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CALCIUM FLUXES IN JUVENILE TILAPIA, *OREOCHROMIS MOSSAMBICUS*, EXPOSED TO SUBLETHAL WATERBORNE Cd, Cu OR MIXTURES OF THESE METALSSYLVIA M.G.J. PELGROM,\* ROBERT A.C. LOCK, PAUL H.M. BALM and SJOERD E. WENDELAAR BONGA  
Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen the Netherlands

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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  $^{45}\text{Ca}$  as tracer. The maintenance of Ca homeostasis in fish is crucial throughout life but is especially important in young fish; Ca is also critical for growth. Single metal exposure had no effect on Ca fluxes after 6 d of exposure. In fish coexposed to  $200\ \mu\text{g Cu L}^{-1}$  +  $70\ \mu\text{g Cd L}^{-1}$  however, Ca influx was significantly decreased, whereas Ca efflux was not affected. As a result, the net flux was decreased. Because the effect on Ca fluxes observed in Cu/Cd-coexposed fish could not have been predicted from the effects of single metal exposures, this study underscores the impact of interactions between toxicants. Because natural freshwaters are commonly polluted by mixtures of metals, interactions between toxicants are important in risk assessment of heavy metals.

**Keywords**—Cu/Cd interactions    Ca fluxes    Combination toxicology    Fish    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 [1]. This, together with their crucial role in physiological homeostasis and energy metabolism [2] and their delicate structure, make the gills a sensitive target organ for waterborne pollutants [3]. Many toxicants, such as waterborne heavy metals, not only enter the organism through the gills [4] but also exert their primary toxic effects on the branchial epithelium by interfering with one or more essential physiological processes, such as calcium (Ca) transport [5]. It appears that dysfunctions in branchial ionoregulatory mechanisms underlay most of the physiological effects of sublethal heavy metal exposure [6-8]. 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 [9-15]. However, large differences exist among these studies including differences in water quality, waterborne metal concentrations, and the species studied [7,9,10]. Only for trout has a model for Cd-induced inhibition of  $\text{Ca}^{2+}$  uptake been presented [16,17].

Metal contamination of aquatic environments usually involves mixtures of heavy metals, and therefore it is imperative to study metal interactions in fish to gain insight into 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 [18-20]. In the present study, we investigated metal interactions in vivo to substantiate further the relevance of these interactions for physiological processes in organisms. As a first step toward a more realistic approach to studying the impact of heavy metal contamination [21], 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 coexposure 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-coexposed fish [13,22]. 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 [23]. Calcium is mainly taken up via the gills [24]. Previous studies [10,11,16] have shown that Cd mainly impairs Ca homeostasis, whereas Cu negatively affects sodium (Na) balance in fish and has only minor effects on Ca balance. It is not known what the effects of combined Cu/Cd exposures are on Ca homeostasis in fish.

Recently, Wood [5] 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. This 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 d 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 d after hatching, under artificial freshwater conditions with undetectable Cu and Cd concentrations (detection levels below 0.1 and  $0.01\ \mu\text{g L}^{-1}$  respectively). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM  $\text{HNO}_3$ , 0.5 mM  $\text{CaCl}_2$ , 0.06 mM KCl, and 0.2 mM  $\text{MgCl}_2$ , 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 cl: Acute toxicity for fish). Water was continuously aerated,

\* To whom correspondence may be addressed.

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% dry weight/wet weight per day. The Cu and Cd contents of the food were:  $9.86 \pm 0.16 \mu\text{g Cu g}^{-1}$  and  $0.22 \pm 0.01 \mu\text{g Cd g}^{-1}$  dry food (means  $\pm$  SE;  $n = 10$ ) [25].

#### 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% dry weight/wet weight Tetramin); food was eaten within 1 min. During the acclimation period, the water in the flux chambers was continuously aerated and refreshed by means of a flow-through system (flow rate  $0.24 \text{ L h}^{-1}$ ; multichannel peristaltic pump, Watson Marlow). The experimental conditions were the same as tested and described for the whole-body flux experiments in Pelgrom et al. [12]. 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 (3–4 flux chambers per experimental group; 12 flux chambers simultaneously), were raised gradually [13] to 50, 100, or  $200 \mu\text{g L}^{-1}$  Cu; 20, 35, or  $70 \mu\text{g L}^{-1}$  Cd; or  $50 + 20$ ,  $100 + 35$ , or  $200 + 70 \mu\text{g L}^{-1}$  Cu + Cd by flow through (flow rate:  $4 \text{ h } 0.90 \text{ L h}^{-1}$  followed by a flow rate of  $0.24 \text{ L h}^{-1}$ ). Prior to the experiments with combined Cu/Cd exposures, we performed an experiment with 20 and  $70 \mu\text{g L}^{-1}$  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 with a flameless atomic absorption spectrometer (AAS, Philips PU 9200), did not deviate more than 5% of the nominal metal concentrations. After 6 d of metal exposure,  $\text{Ca}^{2+}$  influx and efflux were determined by means of  $^{45}\text{Ca}$ .

For measurement of  $\text{Ca}^{2+}$  influx,  $0.75 \text{ MBq L}^{-1} \text{ } ^{45}\text{CaCl}_2$  (Amersham, England) was added to the flux chambers, and flow-through was stopped. After 45 min of tracer exposure, water samples were taken, and fish were quickly anesthetized with phenoxy-ethanol (1:400). After rinsing, four fish per group were immediately killed in dry ice/acetone for whole body  $\text{Ca}^{2+}$  influx determination.

To investigate  $\text{Ca}^{2+}$  efflux, the remaining five fish of each flux chamber were intraperitoneally injected with  $0.12 \text{ MBq } ^{45}\text{CaCl}_2$  [12]. After recovery from anesthesia, fish were put back in their flux chambers that in the mean time had been rinsed and filled with radiotracer-free experimental water. Subsequently, the flux chambers were continuously flowed through overnight ( $0.24 \text{ L h}^{-1}$ ). During efflux measurement, the water flow was stopped, and tracer appearance in the water was monitored during 4 h to assess  $\text{Ca}^{2+}$  efflux. After this period, fish were anesthetized, and blood was taken from the caudal vessels after severing the tail. Plasma radiotracer concentrations were determined in triplicate for each fish.

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

$\text{Ca}^{2+}$  influx was calculated on the basis of the total body radioactivity per hour of exposure to  $^{45}\text{Ca}^{2+}$ , and the respective

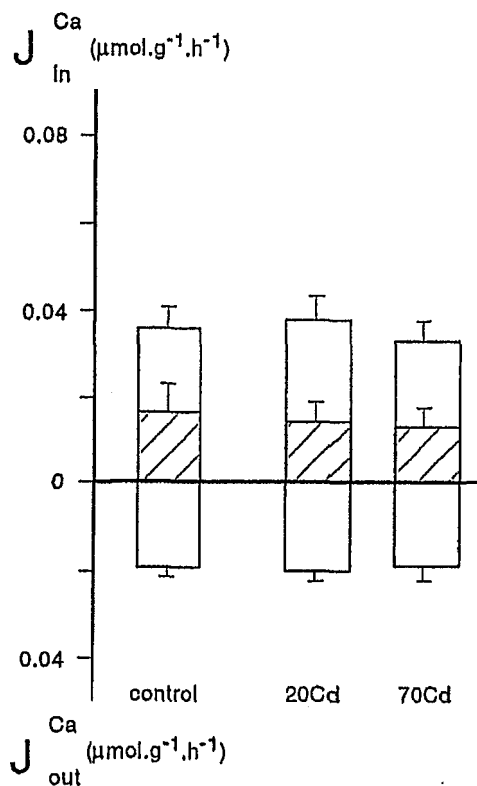


Fig. 1. Ca influx, indicated by  $J_{in}$  (open bars above zero;  $n = 16$ ); Ca efflux, indicated by  $J_{out}$  (open bars below zero;  $n = 4$ ); and net Ca flux (shaded bars;  $n = 4$ ) of fish exposed during 6 d to 20 or  $70 \mu\text{g Cd L}^{-1}$  or no Cd (controls).

mean tracer-specific activities in the water [12].  $\text{Ca}^{2+}$  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 (cresolphthalein complexone method, Sigma Diagnostics), as it has been demonstrated that in young tilapia exposed to Cu and/or Cd under these conditions, the plasma Ca concentrations are affected significantly [13]. The net flux is given as the calculated difference between the average influx and the efflux for each flux chamber. The experiments were not performed simultaneously. Consequently, fish did not originate from one breeding, which may account for the variability between the controls.

#### Statistics

Data are presented as means  $\pm$  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-coexposed fish are indicated by a ( $p < 0.02$ ), whereas significant differences between single Cd- and Cu/Cd-coexposed fish are indicated by b ( $p < 0.02$ ).

## RESULTS

### Cd

Exposure during 6 d to 20 or  $70 \mu\text{g L}^{-1}$  Cd had no effect on  $\text{Ca}^{2+}$  influx, efflux, and net flux (Fig. 1). Concomitantly

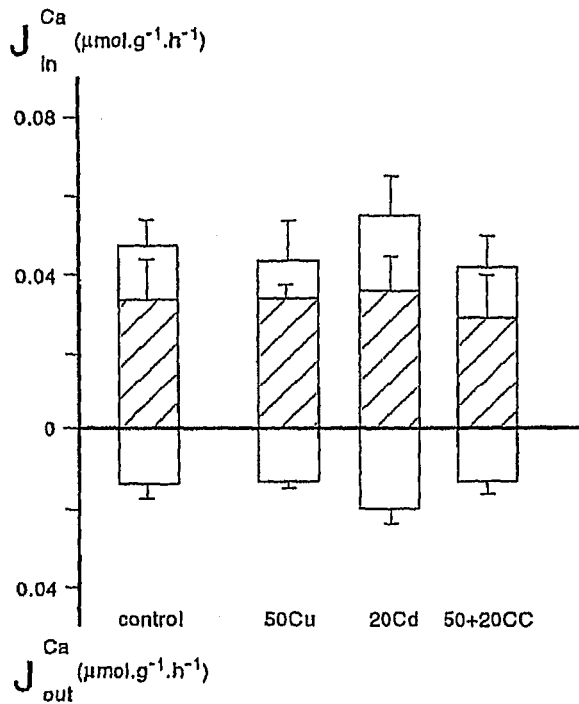


Fig. 2. Ca influx, indicated by  $J_{in}$  (open bars above zero;  $n = 12$ ); Ca efflux, indicated by  $J_{out}$  (open bars below zero;  $n = 3$ ); and net Ca flux (shaded bars;  $n = 3$ ) of fish exposed during 6 d to 50  $\mu\text{g Cu L}^{-1}$ , 20  $\mu\text{g Cd L}^{-1}$ , or 50  $\mu\text{g Cu L}^{-1}$  + 20  $\mu\text{g Cd L}^{-1}$ .

determined Na fluxes in these Cd-exposed fish also showed no effect on Na fluxes (data not shown).

#### Cd and Cu

Figures 2 and 3 show Ca influx, efflux, and net flux of fish after single and combined exposure to 50  $\mu\text{g L}^{-1}$  Cu and 20  $\mu\text{g L}^{-1}$  Cd (Fig. 2) or 200  $\mu\text{g L}^{-1}$  Cu and 70  $\mu\text{g L}^{-1}$  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 flux in Cu/Cd-coexposed fish was 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  $\text{Ca}^{2+}$  influx, whereas exposure to these concentrations of Cu or Cd singly had no effect.  $\text{Ca}^{2+}$  efflux was not affected by Cu/Cd coexposure. Consequently, the Cu/Cd-coexposed 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 severalfold within a relatively short period [26,27]. The observed inhibition of Ca uptake in young tilapia will have consequences for fish development in polluted areas.

#### Cd

In tilapia exposed during 6 d to both sublethal concentrations of waterborne Cd tested, Ca and Na fluxes were not

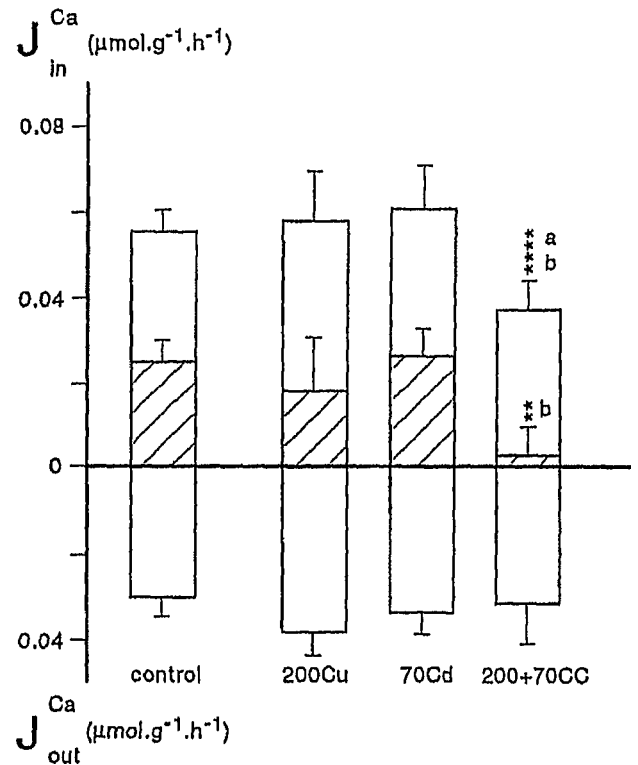


Fig. 3. Ca influx, indicated by  $J_{in}$  (open bars above zero;  $n = 12$ ); Ca efflux, indicated by  $J_{out}$  (open bars below zero;  $n = 3$ ); and net Ca flux (shaded bars;  $n = 3$ ) of fish exposed during 6 d to 200  $\mu\text{g Cu L}^{-1}$ , 70  $\mu\text{g Cd L}^{-1}$ , or 200  $\mu\text{g Cu L}^{-1}$  + 70  $\mu\text{g Cd L}^{-1}$ . 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.

different from control values. Previous studies, however, reported that Cd exposure, at these concentrations, primarily affects Ca homeostasis [10,11,13,15]. Recently, Reid and McDonald [28] and Playle and Dixon [29,30] have shown that (apical) gill binding affinity for  $\text{Cd}^{2+}$  was similar to that for  $\text{Ca}^{2+}$ , 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 [10,16]. In comparing the present results with flux data from other studies, it should be noticed, that the experimental setup in the present study differed in several important aspects: first, the species studied. Cd-induced inhibition of Ca 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 [31], whereas others such as roach, perch, and tilapia can tolerate much higher concentrations [32], as seen in this study. Second, a 3-d acclimation period was used prior to the metal exposure in the present study and not by others [10,27]. It has been demonstrated by Dharmamba and Maetz [33] that during the first 24 h after the introduction of fish into flux chambers, net ion fluxes were negative in control fish. After 24 to 48 h, net ion uptake was observed, indicating acclimation to the experimental conditions [33]. Therefore, a long acclimation period seems a prerequisite for reducing the effects of stress on the flux mea-

surements. 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 [10,27]. Third, 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 to 24 h [10,16], whereas in the present study Ca fluxes were determined after 6 d of exposure, which was initiated gradually rather than acutely. Fourth, the present flux data were obtained with fed fish, whereas Reader and Morris [27] and Reid and McDonald [10] performed their flux experiments after a 5-d 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  $\mu\text{g L}^{-1}$  Cu (personal observations). Therefore, results of 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 d may be interpreted to indicate acclimation. However, acclimation to sublethal Cd concentration did not occur in the presence of Cu because Ca uptake was inhibited in Cu/Cd-coexposed fish. For trout, it has been shown that Cd interacts with Ca uptake mainly through effects on basolateral  $\text{Ca}^{2+}$ -ATPase activity, the Ca pump [17]. 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 d 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 [22]. 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 increased toxicity of these metals, as concluded from the protein content of trout hepatocytes [34] and KB cells [18,35]. Meshitsuka and coworkers [18,35] 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 gain insight into 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-coexposed fish [13]. 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 [5,36,37]. In addition, whole-body metal accumulation rates [25] and distribution [22] between single metal- and Cu/Cd-coexposed fish were different. Those observations, together with the present results, illustrate the fundamentally different responses in Cu/Cd-coexposed fish compared to single metal-exposed fish. Though the effects observed in the present study on Ca fluxes were at relatively high concentrations of Cu and Cd, these concentrations have been observed in polluted areas. Moreover, tilapia can tolerate high concentrations of Cu and Cd, though previous studies [12,13,22] have demonstrated that physiological homeostasis

is disturbed after exposure to lower Cu and Cd concentrations. Because physiological homeostasis in more sensitive species of teleost fish is disturbed at lower concentrations of Cu or Cd, negative effects of exposure to Cu and Cd in combination may occur at environmentally realistic concentrations.

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