cd.l^{-1} (Cd) or 100 µg Cu l^{-1} + 35 µg Cd.l^{-1} (Cu+Cd). The number of fish per experimental group is shown in the bar. Asterisks indicate significant differences between controls and metal-exposed fish, whereas significant differences between single Cu or single Cd and combined Cu+Cd exposed fish are indicated by* ***a*** *and* ***b*** *respectively.*

Figure 2 to 7. *Primary lamellae of the gills of tilapia; bars represent 1µm*
Fig. 2: Control. *Chloride cell (c) with apical pit (arrow), mitochondria (m) and tubular system (t), p, pavement cell.* ***Fig 3: Cu-exposure.*** *Chloride cell showing necrotic swelling (n) in the basal cell area, and remnants of apoptotic chloride cell (arrowheads); arrow, intact chloride cell.* ***Fig. 4: Cu-exposure.*** *Chloride cell with extensive tubular system (t).* ***Fig. 5: Cd-exposure.*** *Intact chloride cells (c) and apoptotic chloride cell (arrowhead) covered by pavement cell extension (p), the apoptotic cell shows several lysosome-like bodies (arrows).* ***Fig. 6. Cu/Cd co-exposure.*** *Chloride cell showing an extensive tubular system (t) and several lysosome-like bodies (arrows); p, pavement cell.* ***Fig. 7: Cu/Cd co-exposure.*** *Apoptotic pavement cell (arrowhead), c, chloride cell.*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Ultrastructure branchial epithelium

The ultrastructure of the branchial epithelium of tilapia (Fig. 2) has been described before by Wendelaar Bonga and Van der Meij (1989). Investigation of the gill structure at the ultrastructural level showed differences between controls, single metal exposed fish and combined Cu/Cd exposed fish (Figures 2-7). The branchial epithelia were structurally intact in all groups. No differences in the intercellular space were observed between control and metal exposed tissue, and no leucocyte infiltration in metal-exposed groups was found. Compared to controls, in Cu-exposed fish the enlargement of the chloride cells (Fig. 3), and the condensation of the basal labyrinth were most obvious (Fig. 4). In these gills, there were relatively many differentiating chloride cells.

In Cd-exposed fish, the most striking observations were the increased apoptosis of the chloride cells, and the increased number of lysosomes, particularly in the apoptotic chloride cells (Fig. 5) Neither degeneration of the pavement cells, nor changes in their amount of lysosomes were observed

The morphological changes observed in the branchial epithelium of combined Cu/Cd exposed fish were a combination of those observed in fish exposed to Cu or Cd singly, though not in a additive way (Figures 6 and 7) An exception was the amount of lysosomes in the chloride cells, which in Cu/Cd exposed fish was higher than in single Cd exposed fish, although these lysosomes appeared less restricted to apoptotic chloride cells as in the case of the Cd-exposed fish.

Gill metal concentrations

Table 2 shows the Cu and Cd concentrations in whole gills of control and metal exposed fish, and in the purified branchial membrane preparations (P_3), which were used for active ion-transport measurements. The Cu and Cd concentrations were significantly increased in the gills of Cu and Cd exposed fish, respectively The metal concentrations in the combined Cu/Cd exposed fish were increased to the same extent as in the single metal exposed fish. A similar picture was obtained with the P_3, except for a significant reduction in the Cd concentration in the Cu-exposed group The % of Cu recovered in the P_3 did not differ between the groups. In contrast, the % of Cd recovered in the P_3 in Cd and Cu/Cd exposed fish was significantly lower than in controls

	Gills		P_3-fraction		P_3/H_0 recovery	
	[Cu] ($\mu g\ g^{-1}$ ww)	[Cd] ($\mu g\ g^{-1}$ ww)	[Cu] ($ng\ g^{-1}$ fish)	[Cd] ($ng\ g^{-1}$ fish)	[Cu] (%)	[Cd] (%)
control	0 42 ± 0 02	0 027 ± 0 002	6 90 ± 1 36	0 205 ± 0 064	38 6 ± 5 6	14 9 ± 4 3
Cu	1 43 ± 0 11****	0 033 ± 0 005	12 43 ± 1 13***	0 103 ± 0 038*	34 6 ± 15 4	7 7 ± 1 1
Cd	0 46 ± 0 02	4 066 ± 0 537****	7 03 ± 0 67	0 924 ± 0 339***	41 9 ± 10 1	1 4 ± 0 5****
Cu+Cd	2 01 ± 0 30****	3 907 ± 0 371****	10 17 ± 2 06*	0 649 ± 0 102***	23 2 ± 8 6	1 6 ± 0 5****

Table 2 Cu and Cd concentrations in the total gill tissue (gill) and in the purified branchial plasma membrane preparation (P_3) of tilapia exposed for 6 days to 100 μg Cu l^{-1} (Cu), 35 μg Cd l^{-1} or 100 μg Cu l^{-1} + 35 μg Cd l^{-1} (Cu+Cd) The recovery of Cu and Cd in the P_3-fraction is related to the metal concentration in the crude homogenate of the gill soft tissue (n=6 per group) Significant differences between controls and metal-exposed fish are indicated by asterisks

Na^+/K^+-ATPase activity

The total Na^+/K^+-ATPase activity in both H_0 and P_3 in metal exposed fish were not significantly different from controls, although combined Cu/Cd exposure resulted in higher total activities in H_0 and P_3 compared to the activities in Cu-exposed fish (Figure 8) The specific activities, enzyme purification (V_{spec} P_3/V_{spec} H_0) and protein recovery (protein P_3/protein H_0) in metal exposed fish did not differ from control values (data not shown)

Ca^{2+}-transport

Ca^{2+}-transport activities in controls and metal exposed fish are shown in Table 3 Cd exposure, singly and in combination with Cu, resulted in significantly decreased Ca^{2+}-transport activity

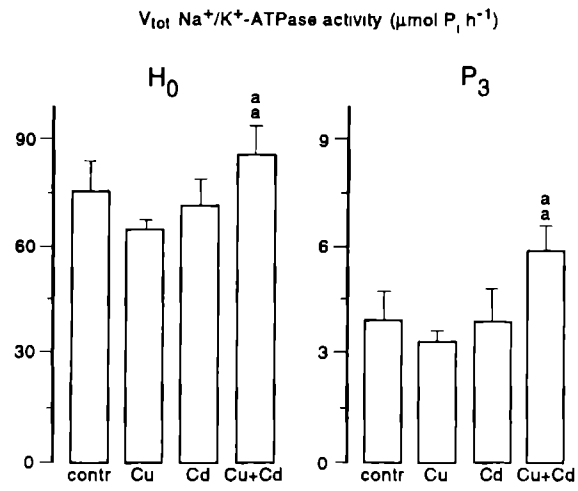

Figure 8. *Total Na^+/K^+-ATPase activity (in µmol P_i h^{-1}) in the crude branchial homogenate (H_0, left panel) and in the purified branchial plasma membrane preparation (P_3 right panel) of tilapia exposed for 6 days to 100 µg Cu l^{-1} (Cu), 35 µg Cd l^{-1} (Cd) or 100 µg Cu l^{-1} + 35 µg Cd l^{-1} (Cu+Cd), with n=6 for each group Significant differences between single Cu exposed fish and combined Cu+Cd exposed fish are indicated by aa*

DISCUSSION

Sublethal waterborne Cu or Cd exposure not only resulted in accumulation, but also in metal-specific structural changes in the gills of tilapia Cu exposure led to an increased number and diameter of chloride cells, while Cd exposure induced an increase of lysosome-like bodies and promoted apoptosis of chloride cells In spite of the summation of the tissue content of both metals in the gills of combined Cu/Cd exposed fish, the structural and physiological effects observed in these fish are complex and do not simply reflect a summation of the effects of the two individual metals This is in particular shown by changes in Na^+/K^+-ATPase activity and chloride cell numbers and diameter These changes formed part of the response of the animals to the challenges applied, which probably contributed to the maintenance of the structural integrity of the gills in all groups

		Ca^{2+}- transport (nmol Ca min^{-1} mg^{-1}prot)
control	(n=6)	4 16 ± 0 76
Cu	(n=6)	4 10 ± 0 86
Cd	(n=6)	2 30 ± 0 51**
Cu+Cd	(n=6)	2 59 ± 0 47*aa

Table 3. *Branchial Ca^{2+}-transport in the purified branchial plasma membrane preparation (P_3) of tilapia exposed for 6 days to 100 µg Cu l^{-1} (Cu), 35 µg Cd l^{-1} (Cd) or 100 µg Cu l^{-1} + 35 µg Cd l^{-1} (Cu+Cd) Significant differences between controls and metal exposed fish are indicated by asterisks, whereas significant differences between single Cu exposed fish and combined Cu+Cd exposed fish are indicated by* ***aa***

Plasma Cu, Cd and ceruloplasmin

Control plasma Cu values of tilapia were in the range as reported for other teleost species (600 1300 µg l^{-1}) (Stagg and Shuttleworth, 1982, Bettger *et al* , 1987) The elevated plasma metal levels of Cu exposed tilapia are in line with studies on Cu exposed carp (Yamamoto *et al* , 1977), flounder (Stagg and Shuttleworth, 1982) and catfish (El-Domiaty, 1987) Data on control values of plasma Cd concentrations in fish are not available due to the low Cd concentrations in plasma, Cd could not be detected by Roberts *et al* (1979) and Kuroshima (1987) in their Cd exposed fish The method used in the present study yielded reproducible plasma Cd concentrations of about 10 µg Cd l^{-1}, which were well above its detection limit In mammals control Cd values in blood of plasma range from 3 µg l^{-1} in humans (Kido *et al* , 1990) to 40 µg l^{-1} in rats (Suzuki *et al* , 1983) In tilapia, plasma Cd concentrations were increased after Cd and Cu/Cd exposure to levels which in some of these fish approached water levels In combined Cu/Cd exposed fish, plasma concentrations of Cd and Cu increased to the same levels as in single metal exposed fish, although it should be noted that the total metal burden in combined-metal exposed fish was higher, with both Cu and Cd concentrations increasing

Increased levels of ceruloplasmin after Cu exposure have been demonstrated in plasma of carp (100 µg Cu l^{-1} (Yamamoto *et al* , 1977) and tilapia (200 µg Cu l^{-1}, Pelgrom *et*

al., 1995a). In line with observations in the last study, exposure to 100 μg Cu l^{-1} had no effect in tilapia. Cd exposure, however, induced a significant decrease in plasma ceruloplasmin. There are no data available from other fish studies, but in mammals Cd exposure has also been shown to reduce serum ceruloplasmin levels (Stonard and Webb, 1976; Frieden, 1979). These observations further demonstrate that toxic metals may cause effects, directly as well as indirectly, by interfering with the metabolism of other metals (Task Group om Metal accumulation, 1973; Pelgrom *et al.*, 1994) In the presence of Cu, no Cd-induced decrease of plasma ceruloplasmin concentrations could be detected. Although Cu exposure itself had no effect on this parameter, the presence of a high plasma Cu concentration in these fish may have prevented an effect of Cd on plasma ceruloplasmin.

Chloride cell numbers and diameters

The number of chloride cells was increased in the opercula of both single- and combined-metal exposed fish. Notable, however, was the less pronounced increase in chloride cell numbers in the Cu/Cd co-exposed fish compared to Cu exposed fish This phenomenon was confirmed in several additional experiments, and was also observed in fish exposed to concentrations as low as 20 μg Cu l^{-1} and 5 μg Cd.l^{-1} Proliferation of chloride cells is a well-documented response to exposure to agents known to compromise branchial ion uptake (Wendelaar Bonga *et al* , 1990; Perry and Laurent, 1993), including Cu or Cd (Baker, 1969; Gupta and Rajbanshi, 1981, Parrott and Sprague, 1993, Pratap and Wendelaar Bonga, 1993) Chloride cells function in ion uptake in freshwater teleosts and therefore, an increase in chloride cell numbers is interpreted as an adaptive response to compensate for passive ion losses induced by stressors such as metal exposure (Perry and Laurent, 1993) It has been argued, however, that not the total number of chloride cells (including differentiating, mature and degenerative cells), but only the number of mature chloride cells in contact with the external environment contribute to the ion transport capacity (Wendelaar Bonga *et al.*, 1990, Perry and Laurent, 1993) The mean diameter of the chloride cells in our study as well as the ultrastructural observations suggest that the increase in the number of cells was not caused by a disproportionate increase of small, immature or degenerative cells. Cu exposure even resulted in significantly larger cells, which was also observed in a previous study with several Cu concentrations (Pelgrom *et al.*, 1995a). In the presence of Cd or both Cu and Cd, this chloride cell hyperplasia did not occur. Obviously, the presence of Cd during Cu exposure inhibits the proliferation, and prevents the enlargement, of the chloride cells

Ultrastructure of the branchial epithelium

Also at the ultrastructural level the effects of sublethal Cu exposure are different from those of Cd exposed fish. Whereas Cu induced an increase in the extent of the tubular system of the chloride cells, in Cd-exposed fish those cells showed an increase in lysosome-like bodies and in apoptosis of the cells. Metal induced structural changes in the gills have also been reported before, albeit at much higher concentrations of the metals than used in our study (Baker, 1969; Battaglini *et al.*, 1993). The effects included separation of epithelial layers from the underlying tissues, tissue necrosis, and fusion of secondary lamellae, without apparent differences between Cu or Cd exposed fish. Such branchial damage is generally observed in fish exposed acutely to relatively high concentrations of heavy metals, in particular during prolonged exposure periods (Mallatt, 1985). In our study, however, fish were exposed gradually to relatively low metal concentrations. In our opinion, these experimental conditions have more ecological significance, and they allowed us to demonstrate that waterborne Cu and Cd have different effects on the gill structure. Cd-induced degeneration of chloride cells has previously been observed in tilapia (Pratap and Wendelaar Bonga, 1993). In contrast to that study, in which Cd concentrations were used that were comparable to that of our experiments, we neither observed leucocyte infiltration nor excessive degeneration of the pavement cells in gills of the Cd-exposed fish. This discrepancy may be attributed to experimental differences such as gradual exposure in artificial freshwater, as in our study, and acute exposure in tapwater in the study of Pratap and Wendelaar Bonga (1993). This lends support to the notion that such experimental conditions can markedly influence the effects of experimental challenges, as was concluded by Balm and Pottinger (1993) in a study on the effects of low water pH on rainbow trout.

In the combined Cu/Cd exposed fish proliferation of the chloride cells was less than in the fish exposed to Cu or Cd, but the cytoplasm of these cells contained more lysosomes. Under these conditions, lysosomes may serve as a storage for Cu and Cd. In livers of mammals (Nederbragt *et al.*, 1984) and fish (Bunton and Frazier, 1994) high Cu concentrations have been found in the lysosomes. Several studies have indicated an important role for these organelles in the intracellular availability of metals in mammalian and non-mammalian organisms (Fowler, 1987). In the gills of Cu/Cd exposed fish, both Cu and Cd concentrations were increased, whereas the increase in chloride cell numbers was lower than in the single metal exposed fish, and this may imply the need for the higher numbers of lysosomes per cell.

Branchial metal accumulation

We did not observe Cu/Cd interactions during accumulation of the individual metals at the concentrations used In combined Cu/Cd exposed fish, Cu and Cd accumulated to the same extent as in single metal exposed fish Increased Cu and Cd concentrations in the gills of Cu or Cd exposed fish respectively has been observed in several studies on various fish species (Roberts *et al.*, 1979; Segner, 1987, Harrison and Klaverkamp, 1989; Pelgrom *et al.*, 1995[c]) Absence of Cu/Cd interference during binding of these metals to the gill surface was reported for fathead minnows exposed to low Cu or Cd (Playle *et al.*, 1993). From this study, based on the Cu and Cd binding sites on the gills, it was concluded that Cd binds to gills at least as effectively as Cu This could explain our observations in tilapia, that the gills accumulated more Cd (in $\mu g\ g^{-1}$) at a lower concentration than Cu. In general, an increased heavy metal content in the total gill tissue is mainly caused by increased intracellular metal concentrations rather than by binding to the mucus, as demonstrated by Pilgaard *et al* (1994)

Since waterborne Cu and Cd are able to cross the gill epithelium, a number of enzyme activities are at risk, which may result in functional impairment (Task Group on Metal accumulation, 1973). Branchial ATP-dependent ion transporting enzymes are mainly located in the basolateral membranes of chloride cells in the gill epithelium (Hossler, 1980) Although the purification method used involved metal chelating agents such as EDTA, we nevertheless found a substantial amount of Cu and Cd associated with the final membrane fraction (which contains mainly chloride cell basolateral membranes (Flik and Verbost, 1994) In a recent study on zebrafish, Wicklund Glynn *et al* (1994) provided evidence that accumulation of Cd mainly occurred in the chloride cells This is compatible with our data The observed increase in membrane-associated metal concentrations in the gills of tilapia indicates a high metal-binding capacity of these membranes, which may partially relate to metal-induced changes on the main ion-transporting enzyme systems present in the chloride cell basolateral membranes (Lemaire-Gony and Mayer-Gostan, 1994). In gills of both control and Cu exposed tilapia about 40% of the Cu content was recovered from the plasma membrane fraction In liver and kidney of mammals, rainbow trout and eel however, the greater part of the total Cu was found in the cytoplasm, bound to metal-binding proteins, whereas the microsomal fraction contained a low amount of Cu (less than 10 %) (Ley III *et al* , 1983, Cousins, 1985) In contrast to Cu, the amount of Cd associated with the plasma membrane in control tilapia gills is only 15%, and the greater part of the accumulated Cd in the gills of Cd-exposed fish was located in the cytoplasm Similar observations were made in studies on liver and gills of rainbow trout, carp and scallop, where the greater part of Cd in both control and

Cd-exposed fish was found in the cytoplasm (Kito *et al.*, 1982; Thomas *et al.*, 1985; Evtushenko *et al.*, 1986).

Na^+/K^+-ATPase activity

Although the Cd concentrations in the gills of exposed fish were notably increased, we observed no effect on total and specific Na^+/K^+-ATPase activity. These results may seem to contrast the Cd-induced inhibition of Na^+/K^+-ATPase observed in *in vitro* studies with homogenates and membrane preparations (Tokushiga *et al.*, 1984; Lemaire-Gony and Mayer-Gostan, 1994). However, Tokushiga *et al.* (1984) did not observe inhibition Na^+/K^+-ATPase activity in intact vascular smooth muscle cells (VSMCs) when exposed to Cd concentrations that inhibited Na^+/K^+-ATPase in muscle homogenates and subcellular fractions. They explained the inability of high intracellular Cd concentrations to inhibit the Na^+/K^+-pump in intact cells by binding of the metal to intracellular ligands other than the membrane bound enzymes. The data of our study support this option, as during *in vivo* Cd-exposure only a minor fraction of the Cd content in the gills could be recovered from the membrane fraction.

The observed increase in chloride cell numbers, which was most prominent in the Cu group, was not accompanied by an increase in Na^+/K^+-ATPase activity. Proliferation of branchial chloride cells is a commonly observed compensatory response to agents known to challenge branchial Na uptake (Wood, 1992; McDonald and Wood, 1993; Perry and Laurent, 1993). In response to sublethal metal exposure, however, an increase in chloride cell numbers is not always associated with an increased capacity for Na transport. Even a decrease of the total Na^+/K^+-ATPase activity has been observed in Cd exposed tilapia, in which the chloride cell number had increased (Pratap and Wendelaar Bonga, 1993). In the present study we found that Na^+/K^+-ATPase activity in Cu/Cd exposed fish was higher than in single Cu-exposed fish, although the chloride cell numbers in the former group were lower. These data indicate that the total chloride cell numbers are not an index for branchial Na-uptake capacity. Related to this, the association of Na-transport with chloride cells under freshwater conditions has recently become a point of discussion, since Goss *et al.* (1992) proposed a model in which pavement cells rather than chloride cells are involved in Na-uptake.

Ca^{2+}-transport

In contrast to Na^+/K^+-ATPase activity, Ca^{2+}-transport was inhibited in the purified plasma membrane preparation of Cd and Cu/Cd exposed fish, despite the increased

branchial chloride cell numbers. The inhibition of Ca^{2+}-transport capacity in mature fish exposed to Cd and Cu/Cd is consistent with the Cd-induced disturbed Ca-homeostasis observed by Larsson *et al.* (1981) and Pratap *et al.* (1989), and confirms the inhibitory effect of Cd^{2+} on active Ca^{2+} uptake by the gills of trout (Verbost, 1989). *In vitro* Cd exposure of gill membrane preparations has been shown to inhibit active Ca^{2+}-transport mechanisms (Bansal *et al.*, 1985; Verbost, 1989). There is good evidence that the degree of heavy metal-induced inhibition of Ca^{2+} activated enzyme activity was related to the free heavy metal ions available (Bansal *et al.*, 1985; Verbost, 1989). Although chelating agents were present during membrane purification and Ca^{2+}-transport measurement in our study, the metal-induced inhibition of Ca^{2+}-transport during *in vivo* metal exposure was still present during *in vitro* Ca^{2+}-transport determination. Obviously, there is no reactivation *in vitro* of the *in vivo* inhibited Ca^{2+}-transport mechanisms. Branchial Na^+ and Ca^{2+} transport differ from another in a number of ways, including the hormonal control and specific transport sites (McDonald and Wood, 1993). This also may contribute to the metal- and transport enzyme specific effects of Cu and Cd on transepithelial ion exchange (Nieboer and Richardson, 1980; Reid and McDonald, 1991), and is also in line with previous observations in tilapia, where active Na uptake, but not Ca uptake, was inhibited in fish exposed to 200 $\mu g.l^{-1}$ Cu (Pelgrom *et al.*, 1995[a]).

Heavy metals have a high affinity for nucleophilic groups, in particular for SH-residues of amino acids and proteins, and the heavy metal interaction with ion-ATPases has mainly been attributed to the affinity of these metals for sulfhydryl groups on the ion transporting enzymes (Jacobson and Turner, 1980).

In conclusion, the action of sublethal concentrations of waterborne Cu and Cd on the gills is metal- and transport enzyme specific, resulting in different physiological responses. Notably, the physiological response observed in fish exposed to combined Cu and Cd cannot be predicted on the basis of single metal exposures, as is shown by plasma ceruloplasmin levels, Na^+/K^+-ATPase activity and chloride cell numbers and diameter. Our data provide further evidence for the notion that the subcellular distribution rather than the total concentration of metals determine the toxic action of Cu and Cd. Overall however, there seems to be no apparent relation between metal accumulation in the gills, which in the Cu/Cd co-exposed group was additive, and the physiological response, which was not additive for any of the parameters studied.

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CHAPTER 7

SENSITIVITY OF TILAPIA (*OREOCHROMIS MOSSAMBICUS*) MELANOTROPE FUNCTION TO COPPER AND STRESS

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ABSTRACT

The effects of exposure to various concentrations of waterborne copper (Cu) and to a variable stress protocol on melanotrope function were studied in the teleost tilapia *In vivo* exposure to low (up to 75 μg Cu l^{-1}), but not to high (100 and 200 μg Cu l^{-1}) caused a decline in plasma α-MSH levels, as well as in lower *in vitro* release of α-MSH by the NILs, demonstrating that pituitary endocrine cells are targets for heavy metals The stress protocol also evoked an inhibition of *in vitro* α-MSH release, although it appeared to be more potent than Cu in this respect NIL (Neuro Intermediate Lobe) α-MSH content was elevated by stress but not by Cu When Cu and stress were administered simultaneously, an effect on the relative amounts of the various α-MSH forms was observed The mechanisms involved in the inhibition of the release are therefore different for the stressors studied This is supported by differences in brain Cu and Cd concentrations and hypothalamic ppMCH mRNA (preproMCH mRNA) levels between the experimental groups The data suggest that the observed inhibition of α-MSH release by the different treatments is at least partly communicated by higher brain centres

INTRODUCTION

Activation of the hypothalamus-pituitary adrenal axis (HPA-axis) in response to stressors is an integral part of adaptive regulations (Chrousos and Gold, 1992, Dallman, 1993) In fish, regulatory mechanisms similar to those in mammals have been identified (Donaldson, 1981), and

peptides derived from the prohormone proopiomelanocortin (POMC) appear to play pivotal roles in the regulation of corticosteroid production under stressful conditions Among these, ACTH (adrenocorticotropic hormone) (Sumpter *et al.*, 1986; Balm *et al.*, 1994), N-terminal peptide (O'Connell *et al.*, 1993), β-endorphin (Szalay and Folly, 1992; Balm *et al.*, 1995), but also α-MSH (α-melanocyte stimulating hormone) (Dell *et al* , 1982, Vinson *et al.*, 1983; Lamers *et al.*, 1992; Balm *et al.*, 1995) have been demonstrated to be corticotropic in mammals as well as in fish. In the course of our studies into melanotrope function in tilapia (*Oreochromis mossambicus*) acclimating to environmental challenges (Balm *et al.*, 1987, Lamers *et al* , 1994), preliminary observations indicated an effect of *in vivo* copper (Cu) exposure on α-MSH release. It has been known for some time that Cu is a stress factor in fish (El-Domiaty, 1987), and in a previous study with tilapia we observed increased plasma cortisol levels in Cu-exposed fish (Pelgrom *et al* , 1995[b]) Our observation led to the present study, which characterizes the effects of Cu on melanotrope function

Nowadays freshwater environments are commonly polluted by heavy metals such as copper (Cu), and (neuro-) endocrine organs such as adrenals and brains may accumulate significant amounts of Cu (Solbé and Cooper, 1976, El-Domiaty, 1987)

This study investigates plasma α-MSH levels, neurointermediate lobe (NIL) α-MSH contents, and basal and cAMP-stimulated α-MSH release *in vitro*, in fish exposed to various environmentally relevant concentrations of Cu In addition, HPLC (high performance liquid chromatography) analysis of the various forms of α-MSH released was carried out in the experimental groups, because the degree of acetylation of the hormone, which has been demonstrated to be regulated (Lamers *et al* , 1992), markedly affects its melanotropic, lipolytic, and corticotropic potencies (Rudman *et al* , 1983; Balm *et al* , 1987, Kishida *et al* , 1988)

There are several indications that stress interacts with metal metabolism in mammals as well as in fish, whereas in previous studies with tilapia, we observed Cu and Cd (cadmium) redistribution after metal exposure (Pelgrom *et al* , 1994, 1995[a]) Weber *et al* (1992) demonstrated metal redistribution in response to restrainment stress in largemouth bass In order to examine interactions between two different types of stimuli, the effect of a stress protocol on the melanotropes was compared in control and Cu exposed fish

One of the mechanisms of actions involved in peptide regulation may include the hypothalamus. Among the toxic effects of the metal described in mammals are impaired communication between hypothalamus and the pituitary (Kochman *et al* , 1992) To investigate whether this could also underly the effects of Cu on melanotropes in tilapia, hypothalamic ppMCH (prepro melanin-concentrating hormone) mRNA levels were measured in the experimental fish The neuropeptide MCH regulates both α-MSH release and acetylation (Groneveld *et al* , 1995) and modulates the HPA-response during stress in mammals as well as in fish (Baker, 1991)

MATERIALS AND METHODS

Fish

Tilapia (*Oreochromis mossambicus*) were obtained from our laboratory stock. Fish were kept, from 9 days after hatching, in artificial freshwater with undetectable Cu concentrations (detection levels below 0.1 μg Cu.l^{-1}). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8 Water was continuously aerated, filtered and refreshed by means of flow-through. The light/dark regime was 12/12 h and the water temperature was 25°C. Fish were fed commercial tropical fishfood (Tetramin™) at 2% $^{dw}/_{ww}$ per day.

Experimental design

Cu exposure

Six weeks before the start of an experiment, sexually mature female fish (mean weight 18 gram) were housed in groups of 13 fish in 80 l aquaria containing artificial freshwater which was continuously filtered and refreshed, by means of an Eheim circulation pump and a 16-channel peristaltic pump (Watson Marlow). The experiment started by switching the water supply from the aquaria to reservoirs either filled with artificial freshwater (controls) or with artificial freshwater with a well-defined Cu concentration (added as nitrate, Spectrosol, BDH, England). During the first 6 h, the flow rate was 4.5 l.h^{-1}. This was followed by a flow rate of 1.5 l.h^{-1}. In this way the Cu concentrations in the aquaria were gradually raised, and reached a plateau after 18 h (Pelgrom *et al* , 1994). The measured concentrations (by Atomic Absorption Spectrometry, AAS) in the aquaria deviated maximally 5% from the nominal concentrations (25, 50, 75, 100 or 200 μg Cu l^{-1}) The exposure times lasted 6 days After exposure, blood was collected (Balm *et al* , 1994) and fish were killed by spinal transection

Cu exposure and variable stress protocol

Six weeks before the start of the experiment, fish were divided into 4 groups of each 13 fish (2 controls and 2 Cu groups) Two identical climate rooms each housed one control group and a group exposed to Cu (Control and Cu group). During 6 days, the fish were exposed to 50 μg Cu l^{-1} (as described above) During this period, fish housed in one of the climate rooms were disturbed by either switching off the light, or confining them in a net, or by switching off the oxygen supply, or by chasing them with a net (stressors and Cu+stressors groups). Each disturbance lasted for 10 minutes they were performed in random order and at irregular intervals between 8 am and 5 pm daily Feeding and disturbances ended the day before sacrifice. After

exposure, fish were killed by spinal transsection, blood was taken and pituitary glands and hypothalami were dissected from the brain

Plasma

Blood samples were taken from the caudal vessels and centrifuged at 4°C Plasma glucose was determined spectrophotometrically using a D-glucose kit (Boehringer Mannheim, UV-method) The plasma was stored at -20°C until α-MSH assay

Incubation of the NILs

After a short pre-incubation, NILs (neurointermediate lobes), of the freshly dissected pituitaries, were incubated in 1 ml of a solution of 15 mM HEPES, 142 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ $2H_2O$, pH 7 38, with 0 25% (w/v) glucose and 0 03% bovine serum albumin (BSA, grade V, Sigma), at 28°C in a shaking waterbath (n=10 per group) During 6 h or 15 h of incubation, samples (10-30 μl) were taken from the incubation medium at regular intervals In the case of an initial 6 h period, the remaining medium was replaced by fresh medium with a double concentration of glucose (0 5%) After a subsequent 15 h incubation, the final medium sample was taken Samples were stored at -20°C until HPLC analysis and/or α MSH assay

Superfusion of the NILs

After static incubation, NILs were placed on a filter in a superfusion chamber (volume 10 μl) Two NILs per chamber were superfused with incubation medium (n=5 per group), at a continuous flow rate of 30 μl/min (Watson Marlow peristaltic pump) Fractions were collected at varying periods (Iso fraction collectors) After reaching a basal level of release, the medium was replaced by a medium containing 10^{-3} M 8-Br cAMP (8 bromoadenosine 3' 5'-cyclic monophosphate, Sigma) during 30 minutes Fractions were stored at -20°C until α-MSH assay

Preparation of NIL extract

After incubation and/or superfusion, NILs were homogenized in 500 μl ice-cold HCl (0 1 M) with a glass to-glass potter homogenizer The homogenates were centrifuged for 5 min at 10,000 g in an Eppendorf centrifuge The supernatants were stored at 20°C until α MSH assay

HPLC

Equal volumina of pooled NIL incubation media were subjected to reversed phase high performance liquid chromatography (HPLC, Spectra Physics SP8700, Eindhoven, The Netherlands), using a Spherisorb ODS column (Chrompack B V , Middelburg, The Netherlands) (Martens *et al* , 1982) α-MSH was eluted with a 1 propanol gradient (10-16%) in a buffer solution of 0 5 M formic acid/ 0 14 M pyridine (pH 3) The flow rate was 2 ml/min To the

fractions collected, 10μg BSA was added and the samples were dried in a Savant Speedvac concentrator and stored at -20°C until α-MSH assay. Shortly before the α-MSH assay, peptides were dissolved in 0 1 N HCl/MeOH (1·1, v/v) at 4°C The identification of the three forms of α-MSH has been described previously, and the recovery of α-MSH after HPLC was equal for all three forms of α-MSH (Lamers *et al* , 1991) The di/mono ratios are expressed as the ratio of the peak areas of diacetyl-α-MSH to monoacetyl-α-MSH

α-MSH RIA

Concentrations of α-MSH were determined in duplicate in a radioimmuno assay (RIA). The antiserum was raised against synthetic mono-acetyl α-MSH (Sigma, M4135, St. Louis, M.O , USA) and characterized in our laboratory (Van Zoest *et al* , 1989) The cross-reactivity with des- and di-acetyl α-MSH was 100%, with ACTH less than 0 5% The antiserum was used in a final dilution of 1 60,000 Bound and free labelled α-MSH were separated by polyethylene glycol precipitation of the immuno complex For plasma α-MSH measurement a secondary antibody was used for this species in a RIA system, described in detail by Balm *et al* (1995)

ppMCH mRNA measurement

Hypothalamic ppMCH mRNA levels were measured by dot blot analysis as described by Groneveld *et al* (1995) Briefly. total RNA was isolated by the acid guanidinium-thiocyanate phenol chloroform procedure (Chomczynski and Sacchi, 1987) RNA was resuspended in 5 x SSPE (1 x SSPE is 0 18 M NaCl, 0 01 M NaH_2PO_4, pH 7 4, 0 001 M EDTA), 7 4% formaldehyde solution, and blotted on nitrocellulose using a dot blot apparatus (BioRad) Dot blots containing a sense tilapia pMCH cRNA standard dilution series were hybridized with ^{32}P labelled antisense cRNA probe (Groneveld *et al* , 1993) Hybridization signals were quantified by densitometric scanning of the autoradiograms The values were converted by the sense pMCH cRNA standard curve to pg ppMCH mRNA per tissue

Statistics

Data are presented as means $\pm$ SEM To test differences between experimental groups, data were analyzed by the Mann-Whitney *U* test Significant differences between control and Cu exposed fish are indicated by asterisks * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, and ****: $P < 0.001$ Effect of the variable stress protocol is indicated by circles Significant differences between controls and stressed fish under control water conditions (control vs stressors) are indicated by closed circles (●), whereas open circles (○) indicate significant differences between Cu and Cu+stressors fish

RESULTS

During the experiments, no mortality was observed, and there were no differences in body weight and feeding behaviour between control and experimental groups

Plasma α-MSH concentrations were decreased in fish exposed to low Cu concentrations (25, 50 and 75 μg Cu.l^{-1}). Higher Cu concentrations (up to 200 μg.L^{-1}) had no effect (Fig. 1).

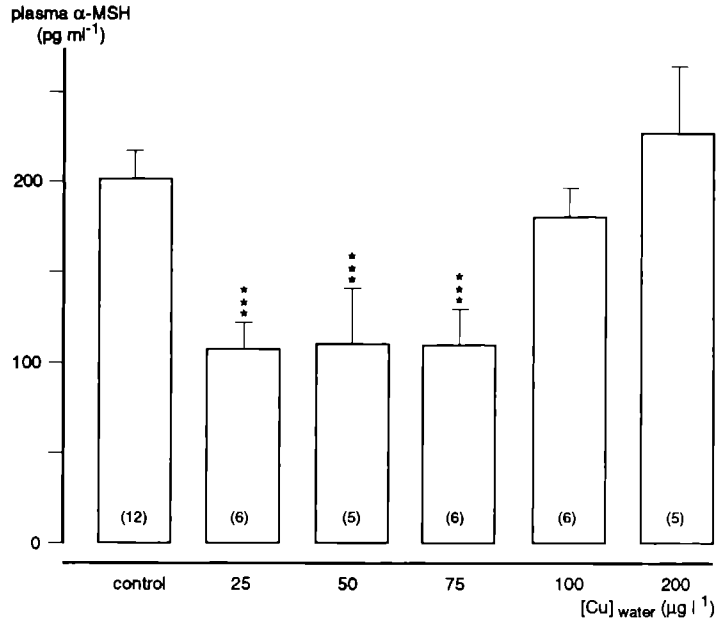

Figure 1. Plasma α-MSH concentrations (in pg per ml) in fish exposed during 6 days to 0 (control), 25, 50, 75, 100 or 200 μg Cu l^{-1} in the water Significant differences between controls and Cu-exposed fish are indicated by asterisks, and the n-values are indicated in the bars

During *in vitro* incubation of NILs, at all time intervals significantly lower amounts of α-MSH were released by the NILs of fish exposed during 6 days to 50 μg Cu l^{-1} compared to controls (Fig. 2). Analysis of the incubation medium after 6 h of incubation by HPLC showed that the profile of α-MSH immunoreactivity released by Cu-exposed fish is comparable to that of control fish (Fig. 3), as demonstrated by the similar ratios of di-acetyl α-MSH / mono-acetyl α-MSH (di/mono). After a subsequent 15 h incubation, the unstimulated release of α-MSH was still lower

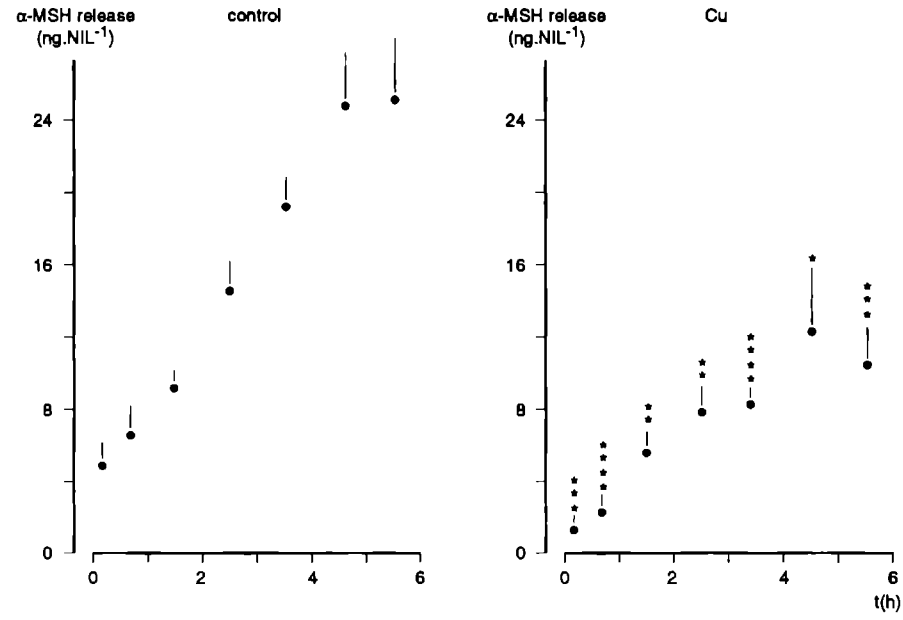

Figure 2. In vitro α-MSH release by NILs of control fish (left panel) and of fish exposed during 6 days to 50 μg Cu.l^{-1} (right panel). Significant differences between controls and Cu-exposed fish are indicated by asterisks, with n=10 for each time point.

($p<0.05$) by tissue from Cu-exposed fish, as shown by superfusion (Fig. 4). Administration of 10^{-3} M 8-Br-cAMP during 30 minutes resulted in an increased α-MSH release by NILs of both control and Cu-exposed fish, but compared to controls, the maximally stimulated α-MSH release by Cu-exposed fish (in pg.min^{-1}.NIL^{-1}) remained lower ($p<0.02$). In controls, α-MSH release returned to baseline levels of α-MSH release within 20 minutes after termination of the pulse. In Cu-exposed fish however, the α-MSH release after 8-Br-cAMP stimulation remained elevated for at least 50 minutes. The total amount of α-MSH released in response to 8-Br-cAMP by controls and Cu-exposed fish (area under the curve) were not significantly different (Fig. 4), nor was the α-MSH content of the NILs after superfusion (Fig. 5). The Cu contents of the brains (central nervous system, CNS) were similar in control and Cu-exposed fish. However, the Cd concentration in the CNS of Cu-exposed fish was significantly higher than in controls (Fig. 5). Cu and Cd concentrations of the pituitary gland could not be determined, since in a pooled sample of 10 pituitaries they were below the detection limit of 0.10 and 0.01 μg.g^{-1}, respectively.

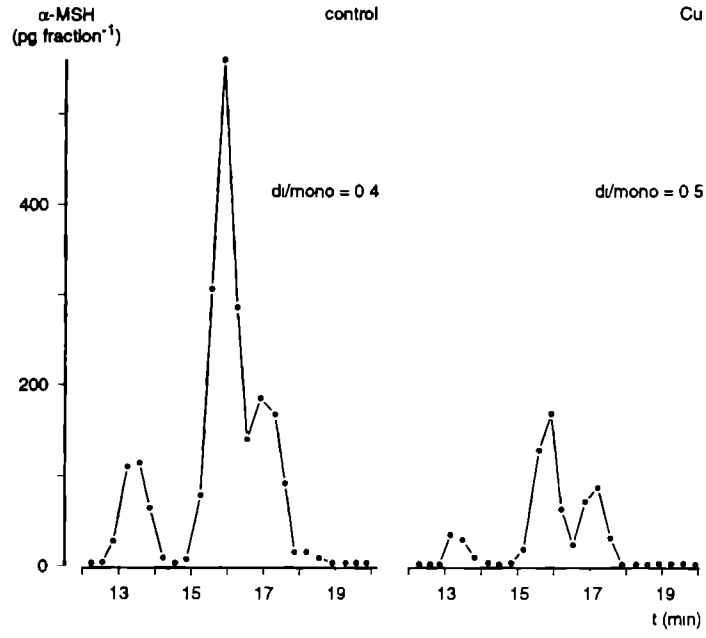

Figure 3 HPLC profiles of the pooled medium after 6 h in vitro incubation of NILs of control fish (left) and of fish exposed during 6 days to 50 µg Cu l⁻¹ (right) Ratios of di-acetyl α-MSH/mono-acetyl α-MSH are indicated in the figure by di/mono

The effects of a stress protocol were compared in controls and Cu-exposed fish Under control and Cu conditions, the amounts of α-MSH released by the NILs after 15 h of *in vitro* incubation, as well as after 6 h of incubation (data not shown), were significantly lower than in non-stressed fish (Table 1) The reverse was found for the NIL contents Stress in control fish resulted in higher Cd concentrations in the brain compared to non-disturbed control fish, whereas in Cu+stressors fish the Cd concentration were lower than in Cu-exposed fish left undisturbed. No differences in plasma glucose concentrations were observed between the groups (Table 1) HPLC analysis of α-$MSH_{1,7}$ released after 6 h of incubation by the NILs, showed that Cu+stressors resulted in relatively more release of di-acetyl α-MSH, as indicated by a shift of

the di/mono ratio compared to the other groups (Fig 6) Exposure to the variable stress protocol had no effect on total ppMCH mRNA content in the hypothalamus of control fish, but markedly reduced total ppMCH mRNA contents in Cu treated fish (Fig 7)

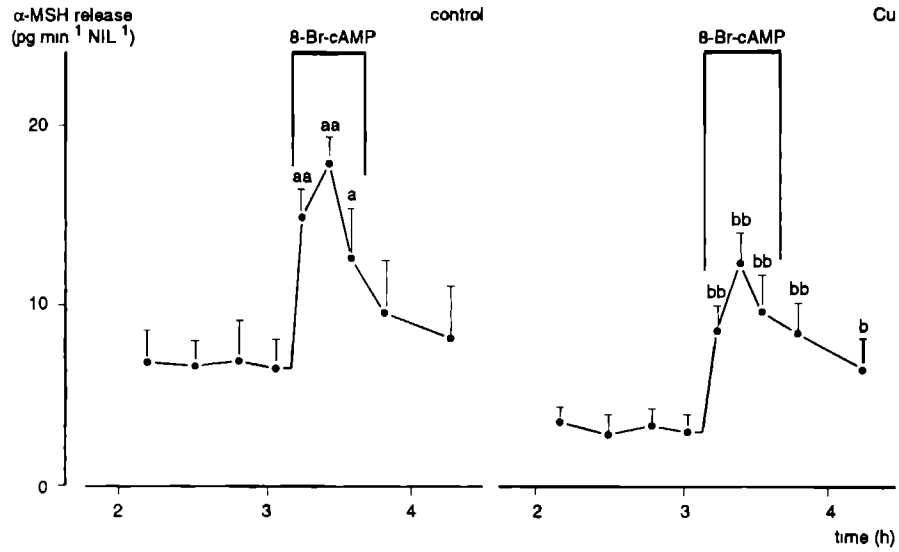

Figure 4 α-*MSH release (in pg per minute per NIL) during in vitro superfusion of the NILs of control fish (left) and of fish in vivo exposed to 50 μg Cu l^{-1} (right) Compared to controls, the basal release in Cu-exposed fish was significantly lower Secretagogue stimulated* α-*MSH release was investigated by means of stimulation during 30 minutes with 10^{-3} M 8-Br-cAMP Significant differences between basal release and 8-Br-cAMP stimulated release are indicated in the controls by* **a**, *and in the Cu-group by* **b**, *with* $n=5$ *for each point*

DISCUSSION

A decline in plasma α-MSH levels, observed in fish exposed to 50 μg Cu l^{-1}, was accompanied by lower (unstimulated) α-MSH release by the NILs Initially, it has been shown that in fish (Sumpter *et al* , 1986, Lamers *et al* , 1992) and in mammals (Carr *et al* , 1990) stressful situations resulted in an increased α-MSH release Recently, however, Balm *et al* (1995) and

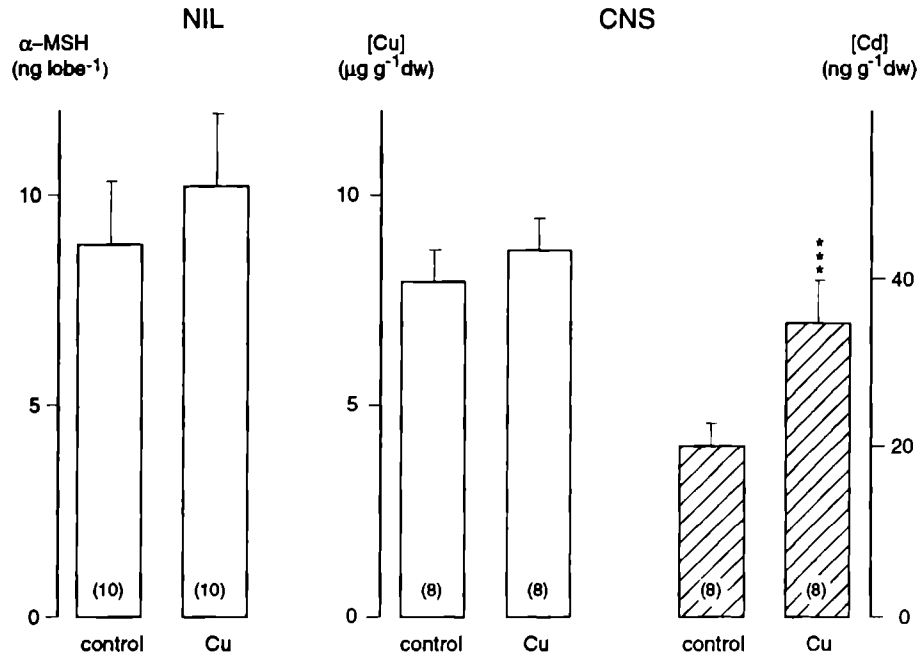

Figure 5 α-MSH contents of the NILs (in ng per lobe) of control fish and of fish exposed to 50 µg Cu l⁻¹ during 6 days (left panel) Cu concentration (in µg per gram dry weight open bars) and Cd concentration (in ng per gram dry weight, shaded bars) in the CNS (brains) of control fish and fish exposed during 5 days to 50 µg Cu l⁻¹ Significant differences are indicated by asterisks, and n values are indicated in the bars

Balm and Pottinger (1995) observed lower plasma α MSH levels in confined tilapia and trout respectively These observations, together with our present results, indicate that the response of the MSH cells to environmental challenges is stressor-specific, rather than an uniform response HPLC profiles of the α-MSH release did not show differences between Cu exposed and control fish It has been demonstrated previously that tilapia, in response to environmental challenges, can not only modulate the quantity but also the quality of the α-MSH released by the NIL, whereas total, and in particular di-acetyl α-MSH levels in the plasma, were increased (Lamers *et al* , 1992) Acetylation of α-MSH has been shown to enhance its potency with respect to its lipolytic, melanotropic and corticotropic actions (Rudman *et al* , 1983, Balm *et al* , 1987, Kishida *et al* , 1988, Lamers *et al* , 1992) In our study, we did not observe a shift of the mono/di acetylation ratio of α-MSH to the more potent di-acetylated form in Cu-exposed fish.

		control	stressors	Cu	Cu +stressors
NIL α-MSH release	(ng)	17 3 ± 4 6 (10)	●●●● 0 3 ± 0 1 (8)	*** 11 5 ± 5 0 (10)	○○○○ 0.4 ± 0 2 (7)
NIL α-MSH content	(ng)	9 09 ± 4 2 (8)	● 20 2 ± 3 3 (7)	11 7 ± 3 1 (10)	○ 23 9 ± 7 3 (7)
$[Cu]_{CNS}$	($\mu g\ g^{-1}$ dw)	7 6 ± 0 5 (8)	8 0 ± 0 6 (7)	7 0 ± 0 5 (8)	** 6 3 ± 0 4 (7)
$[Cd]_{CNS}$	($ng\ g^{-1}$ dw)	22 0 ± 0 4 (8)	●●●● 45 0 ± 6 0 (7)	**** 49 0 ± 5 0 (8)	* ○○○ 30 0 ± 6 0 (7)
$[glucose]_{plasma}$	(mg %)	77 4 ± 7 7 (8)	67 6 ± 5 8 (8)	82 3 ± 11 6 (7)	80.1 ± 16 2 (8)

Table 1. *Effects of a stress protocol were investigated on α-MSH release by the NILs, the α-MSH contents of the NILs, Cu and Cd concentrations in the CNS and plasma glucose concentrations in control fish and fish exposed to 50 μg Cu l^{-1} during 6 days Significant differences between controls and Cu-exposed fish and between stressors exposed fish and Cu+stressors exposed fish are indicated by asterisks Significant differences between controls and the stressed group are indicated by closed circles (●), whereas open circles (○) indicate significant differences between Cu and Cu+stressors exposed fish*

Although the melanotropes in Cu-exposed fish were inhibited, Cu-exposure did not result in desensitization of the NILs to 8-Br-cAMP. A comparable phenomenon was observed in tilapia after *in vivo* treatment with interleukin-1, where inhibition of the basal α-MSH release was not accompanied by desensitization of the NILs to TRH (Thyrotropin Releasing Hormone) stimulation (Balm *et al.*, 1993). Our observation that α-MSH after Cu-exposure was released to the same extent as in controls in response to a secretagogue, whereas plasma and basal *in vitro* α-MSH levels were lower, may point to a Cu-induced inhibition of α-MSH release rather than an inhibited production of the peptide. This is supported by our observation that the α-MSH content of the NILs of Cu-exposed fish did not differ from that of controls.

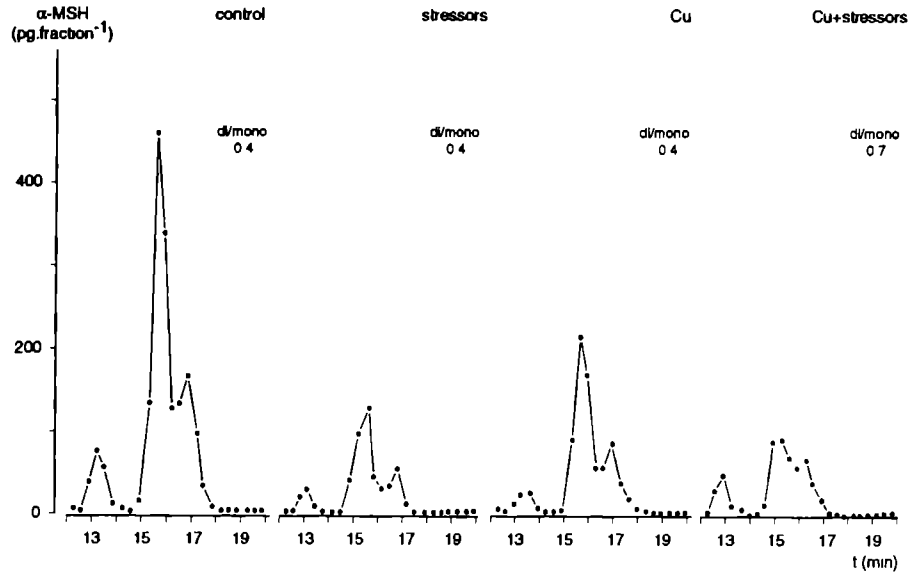

Figure 6. *HPLC profiles of the pooled medium after 6 h of in vitro incubation of NILs of control fish, stressors exposed fish, Cu-exposed fish (50 μg.l⁻¹; 6 days) and Cu+stressors exposed fish. Ratios of di-acetyl α-MSH/mono α-MSH are indicated in the figure by di/mono*

Several studies have demonstrated that an increased heavy metal burden adversely affects fish (Buckley *et al.*, 1982; Pelgrom *et al.*, 1995[b]) as well as mammals, although the route of uptake is different. However, there are few studies on the (toxic) effects of heavy metals on the pituitary in fish and mammals. Studies have mainly focused on the functional and structural changes in the gonads and adrenals (Walker and Cooper, 1992), which likely result from pituitary dysfunction. Several mechanisms may be involved in Cu-induced effects on pituitary peptide regulation. First, Cu plays an important role in the mechanisms of action of brain peptides, by mediating the interaction between the peptide hormone and its receptor molecule. Kochman *et al.* (1992) showed that Cu complexed with luteinizing hormone releasing hormone (LHRH) resulted in a higher release of luteinizing hormone (LH) than LHRH alone, which illustrates the necessity of well-controlled Cu concentrations in the brain. In our Cu-exposed fish however, Cu did not

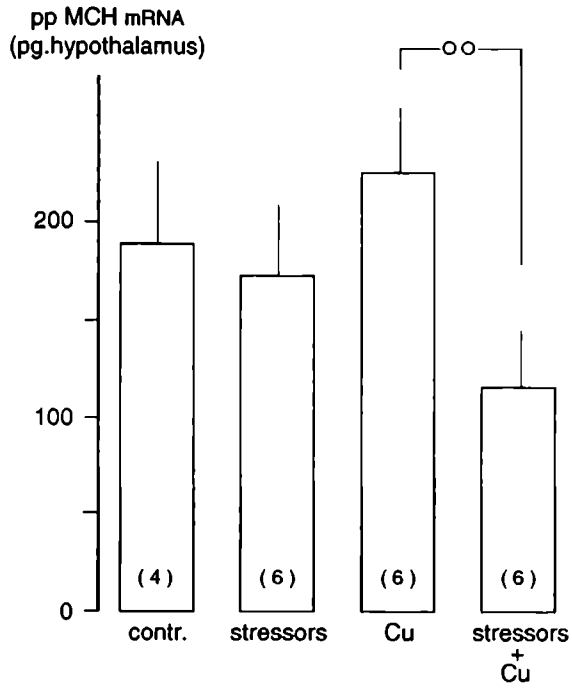

Figure 7. Hypothalamic ppMCH mRNA levels (in pg per hypothalamus) of controls, stressors exposed, Cu-exposed (50 µg.l^{-1}; 6 days) and Cu+stressors exposed fish. Significant differences between Cu-exposed and Cu+stressors exposed fish are indicated by open circles (○) The n-values are indicated in the bars.

accumulate in the brain. However, the brains of Cu-exposed fish contained more Cd, which may be a second interfering mechanism with neuro-endocrine communication. It has been suggested that a primary action of Cd on cellular function involves alteration in the calcium flux across the membrane and subsequently the intracellular messenger system involved in the stimulus-coupled pituitary hormone release (Cox and Harrison, 1983). Shibuya and Douglas (1992) demonstrated that Ca channels in rat melanotropes are permeable to Cd. A negative effect of Cd on the pituitary has been reported by Winstel and Callahan (1992), who showed that Cd *in vitro* inhibited TRH-induced prolactin secretion. Therefore, metal exposure probably affects pituitary function, and the effect may not be limited to the melanotropes. In our *in vivo* Cu studies we also observed that pituitaries from fish exposed to Cu (which possessed increased brain Cd concentrations) released lower amounts of prolactin *in vitro* than pituitaries from control fish (Balm, Pelgrom and Prunet; unpublished observations). A third factor that may play a role in Cu-

induced effects on pituitary peptide regulation is the involvement of the Cu-binding metalloprotein ceruloplasmin (Schreiber and Přibyl, 1980) Plasma ceruloplasmin levels are not only affected by exposure to Cu (Pelgrom *et al* , 1995[a]), but also by various hormonal factors (Evans *et al* , 1970). Schreiber and Přibyl (1980) suggested that ceruloplasmin at the level of the hypothalamus plays a role by inactivation of dopamine, a potent inhibitor of the α-MSH release from the NILs in mammals (Tilders *et al* , 1985) and fish (Lamers *et al* , 1991) In this way, and by binding of excess Cu at higher concentrations during Cu exposure, ceruloplasmin may play a protective role in the neurotoxic effect of Cu at higher Cu levels This may, partly, explain the absence of lower plasma α-MSH levels at higher Cu concentrations, since ceruloplasmin is induced at Cu concentrations higher than 50 $\mu g.l^{-1}$

In both control and Cu-exposed fish the variable stress protocol inhibited *in vitro* α-MSH release by the NILs, even more pronounced than Cu exposure alone Since the NILs of the stressors exposed fish contained significantly more α-MSH than the non stressed fish, the stressors likely primarily affect the release rather than the synthesis of the peptide, which makes a difference between the melanotrope response to Cu exposure alone In contrast to our results, studies on the α-MSH response to short-term disturbance stress in trout resulted in no effect (single stressor) or increased plasma α-MSH levels (combination of stressors) (Sumpter *et al* , 1986) Perhaps the exposure regime in our study may account for the observed inhibition of the α-MSH release Unlike acute exposures to extreme conditions, the present stress-protocol was moderate and chronic Unchanged plasma glucose levels corroborate the relative moderate response to the stress-protocol in this study

Analysis of $\alpha\text{-MSH}_{1-r}$ released by the NILs showed that, although the direction of the α MSH response to the variable stress protocol in the presence and absence of Cu was similar, the secretory signal differed between the groups stressors together with Cu, but not under control conditions, resulted in a shift of the di/mono acetyl-α MSH ratio This indicates that the regulating mechanisms underlying the inhibition of the α-MSH response in stressors exposed and Cu+stressors exposed fish is of different origin This suggestion is supported by the results on Cu and Cd concentrations in the CNS Whereas the variable stress protocol, or Cu-exposure, had no effect on the Cu concentration in the CNS, interaction between Cu and stressors resulted in lower Cu levels in the CNS of Cu+stressors exposed fish Interaction between Cu and the variable stress protocol was also observed in the Cd content of the CNS, where the Cu or the stressors singly increased the Cd concentration whereas such an increase was not observed in the Cu+stressors exposed group We propose, that it is not unlikely that the stressors-induced changes in Cu and Cd concentrations in the CNS interferes with the control of α-MSH release by the NILs as discussed above

The observed lower ppMCH mRNA levels in the hypothalami of Cu+stressors exposed fish

are consistent with a role of the CNS in stressor induced effects on the melanotropes. From several studies it is known, that MCH is involved in the regulation of the stress response of the HPA-axis (Baker *et al* , 1985, 1986, Baker, 1991), possibly through regulating the release of α-MSH by the pituitary (Barber *et al* , 1987) Interestingly, MCH *in vitro* also has an effect on acetylation (Groneveld *et al* , 1995), and Cu-exposure in combination with exposure to the variable stress protocol appeared to be the only treatment that affects both ppMCH mRNA levels and di/mono α-MSH ratio In accordance with observations of others, this study shows that the variable stress protocol probably has a multifactorial effect on the MCH neurons, and the responses are dependent on the regime of stressor exposure

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CHAPTER 8

THE PHYSIOLOGICAL RESPONSE OF TILAPIA (*OREOCHROMIS MOSSAMBICUS*) TO (NON-METAL) STRESSFUL STIMULI IS COMPROMISED BY WATERBORNE HEAVY METALS

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ABSTRACT

To study the physiological responses of fish to a combination of environmental challenges, we exposed tilapia (*Oreochromis mossambicus*) for 6 days to a various stress protocol (stressors) and to waterborne heavy metals In fish exposed to either the various stress protocol or to heavy metals, we did not observe changes in plasma Na, Cl, Ca, glucose or cortisol concentrations. In fish exposed to the stressors + metals, however, these parameters were significantly changed.

Plasma Cu and Cd concentrations were not different from control values in either stressors exposed or metals exposed fish, but were lower in fish exposed to stressors + metals

Not only heavy metals exposure affected tissue Cu and Cd concentrations, but in response to the variable stress protocol we also observed redistribution of Cu and Cd in the kidneys, brain and head kidney In the fish exposed to the stressors + metals, the Cd concentration in all organs studied, except for the gills, was higher than in the metals exposed fish, which indicates that exposure to the variable stress protocol affected metal uptake/redistribution during metal exposure

Metal exposure resulted in lower branchial Ca^{2+}-transport activity only, whereas exposure to the stressors and to stressors + metals resulted in lower branchial Ca^{2+}-transport activity and Na^{+}/K^{+}-ATPase activity

Our results indicate that the physiological response in fish exposed to a combination of environmental challenges can not be predicted from results obtained from studies on fish exposed to the challenges singly

INTRODUCTION

In freshwater fish, physiological homeostasis is adversely affected by stressors, such as predators, capture and netting. The response to stressors is characterized by an increased activity of the hypothalamus-pituitary-interrenal (HPI) axis (primary response), which in turn affects energy mobilization, acid-base and hydromineral balances, growth, and reproduction (secondary response; Eddy, 1981; Donaldson, 1981; Schreck, 1981; Heath, 1991). These responses enable the fish to compensate for the adverse effects of a sublethal stressor (Mazeaud *et al.*, 1977; Schreck, 1981). In salmonid fish, stressful stimuli have been shown to initially result in decreased plasma sodium and chloride concentrations (McDonald and Robinson, 1993), which are accompanied by increased levels of circulating epinephrine (Mazeaud and Mazeaud, 1981). Epinephrine has been shown to have a profound effect on diffusional losses of NaCl across the gills, by changing the branchial haemodynamics and by increasing the gill ionic permeability (McDonald and Rogano, 1986). However, in spite of compensating mechanisms, in the period between impaired homeostasis and compensation the performance capacity of the fish is generally reduced (Schreck, 1981). A stressor may not only challenge the capacity of the exposed fish to maintain homeostasis, but also reduce their ability to successfully counteract a second stressor. This phenomenon has been demonstrated in studies with fish exposed to a combination of stressors (Larsson *et al.*, 1984; Barton *et al.*, 1985; Gill and Epple, 1993; Pilgaard *et al.*, 1994). To further improve the understanding of the effects of combined stressors fish may experience in real life, the present study investigates several aspects of the performance capacity of fish faced concurrently with two different types of stressors: simultaneous exposure to a combination of non-metal stressors (further called the variable stress protocol) and to sublethal concentrations of metals. Nowadays, freshwaters are commonly contaminated by heavy metals such as copper (Cu) and cadmium (Cd). When the concentrations of waterborne metals have reached critical concentrations, they influence the structural organisation of the gill tissue, and challenge osmotic- and ionic regulation (Eddy, 1981; Stagg and Shuttleworth, 1982; Pratap and Wendelaar Bonga, 1993; Pelgrom *et al.*, 1995[a]). Since both the non-metal stressors as well as the heavy metals have been shown to challenge the hydromineral balance (Soivio and Oikari, 1976; Wendelaar Bonga and Lock, 1992; Pelgrom *et al.*, 1995[a,b]), the gills may be implicated as the target organ for interaction (Postlethwaite and McDonald, 1995). Branchial ion-transport are regulated by hormones such as cortisol, an interrenal hormone released in the stress response (Mazeaud *et al.*, 1977).

Although heavy metals have been shown to induce signs of stress under certain conditions (acute exposure to relatively high concentrations, immature fish), we applied conditions of waterborne metals that do not result in a stressed state, as concluded from earlier studies with

mature tilapia. To study the response of the experimental fish, we measured chloride cell numbers, active Ca^{2+}-transport capacity, and Na^+/K^+-ATPase activity in the gills. Since not only heavy metal exposure but also stress induced by non-metal stressors affect heavy metal concentrations in, and hence functioning of, organs of fish (Weber *et al.*, 1992; Pelgrom *et al.*, 1995[c]), we studied interactions between the stress treatment and metals on blood composition and heavy metal concentrations in gills and other selected organs of tilapia.

MATERIALS AND METHODS

Fish

Tilapia (*Oreochromis mossambicus*) were obtained from our laboratory stock. Fish were kept, from 9 days after hatching, in artificial freshwater with undetectable Cu and Cd concentrations (detection levels below 0.1 and 0.01 $\mu g.l^{-1}$, respectively). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8. Water was continuously aerated, filtered and refreshed by means of flow-through. The light/dark regime was 12/12 h and the water temperature was 25° C. Fish were fed commercial tropical fishfood Tetramin™ at 2% $^{dw}/_{ww}$ per day. The Cu and Cd contents of the food were: 9.86 ± 0.16 μg $Cu.g^{-1}$ dry food and 0.22 ± 0.01 μg $Cd.g^{-1}$ dry food (means ± SE; n = 10).

Experimental design

Six weeks before the start of the experiment, fish (mean weight 18 gram) were divided into four groups of 13 fish (2 controls and two metal groups). Two identical climate rooms each housed one control and one metals treated group in 80 l aquaria. The artificial freshwater was continuously refreshed by means of a multi-channel peristaltic pump (Watson Marlow). The experiment started by switching the water supply from the aquaria reservoirs either filled with artificial freshwater (controls) or with artificial freshwater with well-defined Cu and Cd concentrations (added as nitrate, Spectrosol, BDH, England). During the first 6 h, the flow rate was 4.5 $l.h^{-1}$, followed by a flow rate of 1.5 $l.h^{-1}$. In this way the Cu and Cd concentrations in the aquaria were gradually raised, reaching a plateau after 18 h (Pelgrom *et al.*, 1994). The daily measured concentrations (by Atomic Absorption Spectrometry, AAS) in the aquaria deviated maximally 5% from the nominal concentrations (50 μg $Cu.l^{-1}$ and 20 μg $Cd.l^{-1}$). Exposure lasted 6 days, and during this period the fish housed in one of the climate rooms were subjected to a variable stress protocol in which the fish were intermittently stressed by either switching off the

light, confining them in a net, by switching off the oxygen supply or by chasing them with a net. Each treatment lasted 10 minutes and was performed daily in random order and at irregular intervals between 8^{am} and 5^{pm}. In this way, we created 4 experimental groups of fish: 1: controls (fish neither exposed to the stress protocol nor to the metals), 2: stressors (fish exposed to the variable stress protocol), 3: metals (fish exposed to Cu/Cd), 4: stressors + metals (fish exposed to the variable stress protocol and to Cu/Cd). Feeding and stress treatment ended the day before sacrifice. After 6 days of Cu/Cd exposure, fish were killed by spinal transsection, blood was taken and organs were dissected. Unstressed groups of fish in climate room 1 were sampled first, one control and one Cu/Cd exposed fish taken alternately. Thereafter, blood was sampled in the same manner from the stressed groups of fish in climate room 2. From all fish, the left opercula were prepared for chloride cell counting after incubation with the fluorescent dye DASPEI (ICN Biochemicals Inc., Plainview, NY; Wendelaar Bonga *et al.*, 1990) The opercular epithelium is an extension of the epithelium covering the gills, and the opercular chloride cell numbers reflect the gill chloride cell number (Wendelaar Bonga *et al.*, 1990) The gills were prepared for either Cu and Cd measurement or plasma membrane isolation

Plasma

Blood samples were taken from the caudal vessels by means of heparinized capillaries, centrifuged at 4°C, and haematocrit values (%) were determined Concentrations of plasma Na were measured with a flame-photometric Auto Analyzer Model IV, Technicon), while the Cl concentrations were determined spectrophotometrically by the forming of ferrothiocyanate. Plasma protein concentrations were determined by means of a reagent kit (Biorad) with BSA (Sigma) as a reference. Plasma glucose was determined spectrophotometrically using a D-glucose kit (Boehringer Mannheim, UV-method). The cresolphtalein complexone method (Sigma Diagnostics) was used for the determination of total plasma Ca concentrations Cortisol levels were determined by Radio Immuno Assay (RIA) as described by Balm *et al.* (1994)

Cu and Cd measurement

Plasma, gills, kidneys, brains and head kidneys were weighed and lyophilized. After determination of the dry weights, the tissues were digested with nitric acid (65% HNO_3 Ultrapur, Merck), and stored in 0.2% HNO_3 at 4°C until analysis (see Pelgrom *et al* , 1994, 1995°). Water samples were acidified to a final concentration of 0 2% HNO_3 Cu and Cd concentrations were determined with a flameless Atomic Absorption Spectrometer (AAS, Philips PU 9200) connected to an electrothermal atomizer (Philips PU 9390X)

Isolation of plasma membranes

Plasma membranes of the branchial epithelia were isolated at 4°C based on the method described by Flik *et al.* (1985), with some adjustments. This procedure leads to a good enrichment of the Na^+/K^+-ATPase and Ca^{2+}-ATPase: the membrane vesicles are leaky and the degree of mitochondrial contamination is low. Briefly, the soft tissue of the gills was scraped off with a glass microscope slide, and carefully homogenized with a glass-to-glass Dounce homogenizer (10 strokes) in an isotonic buffer containing 250 mM sucrose, 12.5 mM NaCl, % mM HEPES/TRIS pH 7.5, 0 1 mM EDTA, 100 U.ml^{-1} aprotinin (Sigma) and 50 U ml^{-1} heparin. Nuclei and cellular debris (pellet P_0) were separated from membrane fractions (supernatant H_0) by centrifugation during 10 minutes at 550 g (Hereus). After centrifugation of the supernatant H_0 (50,000 rpm, 30 minutes, Beckmann Ultracentrifuge, Ti 70 rotor), membranes were collected in a fluffy pellet (P_1) This pellet was resuspended with a glass-to-glass Dounce homogenizer (100 strokes) in an isotonic sucrose buffer containing 250 mM sucrose, 5 mM HEPES/TRIS pH 7.5 and 5 mM $MgCl_2$. The membrane suspension was centrifuged differentially. 10 minutes at 1,000 g followed by 10 minutes at 9,500 g (Sorval RC-5B). Finally, the supernatant was centrifuged during 15 minutes at 20,000 g, resulting in the final membrane fraction, pellet P_3. These pellets P_3 were resuspended by passage through a 23-G needle (10 times) in a buffer containing 20 mM HEPES/TRIS pH 7 4, 1.5 mM $MgCl_2$ and 150 mM KCl (for Ca^{2+}-transport study) or 150 mM NaCl (for Na^+/K^+-ATPase study) Membrane preparations P_1 and homogenates H_0 were quickly frozen in cold CO_2/acetone, and used the next day for determination of protein content, enzyme- and transport-activity

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity in the H_0 and P_3 gill membrane fractions were determined by the method described by Flik *et al* (1985) Routinely, 0.20 mg ml^{-1} saponin was added to optimize substrate accessibility Membrane protein content was determined with a reagent kit (Biorad), using Bovine Serum Albumin (BSA, Sigma) as reference Vesicles were incubated during 10 minutes at 37°C with medium containing 100 mM NaCl, 30 mM Imidazole, 0.1 mM EDTA, 5 mM $MgCl_2$ and either 15 mM KCl or 1 mM ouabain Na_2ATP was added in a final concentration of 3 mM The reaction was stopped by adding ice-cold TCA-solution. Inorganic phosphate (P_i) production was measured by the colorimetric Fiske-Subbarow technique using a commercially (Sigma) phosphate standard

Ca^{2+}-transport

ATP-dependent Ca^{2+}-transport was determined by means of a rapid filtration technique as described by Van Heeswijk *et al.* (1984) Ca^{2+} and Mg^{2+} concentrations were calculated according to Schoenmakers *et al* (1992) using the computer program CHELATOR. Ca^{2+}-

transport was measured at a Ca^{2+} concentration of 10^{-6} M (V_{max}) Uptake of $^{45}Ca^{2+}$ into membrane vesicles (P_3-fraction) was determined during 1-minute incubations in the absence or presence of 3 mM ATP (Tris-ATP). The reaction was stopped in ice-cold isotonic medium containing 0.1 mM $LaCl_3$, and the suspension was filtered (Schleicher & Schull ME 25, pore size 0 45 μm) Filters were rinsed twice with ice-cold medium and transferred to counting vials and dissolved in Aqualuma® ^{45}Ca was determined in a Pharmacia Wallac 1410 liquid scintillation counter

Statistics

Results are presented as means ± SE Differences between groups were tested by the Mann-Whitney *U*-test Significant differences between controls and stressed fish or between controls and metal-exposed fish are indicated by asterisks Significant differences between stressors and stressors + metals groups are indicated by closed circles (●), and between metal-exposed fish and stressors + metals exposed fish are indicated by **a**

* (● **a**) $P<0$ 05, ** (●● **aa**) $P<0$ 02, *** (●●● **aaa**) $P<0$ 01, **** (●●●● **aaaa**) $P<0$ 001

RESULTS

During the experiment no mortality and no differences in body weight and feeding behaviour between control and experimental groups were observed

Compared to controls, neither the variable stress protocol (stressors) nor metal exposure had effects on plasma Na and Cl concentrations, whereas these levels were significantly lower in stressors + metals exposed fish (Fig 1A and 1B) Exposure to the variable stress protocol resulted in higher plasma protein levels (Fig 1D) and an increase in the opercular chloride cell numbers (Fig 1C), in the absence as well as in the presence of metals, whereas metal exposure alone had no effect on these parameters In metal exposed fish, the mean diameter of the chloride cells was smaller than the cells in control fish (10 06 ± 0 14 μm and 10 63 ± 0 22 μm, respectively, with $n=6$ per group, $P<0$ 05)

Exposure to the variable stress protocol or to waterborne metals had no effect on blood haematocrit values and plasma concentrations of cortisol, glucose, total Ca, Cu and Cd (Table 1) Exposure to the variable stress protocol + metals resulted in an increase of the haematocrit and plasma Ca concentration and a lower plasma cortisol level Cu and Cd concentrations in the plasma were significantly lower in this group compared to the stressed group (plasma Cd) or

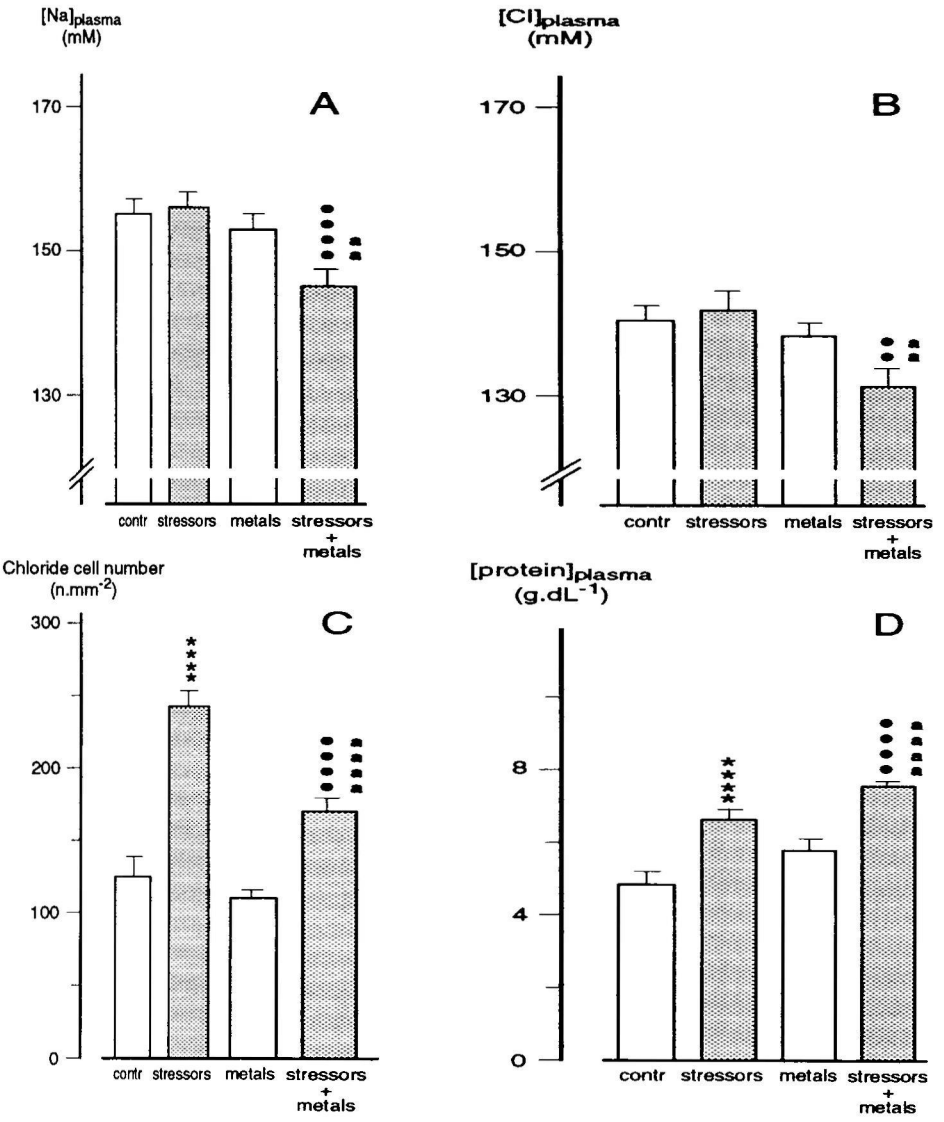

Figure 1. Plasma levels of sodium (A), chloride (B) and protein (D) (n = 12 per experimental group), and the opercular chloride cell number (C) (n = 6 per experimental group) of fish exposed to a variable stress protocol under control and waterborne metal conditions (6 days, 50 µg $Cu.l^{-1}$ + 20 µg $Cd.l^{-1}$, Cu/Cd). Significant differences between controls and stressors exposed fish are indicated by asterisks, between stressors exposed and stressors + metals exposed fish by closed circles (●), and between metals and stressors + metals by a.

metal exposed fish (plasma Cu and Cd).

Metal exposure or the variable stress protocol, singly or in combination, had no effect on the Cu concentrations in the brain (CNS) and head kidneys (Table 2). In the gills of metal exposed fish, an increase in the Cu concentration was observed, which was highest in the group exposed to stressors + metals. Compared to controls, the Cu concentration in the kidneys of the stressed group was significantly lower, whereas the group exposed to stressors + metals had higher Cu levels compared to the Cu content observed in the stressed group.

		control	stressors	metals	stressors+ metals
$[cortisol]_{plasma}$	($ng.ml^{-1}$; n=5)	94.6 ± 26.7	109.0 ± 14.3	86.2 ± 38.5	64.6 ± 14.0 ••
Haematocrit	(%; n=12)	32.6 ± 0.6	31.6 ± 1.2	32.9 ± 1.0	35.1 ± 1.4 ••
$[glucose]_{plasma}$	(mg%; n=5)	66.6 ± 5.5	61.4 ± 7.7	84.6 ± 9.6	84.4 ±11.4
$[Ca]_{plasma}$	(mM; n=8)	3.7 ± 0.2	3.6 ± 0.2	4.0 ± 0.3	6.6 ± 1.0 •[a]
$[Cu]_{plasma}$	($\mu g.l^{-1}$; n=6)	753 ± 71	711 ± 55	746 ± 33	652 ± 29[a]
$[Cd]_{plasma}$	($\mu g.l^{-1}$; n=6)	11.2 ± 1.0	13.9 ± 2.8	12.2 ± 0.8	8.6 ± 1.3 •[a]

Table 1. *Haematocrit values and plasma concentrations of cortisol, glucose, total calcium, copper and cadmium of fish exposed to a variable stress protocol under control and waterborne metal exposure (6 days, 50 μg $Cu.l^{-1}$ + 20 μg $Cd.l^{-1}$, Cu/Cd). Significant differences are indicated as described by Figure 1. The n-values are indicated in the Table.*

In all organs studied, metal exposure resulted in increased levels of Cd (Table 2). Under metal-free conditions, exposure to the variable stress protocol resulted in higher Cd concentrations in the CNS and in the head kidneys. In stressors + metal exposed fish, only the gills accumulated Cd to the same extent as metal exposed fish did, whereas the CNS, head kidneys and kidneys of the stressors + metal exposed fish accumulated significantly more Cd than the organs in metal exposed fish (Table 2).

Ca^{2+}-transport activity was significantly lower in the purified branchial membrane preparation (P_3) of all three experimental groups (Fig. 2). The enzyme purification rate (V_{spec} P_3/V_{spec} H_0) did not differ between the groups, but the protein recovery (prot. P_3/prot. H_0) was

significantly lower in the stressed group ($P < 0.01$, not shown). Metal exposure had no effect on the Na^+/K^+-ATPase activity in the P_3 fraction of the gills, but exposure to the variable stress protocol, under both control and metal containing water conditions, decreased the branchial Na^+/K^+-ATPase activity significantly (Fig. 2).

		control	stressors	metals	stressors+metals
[**Cu**] (µg g $^{-1}$dw)					
CNS	(n=8)	7.55 ± 0.55	8.00 ± 0.62	8.15 ± 0.51	8.01 ± 0.55
head kidney	(n=6)	3.73 ± 0.51	4.32 ± 0.25	2.83 ± 0.33	5.15 ± 0.72
gills	(n=9)	0.74 ± 0.08	1.26 ± 0.28	4.36 ± 0.82 ****	8.44 ± 1.88 •••• ▪▪
kidneys	(n=11)	13.4 ± 2.3	8.0 ± 0.7 *	18.6 ± 3.9	14.8 ± 2.5 •
[**Cd**] (µg g $^{-1}$dw)					
CNS	(n=8)	0.022 ± 0.004	0.045 ± 0.006****	0.041 ± 0.005****	0.083 ± 0.006 •••• ▪▪▪▪
head kidney	(n=6)	0.45 ± 0.04	0.71 ± 0.08 ***	1.13 ± 0.13 ****	1.50 ± 0.07 •••• ▪
gills	(n=9)	0.22 ± 0.05	0.32 ± 0.08	9.00 ± 1.60 ****	9.19 ± 1.09 ○○○○
kidneys	(n=11)	0.78 ± 0.08	0.95 ± 0.18	2.0 ± 0.3 ***	9.1 ± 0.9 •••• ▪▪▪▪

Table 2 *Copper and cadmium concentrations (in µg per gram dry weight) in the CNS (brain), head kidney, gills and kidneys of fish exposed to a variable stress protocol under control and waterborne metal conditions (6 days 50 µg Cu l^{-1} + 20 µg Cd l^{-1}, Cu/Cd). Significant differences are indicated as described by Figure 1. The n values are indicated in the Table.*

DISCUSSION

The results demonstrate that the mechanisms, which compensate adequately for a stressor-induced challenge of physiological homeostasis, apparently are compromized in opposing the adverse effects of a combination of sublethal stressors. In addition to a variable stress protocol, we subjected fish to two metals, since a) natural freshwaters are generally contaminated by metal mixtures, and b) in previous studies we have demonstrated that in particular combinations of metals challenge branchial function (Pelgrom *et al.*, 1994, 1995[b], 1995[c]).

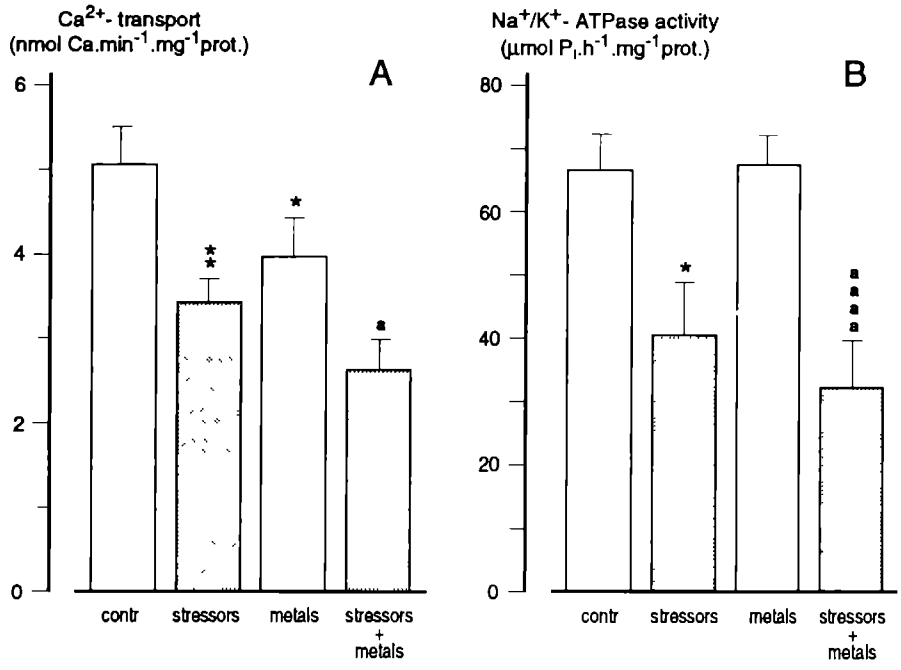

Figure 2. *Active Ca^{2+}-transport (left panel) and specific Na^+/K^+-ATPase activity (right panel) in the purified branchial plasma membrane preparation (P_s) of fish exposed to a variable stress protocol under control and waterborne metal conditions (6 days 50 µg Cu l^{-1} + 20 µg Cd.l^{-1}, Cu/Cd). Significant differences are indicated as described by Figure 1, with n=6 per experimental group.*

The abovementioned aspects are well illustrated by the plasma Na and Cl levels measured in our experimental fish. The regulation and maintenance of ion-homeostasis is very sensitive to stressors, and disturbance of the hydromineral balance is a commonly observed phenomenon in fish exposed to a wide variety of stressors, such as disturbance, confinement or heavy metal exposure (Soivio and Oikair, 1976; Eddy,1981; Pratap and Wendelaar Bonga, 1993; Heath, 1991; Pelgrom *et al.*, 1995[a,b]; Postlethwaite and McDonald, 1995). In the present study it is shown that in freshwater fish, the gills are target during exposure to heavy metals and other stressors, because they play a predominant role in a) maintenance of ion-homeostasis and b) the uptake of waterborne heavy metals during increased concentrations in the water (Stagg and Shuttleworth, 1982; Pelgrom *et al.*, 1995[c]). In response to the variable stress protocol, we

observed an increased number of opercular chloride cells. In freshwater teleosts, chloride cells function in ion uptake, and therefore, an increase in chloride cell numbers has been considered a compensating response to stressor-induced ion-losses (Perry *et al.*, 1992; Perry and Laurent, 1993). This response in our fish may contribute to compensation for stressor-induced ion challenges, since plasma Na and Cl levels were not different from control values in the stressed fish. However, in spite of an increase in chloride cell numbers in the group exposed to stressors + metals, this response was not adequate to oppose the ion losses, since in these fish the ion balance was disturbed. Apparently, the presence of waterborne Cu/Cd during exposure to a variable stress protocol prevented a restoration of the ion disturbance, though Cu/Cd exposure itself had no effect on chloride cell numbers or plasma ion levels. It is conceivable that the observed additional increased Cu and Cd concentrations in the gill tissue interfered directly with the response to the variable stress protocol in this group. However, involvement of the endocrine system can not be excluded. Hormonal control of branchial function enables fish to modify the ion-exchange capacity in the gills in response to stressors (Stagg and Shuttleworth, 1982). The release of cortisol, produced by the interrenal tissue located in the head kidneys of fish, is stimulated as part of the stress response (Mazeaud *et al.*, 1977). The hormone is known to affect ionic regulation and to induce chloride cell proliferation (Perry and Laurent, 1993). In the fish exposed to a variable stress protocol or to waterborne metals, however, we did not observe changes in plasma cortisol levels. Although in many studies, in particular in salmonids, plasma cortisol has been used as indicator of a stress response (Pickering *et al.*, 1986; Sumpter *et al.*, 1986), this has been debated for other species such as red drum (Robertson *et al.*, 1987). For tilapia this parameter is not a reliable sole index of stress. Data on plasma cortisol in this species do not represent unstressed basal levels (several $ng.ml^{-1}$; Balm *et al.*, 1994), since the blood sampling procedure affects cortisol release, because of the extremely fast reaction of these fish to disturbance (Balm *et al.*, 1994). We conclude therefore, that exposure to the variable stress protocol, nor to metals affected the stress responsiveness. However, we measured lower plasma cortisol concentrations in fish exposed to stressors + metals than in other groups, a phenomenon which has also been reported for white sucker stressed by capture and handling during bleached kraft pulp mill effluent (BKME) exposure, and this has been interpreted as a lower stress responsiveness caused by the pollutants (McMaster *et al.*, 1994). The lower circulating levels of cortisol observed in the stressors + metals exposed group may reflect a reduced synthetic capacity of the head kidneys to produce cortisol in response to capture, as suggested by McMaster *et al.* (1994). Other possible explanations are an increased clearance rate of cortisol in fish during long-term stress, as suggested by Redding *et al.* (1984), or the observed higher Cd concentrations in the head kidneys of the fish in our study.

The responses to the stressors + metals exposed fish often differed from those observed in stressors exposed or metal exposed fish. This goes for Na and Cl levels, haematocrit and for

plasma protein and Ca levels Metal exposure did not affect the plasma parameters measured, whereas exposure to stressors only increased plasma protein levels, indicating reduced blood volume, as suggested by Fletcher, 1975 Stressor induced changes in blood composition have been generally observed and include increased haematocrit values (haemoconcentration), and changed plasma protein, Ca, Na and Cl concentrations (Soivio and Oikari, 1976, Pickering *et al* , 1982, Larsson *et al* , 1984, Barton *et al* , 1985, McMaster *et al* , 1994) Our results corroborate the observations of Larsson *et al* (1984) on stressed fish They observed a greater secondary stress response in fish exposed to an acute stressor during metal exposure compared to metal exposed fish In addition, the ability to recover seems to be impaired in stressed fish during metal exposure (Larsson *et al* , 1984) From data of our study, we conclude that the response to chronic stressors (the variable stress protocol) was influenced by waterborne Cu/Cd since the results in this study show that physiological homeostasis was more disturbed in fish exposed to stressors and metals concomitantly

During waterborne metal exposure, Cu and Cd concentrations in the gill tissue increased as a result of heavy metal uptake and accumulation by this organ, which is in accordance with previous studies (Norey *et al* , 1992, Battaglini *et al* , 1993, Pelgrom *et al* , 1995[c]) After uptake of heavy metals by the gills, plasma is generally considered the main transport pathway It is possible, that the combination of higher gill Cu concentrations and lower plasma Cu and Cd concentrations, observed in the stressors + metals exposed group, together reflect an altered metal distribution and/or metal extrusion from the gills Weber *et al* (1992) reported that restrainment stress alone in largemouth bass increased blood serum Cu levels However, their fish were not fed during the experiment, and this may account for the discrepancy with our observations, which indicated the blood Cu concentration in the stressed fish was not different from control values Our fish were fed during the experiment, and feeding conditions influence Cu accumulation and distribution in this species (Pelgrom *et al* , 1994) and other species (Segner, 1987)

In fish exposed to metals only, the Cd concentrations of all organs studied displayed an increase, whereas Cu was only increased in the gill tissue In response to the variable stress protocol, we observed redistribution of Cu and Cd Metal redistribution, induced by non-metal stressors, was also observed in a study with largemouth bass (Weber *et al* , 1992) In our study we observed in the fish exposed to the variable stress protocol lower renal Cu content and higher Cd concentrations in the CNS and head kidneys compared to controls Apparently, uptake and distribution of Cu was better regulated than Cd in our stressors + metals exposed group, which may relate to the fact that Cu is an essential metal, whereas Cd is not

The most striking observation on the stressors + metals exposed group was that in all organs, except for the gills, the Cd concentration was higher than in the organs of metal exposed

fish Obviously, exposure to the variable stress protocol affects metal uptake and/or distribution during Cu/Cd exposure It is conceivable that the observed elevated Cd concentrations in the CNS and the head kidneys are causely related to the impaired stress response, observed in the stressors + metals group in this study

Increased opercular chloride cell numbers were not accompanied by increased Na^+/K^+-ATPase and Ca transport activities A discrepancy between chloride cell numbers and Na^+/K^+-ATPase activities was also observed in studies with fish exposed to Cd or acid water and in submissive fish (Wendelaar Bonga and Lock, 1992) Obviously, our results are in line with the conclusion of that study, that ion-transport capacity is related to the number of chloride cells in contact with the water (mature cells), rather than to the total number of chloride cells It has been demonstrated that the increase in chloride cell number seen in response to acid water was accompanied by a disproportionate increase of immature or degenerating cells (Wendelaar Bonga *et al* , 1990) In our fish, we only observed a decrease in the mean diameter of the chloride cells in the metal exposed fish, possibly relating to a disproportionate increase of immature cells We conclude that this is not the only indication of metal-induced impairment of gill function, since also ultrastructural changes were observed in mature cells of the branchial epithelium of the metal exposed fish, which may interfere with their function in ion transport (Pelgrom *et al* , submitted)

Little is known about the mechanisms of freshwater fish to compensate for stressor-induced challenges of the ion-balance The lower Na^+/K^+-ATPase activity, observed in the fish exposed to the variable stress protocol, and the lower active Ca-transport activity observed in this group and in the metal exposed fish, may be compensated for by the observed increase in chloride cell numbers, since plasma ion levels were only changed in fish exposed to stressors + metals It is possible that in these fish, chloride cell proliferation was not accompanied by a compensatory increase in the number of transport carriers in the gills, a mechanism suggested for stressor-induced ion-imbalance by Postletwaithe and McDonald (1995) It is not unlikely that hormones other than cortisol are involved in stress related ion-disturbances, such as growth hormone, prolactin, or epinephrine, as discussed recently by Postlethwaithe and McDonald (1995) Prolactin is known to control the permeability of the branchial epithelium to water and ions, and may reduce ion efflux during stressor-induced disturbance of ion-homeostasis In this way, prolactin may oppose stressor induced ion-losses and counteract the observed lower active transport activities in the gills In a parallel study on tilapia, we observed that metal exposure stimulated the prolactin-release from the pituitary pars distalis (unpublished observation) Previous study has demonstrated a Cd-induced increase in pituitary prolactin cells in juvenile tilapia (Fu and Lock, 1990)

In conclusion, this study shows that the physiological response in fish exposed to a combination of environmental challenges are not predictable from the responses observed in fish

exposed separately to these challenges. Apparently, compensating mechanisms which function adequately during exposure to a single stressor, can be inadequate to counteract the effects on physiological homeostasis of a combination of stressors

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SUMMARY AND GENERAL DISCUSSION

Summary of the main results:

The purpose of this study was to obtain insight in the effects of combined Cu/Cd exposure on metal accumulation, physiology and endocrine regulation in tilapia. The observed effects in Cu/Cd exposed fish were compared with the effects observed in fish exposed to Cu or Cd singly, in order to investigate whether Cu/Cd-induced effects can be predicted from the effects observed in single metal exposed fish. The main conclusions of this study are summarized as follows:

Cu or Cd not only accumulated during waterborne exposure of tilapia to these metals singly, but also markedly influenced the concentration of the other metal studied in the fish (Chapter 1, 2). The nutritional state of the fish influenced these interactions (Chapter 1). Basically, the degree of Cu and Cd accumulation in fish exposed to combinations of Cu and Cd cannot be predicted from data obtained from fish exposed to Cu or Cd singly.

In mature fish, waterborne Cu and Cd exposures, singly or in combination, resulted in metal- and organ-specific distribution and accumulation of Cu and Cd. Interactions between Cu and Cd appeared organ-specific, and concentration-dependent. Surprisingly, notable amounts of Cu and Cd also accumulated in the intestinal wall of waterborne metal exposed tilapia (Chapter 2).

In fish exposed to waterborne Cu, we observed increased concentrations of Cu in the gill tissue and in the plasma. Proliferation of chloride cells concurred with an increase in the average diameter of these cells. In fish exposed to 200 μg $Cu.l^{-1}$, whole body Na-influx was inhibited, whereas Cu exposure had no effect on whole body Ca-influx; in the plasma of fish exposed to this Cu concentration levels of Na, Cl and ceruloplasmin were changed (Chapter 3). These effects were not observed in fish exposed to lower Cu concentrations.

In fish exposed to combinations of Cu and Cd, the changes in plasma Na and Ca could not be explained by simple synergistic or additive effects of these metals (Chapter 4).

Whole body Ca-influx was significantly decreased in fish exposed to 200 μg $Cu.l^{-1}$ + 70 μg $Cd.l^{-1}$, whereas exposure to these metals separately had no effect (Chapter 5).

Cu exposure resulted in an increased level of plasma cortisol, but in the presence of

waterborne Cd, such Cu-induced elevation of plasma cortisol concentration was not observed (Chapter 4)

During waterborne Cu and Cd exposure, the gills are target for these metals, inducing metal-specific effects Besides accumulation of Cd, increased numbers of lysosomes and apoptotic cells in gill tissue of Cd exposed fish were observed as shown by electron microscopic examination of the gill tissue, and active branchial Ca^{2+}-transport was inhibited In fish exposed to the combination of Cu and Cd, concentrations of both metals increased to the same extent as in single metal-exposed fish However, compared to the single metal exposed fish, we observed different plasma ceruloplasmin levels, branchial Na^+/K^+-ATPase activity and opercular chloride cell numbers These observations indicate that there is no apparent relationship between metal accumulation in the gills and the structural and physiological changes in this tissue (Chapter 6)

Pituitary endocrine cells are target of waterborne Cu, as shown by a decline in plasma α-MSH levels and lower *in vitro* release of α-MSH by the NILs of Cu-exposed fish compared to fish in Cu-free water (Chapter 7) This is the first study demonstrating Cu-induced effects on melanotrope function In fish exposed for 6 days to a stress protocol involving various non-metal stressors, the *in vitro* α-MSH release was inhibited In contrast to Cu-exposed fish, the α-MSH content of the NIL was elevated in the fish subjected to the non-metal stress protocol These results indicate that the mechanisms involved in the inhibition of α-MSH release are different for Cu and non-metal stressors This suggestion may relate to differences in brain Cu and Cd concentrations and hypothalamic ppMCH mRNA levels between Cu-exposed and non-metal stressors exposed fish These results indicate that the observed effects of Cu and/or non-metal stressors on α-MSH may involve higher brain centres (Chapter 7)

In fish exposed to a non-metal stress protocol in combination with waterborne heavy metals, plasma Na, Cl, Ca, glucose and cortisol concentrations were significantly changed, whereas exposure to either the stress protocol or heavy metals had no effect (Chapter 8) The stress protocol resulted in redistribution of copper and cadmium, either in the absence or presence of additional waterborne metals

Discussion

Interactions between Cu and Cd

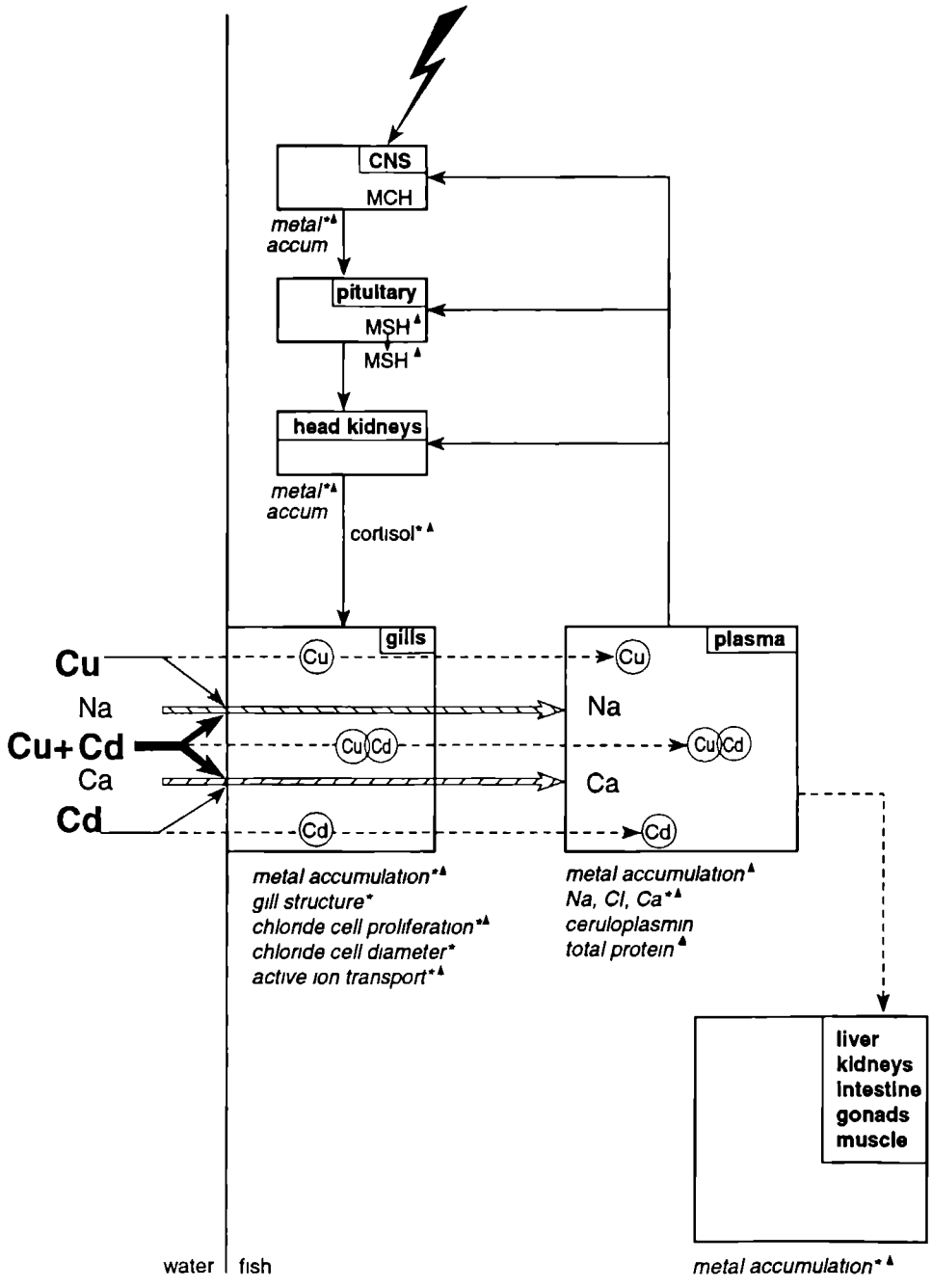
In view of the concern about the increased number of pollutants, including heavy metals in freshwaters, there was particular need to assess the risk of exposure to combinations of heavy metals for fish From studies on single metal exposure, it has become clear that metals taken up by organisms may give rise to impairment of physiological activities Another way by which a

potential toxic metal may cause adverse effects is by changing the metabolism of other, essential, metals Some tissues may be depleted of ligands or metals necessary for enzymatic function even in the absence of increased amounts of the toxic metal in that tissue Many of the observed toxic effects of Cd are thought to be the result of induced deficiencies in essential trace elements, such as zinc, copper and iron (Task Group on Metal Accumulation, 1973, Ashby *et al* , 1980) In studies on rats (Stonard and Webb, 1976, Barański, 1987), it has been shown that Cu metabolism is susceptible to inhibition by Cd, which resulted in changed Cu concentrations in plasma, liver, lungs, brain and intestinal wall, and reduced serum ceruloplasmin levels in Cd exposed rats Our results provide further evidence for Cd-induced effects on Cu metabolism A possible explanation for this phenomenon is the involvement of metallothionein (MT) and ceruloplasmin, the plasma protein involved in donation of Cu to the organs, in the metabolism of Cu Metallothionein has been proposed to function as a reservoir for copper and zinc (Cousins, 1985, Olsson and Haux, 1986), and it has been suggested to participate in the detoxification of heavy metals (Olsson and Haux, 1986), in mammals as well as in fish (McCarter *et al*, 1982, Petering and Fowler, 1986) Concentrations of MT and ceruloplasmin are influenced by heavy metal exposure (Frieden, 1979, Olsson and Haux, 1986) An increased body concentration of Cu or Cd probably impairs the control of essential metal distribution by interacting with the essential metals bound to MT and/or ceruloplasmin

Since natural freshwaters are generally polluted by mixtures of heavy metals, fish have to counteract the adverse effects of more than one metal The reported No Observed-Effect-Level (NOEL) and the Lowest-Observed-Effect-Level (LOEL), however, only apply to exposures to a single metal under well-defined but specific conditions (Foulkes, 1990) There are several ways to classify the action of mixtures (Parrott and Sprague, 1993) It has been proposed by Parrott and Sprague (1993), that the joint action of toxicants, the sum of the fraction of LC_{50s} for individual chemicals, would be the predicted effect of the mixture, resulting in less-than-additive, additive, more-than-additive, independent or antagonistic effect However, the observed effects during combined Cu/Cd exposures were not simple and consistent both increased (Iron and Smith, 1976) and decreased toxicity (Kaji et al , 1992) of combined Cu/Cd exposures have been reported In the present study with tilapia, a limited number of combinations of Cu and Cd concentrations were analyzed after an initial screening of a broader concentration range with immature fish In general, the observed effects on metal accumulation and physiology were not related to the effects observed in the fish exposed to the metals separately Likely, the impact of interactions may increase further if exposure times are prolonged or if additional concentration ranges are considered

Figure 1. Overview of the results obtained with exposure of tilapia to Cu, Cd, Cu/Cd and to non-metal stressors, with special emphasis on interaction between the metals and between metals and stressors. Parameters which are affected are indicated in italics. Effects observed in Cu/Cd exposed fish, which are not a summation of the effects observed in singly Cu or Cd exposed fish (effect Cu/Cd ≠ effect Cu + effect Cd) are indicated by *. ▲ *indicates interactive effects between metals and non-metal stressors. The flash-arrow indicates the sensory component of stressors that act on the HPI-axis directly.*
Exposure to either Cu or Cd primarily affected Na and Ca homeostasis, respectively, although the effects were observed at the highest waterborne metal concentrations tested. Exposure to combined Cu+Cd impaired both Na and Ca balance. This was observed at the branchial level (active ion transport) and in plasma (Na, Ca and Cl concentrations).
During waterborne metal exposure, Cu and Cd are taken up by the gills, and are, partly, distributed to peripheral organs by plasma (interrupted lines). Interaction between Cu and Cd on metal accumulation was observed in the gills, gonads, muscle, CNS (Central Nervous System) and in the head kidneys.
At the branchial level, besides metal accumulation and active ion transport, also gill structure, chloride cell proliferation and cell diameter were affected by metal exposure. In Cu/Cd exposed fish, the observed effects on those parameters were significantly different from the effects observed in the fish exposed to the metals singly.
Heavy metal exposure impaired neuro-endocrine regulatory systems. Metal-induced effects were not only observed at the level of head kidneys (cortisol), but also at the level of the pituitary (MSH) and of the CNS (prepro-mRNA MCH). Exposure to non-metal stressors during metal exposure resulted in interactive effects at the gills (metal accumulation, chloride cell proliferation and active ion transport), plasma (metal accumulation, concentrations of Na, Ca, Cl and total protein), kidneys (metal accumulation) and HPI-axis (metal accumulation in CNS and head kidneys, MSH in the pituitary and cortisol in the head kidneys).

The results obtained substantiate the contention, that the organ of highest accumulation is not necessarily the critical organ for toxic action of the metal, nor are blood metal concentrations good indicators for accumulation in the critical organ. This may especially be true when studying fish exposed to combinations of Cu and Cd. Comparison of metal-metal interaction patterns between organs indicates that each organ relies on different mechanisms for sequestering metals, and the interaction patterns are concentration-, time-, and species dependent (Olsson *et al.*, 1990; Gill *et al.*, 1992; this study). Therefore, Cu and Cd concentrations in whole body or organs are no reliable indicators for metal toxicity, and tell little about the mechanisms of action of the metals.


CNS
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Ca
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chloride cell proliferation*▲
chloride cell diameter*
active ion transport*▲
metal accumulation▲
Na, Cl, Ca*▲
ceruloplasmin
total protein▲
liver
kidneys
intestine
gonads
muscle
metal accumulation*▲
water
fish

Cu and Cd effects on the gills

During waterborne exposure to Cu and Cd, the metals are taken up by the gills (McDonald and Wood, 1993, Kraal *et al* , 1995, Chapters 2 and 6), and are distributed to other organs by plasma (Chapters 3 and 6) In freshwater fish, waterborne heavy metals exert their, initial, toxic action by interfering with the physiological regulation of the major electrolytes (Fu *et al* , 1989, McDonald *et al* , 1989, McDonald amnd Wood, 1993, Pratap and Wendelaar Bonga, 1993, Figure 1) As a result, in freshwater fish Cu and Cd accumulation in metal storage organs, such as liver and kidneys, only become important to survival if the gills are able to withstand the metal-induced effects (Laurén and McDonald, 1987)

Several studies have postulated that the mechanisms of action of Cu and Cd are different, with Cd primarily affecting Ca-homeostasis (Reid and McDonald, 1988, Fu *et al* , 1989, Chapter 5), and Cu mainly disturbing Na-balance (Laurén and McDonald, 1987, Reid and McDonald, 1988, Playle *et al* , 1992, Chapter 3) In fish exposed to Cu and Cd in combination, increased concentrations of both Cu and Cd were observed (Chapters 2 and 6, Figure 1) However, the observed effects of combined Cu/Cd exposures were not a summation of the effects observed in gills of fish exposed to Cu or Cd separately (Chapters 5 and 6) In a study with fathead minnows, Playle *et al* (1993) showed that neither Cu nor Cd interfered with binding of the other metal on the gills, suggesting different gill binding sites for Cu and Cd This indicates, that the observed Cu/Cd interactions on gill morphology, chloride cell number and size, and Na^+/K^+-ATPase activity in tilapia occurred in the gill tissue These interactions may result from competition for carriers, metabolic changes, displacement of metals from metal binding proteins and membrane alterations Although both Cu and Cd are stimulators of metallothionein (MT) synthesis in the liver, in the gill tissue of metal exposed fish such an increase in MT levels is not consistently observed Wicklund Glynn and Olsson (1991) measured in Cd exposed fish significant levels of Cd in the non-MT fraction, whereas the MT content of the gills was sufficient to sequester all Cd taken up from the water This observation is in line with our observations in tilapia exposed to Cu and Cd In the branchial membrane fraction we measured significant amounts of Cu and Cd, indicating that not all of the metals taken up was bound to cytosolic proteins, such as MT Moreover, in combined Cu/Cd exposed fish Cd probably displaced Cu from metal-binding sites on MT In this way the binding potency for Cu was changed, which may partly explain the unexpected effects of Cu/Cd exposure on tilapia

Generally, morphological adjustments to sublethal environmental changes in the gills are considered to be adaptive (Perry and Laurent, 1993) A commonly observed response to agents which impair ion homeostasis is proliferation of chloride cells (Fu *et al* , 1989, Pratap and Wendelaar Bonga, 1993, Chapters 3,6 and 8) This response is considered to be important for restoration of the ion homeostasis by increasing the ion-uptake capacity (Perry and Laurent, 1993) In combined Cu/Cd-exposed fish, the increase in chloride cell number was significantly

lower compared with the response observed in fish exposed to the metals singly This may be caused either by a direct effect of Cu and Cd on the gills, or relates to an indirect effect of the metals on the endocrine control of this response (Stagg and Shuttleworth, 1982, Fu *et al* , 1989) Cortisol is known to induce chloride cell proliferation in gill and opercular epithelia of freshwater teleosts (Foskett *et al* , 1983) which may be one of the major actions to stimulate ionic uptake (Laurent and Perry, 1990) It is conceivable, that Cu/Cd exposure interferes with this endocrine regulation system, as indicated by the observed absence of a cortisol response in Cu/Cd exposed fish, whereas plasma Na, Cl and Ca levels were decreased in these fish (Chapters 4 and 5)

Cu and Cd effects on the HPI-axis

There are many indications that exposure to heavy metals affects (neuro-)endocrine regulation mechanisms Several studies demonstrated metal-induced changes in concentrations of prolactin, cortisol, LH and TSH (Fu *et al* , 1989, Fu and Lock, 1990, Walker and Cooper, 1992, this study) Other observed responses to metal exposure, indicating involvement of the neuro-endocrine system, were cessation of feeding (Lett *et al* , 1976), attraction to water containing metal in the concentration to which they have been previously exposed (McNicol and Scherer, 1993), and behavioural reactions (Gardner and LaRoche, 1973) The changes in hormonal and neural control mechanisms may be part of the stress response in fish (Muñoz *et al* , 1991), and are considered to be adaptive, aimed at restoring metal-induced disruptions of ion homeostasis and/or reproductive failure (Pickering *et al* , 1977, Giles, 1984, Hutchinson and Sprague, 1986, Fu *et al* , 1989, Franklin *et al* , 1992) According to these authors, disturbed ion balance or disturbed reproductive processes are considered to be mediators for increased plasma hormone concentrations Another possible site of hormonal interference by heavy metals may include their action on the HPI-axis, although no direct relationships are apparent between metal accumulation in tissues of this neuro-endocrine regulatory system, exposure concentration of the metal, and HPI-axis activity (Smith *et al* , 1976, this study) However, it has been demonstrated that regulation of essential metals, such as Cu, is important in the biochemistry, and consequently action, of neuro peptides (Kochman *et al* , 1992) It might be anticipated that interference with Cu metabolism in the tissue of the HPI-axis, either by Cu itself or by interaction with Cd with Cu (Chapter 7), may result in impaired hormonal control This may occur even in the absence of overall metal accumulation in the brain In this respect, Cu is of particular interest, because Cu-transporting protein ceruloplasmin may also be involved, since circulating concentrations of ceruloplasmin are not only influenced by heavy metals, but are also under hormonal control, but more importantly, ceruloplasmin exerts control over endocrine mechanisms (Frieden, 1979, Schreiber and Přibyl, 1980) The observed absence of increased plasma cortisol levels in Cu/Cd-exposed tilapia (Chapters 4 and 8) and in BKME (bleached kraft pulp mill effluent) exposed

white sucker (McMaster *et al.*, 1994) may be related to interference of the exposure agents with hormonal control mechanisms. Studies on the toxic effects of heavy metals on the neuro-endocrine system has mainly focussed on hormones produced by the gonads and adrenals (Walker and Cooper, 1992). However, also metal action on hormones and peptides originating from regulating centres (pituitary and hypothalamus) may be involved in metal-induced hormonal impairment (Chapters 7 and 8; Figure 1).

Effects of additional stressors during Cu/Cd exposure

Under natural circumstances, fish are faced with several stressful stimuli, such as predators and low oxygen. Such stressors have been shown to challenge plasma ion balance (McDonald and Robinson, 1993). Fish possess compensating mechanisms to counteract stressor-induced impairment of physiological homeostasis, by increasing the activity of the HPI-axis (Mazeaud *et al.*, 1977; Donaldson, 1981).

In freshwaters polluted by heavy metals, fish will also be exposed to stressors such as predators and capture. As for heavy metal exposure, also stressors impair ion homeostasis, and metals and other stressors have been shown to affect heavy metal concentration in fish, branchial ion-transport mechanisms and hormonal control mechanisms (Chapter 8; Figure 1). By affecting the same systems or interfering with each other, exposure to more than one stressful stimulus may result in effects which are not simply additive. Compensating mechanisms, which will function adequately during exposure to a single stressor can be inadequate to counteract the effects of combinations of sublethal, stressful stimuli (Chapters 4 and 8).

In conclusion, in the present study on tilapia, exposure to Cu or Cd separately, disturbance of physiological homeostasis was observed only in fish exposed to relatively high concentrations of these metals, suggesting adequate compensating mechanisms during exposure to lower concentrations of Cu or Cd. Combined Cu/Cd exposure, however, resulted in impaired homeostasis already at low, environmentally realistic concentrations. The effects were not predictable from the effects observed in the single metal-exposed fish. Interactions between Cu and Cd was observed on metal accumulation, physiology and endocrine regulation. Notably, the observed impaired homeostasis in Cu/Cd exposed fish was not related to water metal concentrations, nor was the metal accumulation in the organs related to the observed effects. Predictions concerning toxic actions of metal mixtures, based on studies on single metal exposures, are therefore difficult to make.

In the present study, the effects of a 6-day exposure period was investigated. However, it is conceivable that chronic exposure to sublethal combinations of Cu/Cd or to other combinations of sublethal stressors will result in exhausted or inadequate compensating mechanism,s resulting in impaired immune reaction and reproduction, which may affect fish populations.

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CURRICULUM VITAE

Sylvia Pelgrom werd geboren op 18 april 1962 in Velp (GLD). In 1981 behaalde zij haar VWO diploma aan het Thomas à Kempis College te Arnhem. In datzelfde jaar begon zij haar studie Biologie aan de Katholieke Universiteit te Nijmegen (oud curriculum, B_4-richting). In november 1988 behaalde zij haar doctoraalexamen met als hoofdvak Biochemie (vakgroep van Prof. de Pont, o.l.v. Dr. Peter Willems; vakgroep van Prof. Hoenders, o.l.v. Dr. Tini van Boekel) en als bijvakken Dierfysiologie (vakgroep van Prof. Wendelaar Bonga, o.l.v. Dr. Paul Balm) en Haematologie (vakgroep Prof. Haanen, o.l.v. Dr. Martin Salden, Radboud ziekenhuis). Tijdens haar studie periode behaalde zij haar 1^e graads onderwijsbevoegdheid Biologie. Van mei 1989 to mei 1993 was zij werkzaam als Onderzoeker in Opleiding (OIO) bij de vakgroep Experimentele Dierkunde aan de Katholieke Universiteit Nijmegen. De verkregen resultaten staan beschreven in dit proefschrift. Daarnaast leverde zij een bijdrage aan het biologie onderwijs, volgde met goed gevolg de cursus Immunotoxicologie en behaalde de aantekening proefdierdeskundige (ex. art. 9 van de Wet op Proefdieren).

zware metalen

Een tweede veronderstelde vervuiling van vijverbo zware metalen Da al als vijvers bu naast park daan het vlakt

DE GELDERLANDER/DINS

Duits bedrijf belooft minder lozingen Rijn

M (ANP) Het Duitse ng und Rohelsen zingen van fors dat et

den bereikt door veranderingen van het lozingssysteem en de bouw van een afvalwaterzuiveringsinstallatie DK verwerkt jaarlijks 400 000 ton ijzerhoudende afvalstoffen die worden omgezet in ruwijzer voor de gietindustrie

Het convenant is gesloten in het kader van het Project O Rijn (POR)

Onderzoek naar vervuiling Maas

DEN HAAG (ANP) – Staatssecretaris Gabor van Natuurbeheer laat onderzoek doen naar de vervuiling van de rivier de Maas door met name cadmium

De bewindsman wil onder meer weten welke gevolgen de verontreiniging heeft voor consumptie en agrarische produkten van uiterwaarden.

De Maas is van alle

Luiveringsschap tikt vuilverbranding Duiven op vingers

Cadmium

Beken moeten we stromen en slingeren

Door onze verslaggever

ARNHEM De laatste, kwetsbare anten als de waterranonkel en de e egelskop dreigen te verdwij moerasgebieden langs de rdrogen in een alarme en de rivierkreeft is r in enkele Gelderse De natuur in de aan een zijden

voor een goed beheer van de beek dalen gebruikt kunnen worden." Beekdalen zijn te vinden op alle zandgronden in Nederland. Spre kende voorbeelden zijn de Geul, de Overijsselse Vecht, de sprengen en beken op de Veluwe, de Regge en de Dinkel. Maar alleen de Winters wijkse beken, de Drentse Aa en de kleine beekjes in het Mijnwegge bied by Roermond vertonen nog een natuurlijk troon

De Baakse Beek: te weinig koper

Koos van Zomeren, schrijver

„In het milieu staat de mens centraal, in de natuur kan hij gemist worden."

Waterkwaliteit van stadsvijvers gecontroleerd

Door onze verslaggever

APELDOORN – Het Zuiveringsschap Veluwe (ZV) gaat volgend jaar een onderzoek houden naar de water en bodemkwaliteit van stadsvijvers Van de tweehonderd veertig vijvers in stedelijke gebieden die daarvoor in aanmerking komen zullen er zestig worden . hemonsterd

zware metalen in de atmosfe

heide blijkt ernstig vervuild

Riza onderzoekt vervuiling slib

Cadmium, niet alleen ook voor vissen

Lood en cadmium

DE GELDERLANDER

Billiton moet terrein saneren

Nader onderzoek verontreiniginge

Door onze verslaggeefster

ARNHEM – Het bedrijfsterrein van Billiton aan de dijk in Arnhem moet worden gesaneerd. Na uitgewezen dat vooral het grondwater zware metalen, dat een grondige schoo

Dat het bedrijfsterrein van Billiton is vervuild, was al eerder bij e inventarisatie duidelijk gewo Maar de ernst van de ver ging was eerder nog steld. Uit een ond milieuadviesbur dat het gro tot tien

Bezwaar tegen metaallozingen loor Billiton

euwe vergunning

nze verslaggeefster

M – De Gelders
rnhem hebb
unninga
ozen

Zware metalen: loodzware kost

Door ing. Geertje Huybers

Berichten over zware metalen (kwik, lood, cadmium) en andere stoffen in ons eten zijn altijd weer goed voor grote koppen in de krant en veel onrust. Dat bleek onlangs maar weer eens bij de loodvergiftiging van koeien in het noorden van het land en de berichten over nitraat in groente.

De grote koppen en de onrust zijn ergens ook wel terecht. Schadelijke stoffen als zware metalen horen niet, of in ieder geval zo min mogelijk in ons voedsel voor te komen. Vandaar weer mo discussie. „Chloor een beetje

ijzer, calcium, zink, ko
sium, die (weliswaar
heden) noodzakelijk
gezondheid te behou

Levensmidd

Omdat cad
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gaat het om 368
van het zwaar
in de loop van
f werd vastge-
al uiberhaupt
r jaar."
ers erkennen
de metaal-

Geuldal vervuild

UTRECHT – Het Geuldal is zo vervuild met zware metalen (lood, cadmium, zink) dat er risico's bestaan voor de volksgezondheid. Kamperen met kleine kinderen in het dal moet worden afgeraden, net als de teelt van consumptiegr

De fysisch geograaf H.
stelt dat in een proef
hij volgende week
de Rijksu

Ministerie pakt cadmium aan

DEN HAAG – Het ministerie voor Milieubeheer (VROM) heeft besloten een lijst openbaar te maken waarop met naam en toenaam de produkten staan waarin te veel cadmium zit.

Cadmium wordt meestal gebruikt als kleurstof.

De maatregel is genomen naar aanleiding van een onderzoek door het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM). Dat onderzoek dateert uit augustus 1988. Volgens het ministerie is het cadmiumgebruik

Rijkswaterstaat wil opheldering over 600 kilo cadmium in Maas

Mogelijk in België calamiteit

Door onze Haagse redactie

DEN HAAG – Rijkswaterstaat wil opheldering van de Belgische autoriteiten over een golf cadmium van 600 kilo die half januari is aangetroffen in het Maaswater.

Bij het meetstation in Eijsden
richt werden vrijdag 18
de namiddag en zater-
in de avond opval-

lend
gecon
aan h
kend g
cadmiu
veelhe
België
helderi
terie va
men.

Het
aren sc
ds
u

Helft cadmium kan uit milieu door statiegeld

Door onze Haagse redactie

DEN HAAG – Een statiegeld
gulden per cadmiumbatte
komt op den duur dat min
lijf van het zwaar gif oodverb
in het milieu be
batterij die nu ongeveer
gulden kost, zou belast moe
worden met één gulden statie

Dat blijkt uit een onderzoek dat
Leids en een Brussels onder
zoeksinstituut voor de Europese
Gemeenschap hebben verricht. Nu
komt jaarlijks nog acht miljoen
kilo cadmium in het milieu te
recht. Volgens zal dat door het s
ker G. Huppes zal dat door het
tiegeld over tien jaar minste
helft minder zijn.
Huppes pleit voor een
voering van statieg
land loopt
erder v
laat

'Gebruiksartikelen vol zware metalen'

DEN HAAG (ANP) – Veel huishoudelijke gebruiksvoorwerpen van kunststof, zoals afwasborstels, emmers en kleerhangers, horen in feite bij het huishoudelijk chemisch afval. Dit blijkt uit de gisteren bekendgemaakte resultaten van een onderzoek van de Consumentenbond.

In één op de drie onderzochte kunststof artikelen zitten te veel zware metalen, zoals cadmium, kwik, chroom, antimoon en/of selenium. Daarnaast is in veel verpakkingen nog steeds pvc verwerkt.

Crematie-as vervuilt bodem en grondwater

NIJMEGEN (ANP) – Overbemesting door mineralen en ophoping van giftige zware metalen als koper en zink. De mens, milieuvijand nummer één, blijkt ook na zijn dood nog een gevaar voor de omgeving.

Het verstrooien van de as na een crematie kan, zo blijkt uit onderzoek, de kwaliteit van de bodem aantasten en het grondwater vervuilen.

Het onderzoek is uitgevoerd door het gerenommeerde ingenieursbureau Haskoning in Nijmegen. De resultaten van het rapport worden vandaag aangeboden aan P. Verkerk, hoofdinspecteur Volksgezondheid en Milieuhygiëne van het ministerie van VROM. Dat alles tijdens een feestelijke receptie ter gelegenheid van onder meer het 25-jarig bestaan van het crematorium Ockenburgh.

Haskoning onderzocht de zogeheten strooivelden van de crematoria in Utrecht, Dieren, Beuningen en Leeuwarden. 'Afhankelijk van de gebruiksduur van het strooiveld en de intensiteit van strooien' treedt bodemvervuiling op. Mineralen komen in grote hoeveelheden in crematie-as voor. Verder treedt een opeenhoping op van zware metalen als koper en zink.

Volgens Haskoning is het beter voor het milieu om de crematie-as boven zee te verstrooien. Het bureau komt met een 'pakket van maatregelen' om de 'milieuhygiënische bezwaren' van het verstrooien van as te verminderen. Zo is het raadzaam de bovenste laag van strooivelden regelmatig af te graven. Het doorlekken van de vervuilende stoffen die een gecremeerd lichaam bevatten, valt tegen te gaan door het aanbrengen van een 'afsluitende laag'.

De Gelderlander
24-09-91

Rhedense Hei is vervuild

DE STEEG – Op de Rhe
Hei en op het Rozendaalsch
is lood en cadmium aange
Volgens de gemeente Rhe
het om een lichte veront
van de ongeveer tien c
dikke humuslaag. Het p
de hei, waarmee de
moet worden teger
voorlopig stopgezet.

Volgens de gem
heideplant de lucht
kunnen vasthoude
rie van Landbou
noemt de aanwezighei
metallurgische industrie in de o
geving als mogelijke boosdoener.

Te hoge cadmiumlozingen KLM mogen toch doorgaan

LEIDEN (ANP) — De KLM loost te veel cadmium in het afvalwater. Toch hoeft het bedrijf geen extra investeringen te doen om dit te voorkomen, voordat de nieuwe zuiveringsinstallatie die het bedrijf wil bouwen klaar is.

De afdeling voor bestuursgeschillen van de Raad van State heeft dit beslist in een geschil tussen de KLM en het Hoogheemraadschap van Rijnland. Hoewel het schap blij is dat het gelijk heeft gekregen, betreurt het dat de cadmiumlozingen voorlopig door blijven gaan.

Het zware gif cadmium is een van de stoffen die volgens EG-normen niet meer geloosd mogen worden.

Volkskrant 20 17/6/92

STELLINGEN

De aanduiding: "data not shown" in een publicatie is dubbelzinnig.

Anders dan in de arbeidsovereenkomst wordt aangegeven heeft een AIO baan in de praktijk een laag "Dolly Parton gehalte".

De voormalige kweekreactor KALKAR gaat alsnog een stralende toekomst tegemoet

Het gezegde: "het is vlees noch vis" is op dit proefschrift niet van toepassing.

Het eerste resultaat van een vruchtbare samenwerking tussen Nijmegen en Wageningen binnen de onlangs erkende M & T onderzoeksschool wordt reeds in januari '96 verwacht.

Niets is zeker en zelfs dat niet.

Promoveren als a.s. moeder betekent voor de promovenda een dubbele aanslag op haar draagkracht.

De uitdrukking: "iemand om zeep helpen" wekt ten onrechte de indruk dat het hierbij gaat om een daad die van reinigende aard is en ook het hulpverlenende aspect is hierbij niet aan de orde.

De term "samenstelling" suggereert dat er sprake is van een stelling die gevormd is uit componenten.

Beleefdheid is geen vanzelfsprekende houding van mensen die veel beleefd hebben.

Ondanks het standpunt van Michelangelo dat een beeldhouwer geen beelden schept maar slechts beelden vrijmaakt die vooraf al vaststaan, wekt de uitdrukking dat iemand ergens "gebeiteld" zit ten onrechte de indruk dat deze persoon voorbestemd was om daar te zitten

Uitgaande van resultaten verkregen met blootgestelde vissen aan òf koper òf cadmium is het onmogelijk een uitspraak te doen betreffende de te verwachten effecten in gecombineerd koper/cadmium blootgestelde vissen
(dit proefschrift)

De plasma cortisol concentratie is geen goede indicator voor stress in tilapia
(Robertson *et al* 1987, *The Progressive Fish Culturist* **49,** 1-12, dit proefschrift)
(Contra Pickering *et al* 1986, *Gen Comp Endocrinol* **64,** 206-211)

Uit de concentraties koper of cadmium in organen van vissen kunnen niet de concentraties metalen in het water waar de vissen aan zijn blootgesteld worden afgeleid
(Handy, R D 1992, *Arch Environm Contam Toxicol* **22**, 74-81) Dit is zeker het geval wanneer vissen tegelijkertijd zijn blootgesteld aan koper en cadmium
(dit proefschrift)

Sylvia Pelgrom
Nijmegen, 21 november 1995

This is a man's world, but it would be nothing without a woman
(James Brown)