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Effect of Water pH on Copper Toxicity in the Neotropical Fish, *Prochilodus scrofa* (Prochilodondidae)

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Copper, a trace element, plays an important role in the cellular metabolism, but a high concentration of this element in the organism is toxic. Increased copper concentrations in aquatic environments resulting from industrial development favor copper uptake by aquatic animals, including fish. Copper bioavailability in water and its toxicity for fish depends on several physicochemical characteristics of water, among which pH is particularly important, especially in soft and ion-poor waters having low buffering capability, as is the case of most continental waters of Brazil (CETESB 1992-2002). However, studies of copper toxicity in Brazilian fish species have so far been restricted to 96h-LC_{50} in water pH 7.3 at 25°C in curimbatá, *Prochilodus scrofa* (Mazon and Fernandes 1999) and to changes in the gill tissue after exposure to copper (96h-LC_{50}) (Mazon et al. 2002) and subsequent recovery in clean water (Cerqueira and Fernandes 2002). This species is highly sensitive to copper and is therefore a potential aquatic vertebrate indicator for environmental monitoring.

Occasional accidents have contributed to the sudden introduction of large quantities of metals into aquatic systems, which may be accompanied by changes in water pH, depending on the type of industrial effluent in question. This study determines the effect of water pH on copper toxicity (LC_{50}) in *Prochilodus scrofa*, an important food fish in Brazil. The lowest and highest mean values of water pH recorded pursuant to occasional ecological accidents in the rivers of Southeastern Brazil have been 4.5 and 8.0 (CETESB 1992-2002). Differential copper accumulation in the gills, liver, kidneys and plasma, as well changes in blood glucose and lactate, and liver and muscle glycogen and lactate, have also been determined during periods of copper exposure, in evaluations of the disruption of metabolic energy during copper exposure.

MATERIALS AND METHODS

P. scrofa (W = 15-8 g; L = 10-5 cm) were taken from the Hydrobiology and Aquaculture Station at the Furnas Hydroelectric Power Plant in Furnas, MG, Brazil. The fish were kept at 25 ± 1°C, pH 7.3, in aquarium with continuous dechlorinated water flow and aeration for at least one month. The laboratory photoperiod was 12D:12L and the fish were fed with balanced fish food. Feeding

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was suspended 24 h prior to the experiments.

Experiments with two replications (30 fish in each copper concentration/pH and controls in each pH) were carried out in static systems with continuous aeration (100% O_2 saturation), constant temperature (25°C), pH (4.5 or 8.0) and total hardness ($CaCO_3$ = 24 mg L^{-1}) for 96 h. Fish (n = 10 each aquarium, not exceeding 1g fish L^{-1}) were placed in 200-liter glass aquariums having water with pH 4.5 or 8.0. The copper agent was $CuSO_4.5H_2O$ and the concentration of copper in water was determined, by Atomic Absorption Spectrophotometry, as less than 1% of the nominal concentration (7-9 nominal levels) at the beginning of experiments and 6% over the 4 days of exposure in each water pH. The water pH was adjusted using ultra pure H_2SO_4 and/or NaOH and was continuously monitored with adjustments made as required. No copper was added to the aquariums containing the control fish with pH 4.5 and 8.0. After the introduction of copper into the water, the dead fish in each aquarium were removed and counted every 12 hours and the behavior of the fish was monitored during the experiments. The 96-h LC_{50} was calculated based on the Trimmed Spearman-Karber method and the LC_{50} Program JSpear test (Hamilton et al. 1977, 1978), with 95% confidence limits.

After the determination of 96h-LC_{50} in water with pH 4.5 and 8.0, a new series of experiments were made to determine the copper accumulated during 96h-LC_{50} exposure to copper and the changes in glucose and lactate concentrations in plasma and glycogen and lactate in the liver and white muscle during the period of exposure. The control and copper groups were placed in 200-liter glass aquariums (n = 10 in each aquarium for each water pH), as described above, and copper [nominal 96h-CL_{50} in water pH 4.5 (200 μgCu L^{-1}) and pH 8.0 (15 μgCu L^{-1})] was added to the aquariums. No copper was added to the aquariums of the control groups in the two water pHs. Fish from each group were sampled without copper in water (time 0) and 6, 12, 24, 48 and 96 h after the addition of copper.

Fish were anaesthetized (0.01% benzocaine) and blood was taken from the caudal vein. Part of the blood samples was centrifuged and the plasma removed for copper analyses. Gill, liver, kidney and white muscle samples were washed in distilled water, weighed and dried at 60°C. Both plasma and dried tissues were digested in ultra pure H_2SO_4 and H_2O at 60°C and analyzed for copper, using an Atomic Absorption Spectrophotometer (AA 12/1475 GEMINI). The remaining blood samples were used to determine glucose and lactate, using commercial kits (Labtest kit 34 and Sigma kit 826, respectively, for glucose and lactate determination). Liver and white muscle samples were used to determine glycogen, using the method described by Dubois et al. (1956) and lactate (Sigma, kit 826).

The results are presented as mean ± S.E.M generated from individual fish from each replicate aquarium and treatment. For clarity, the copper accumulation data of the control group (n = 8 each time) and time 0 are shown all together in each water pH, since no significant differences were found during the experiments between the controls and time 0 in each water pH. The Pearson r correlation was

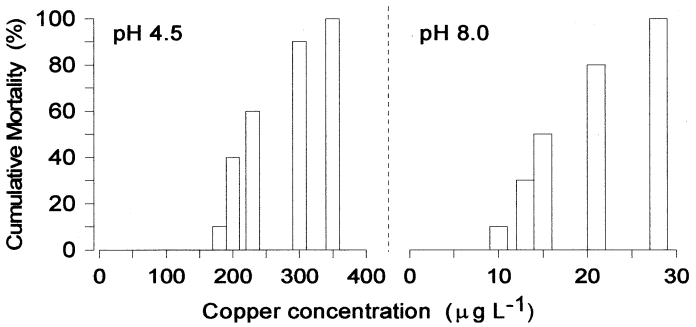

Figure 1. Cumulative percentage mortality of *P. scrofa* during 96h exposure to a range of copper concentrations in water with pH 4.5 and 8.0

applied to correlate the mean values of copper accumulation and exposure time. The Mann-Whitney nonparametric test with 95% confidence limits was applied to compare the ranking of metabolic data from each aquarium and treatment when significant differences were detected.

RESULTS AND DISCUSSION

No fish from the control groups (water pH 4.5 and 8.0) died during the experimental period, revealing the high tolerance of *P. scrofa* to changes in water pH. Maintenance of internal pH and ion regulation are usually the main problems for fish in low and high water pH, since most metabolic processes require an optimum pH for high performance. (Yesaki and Iwama 1992).

The 96h-LC_{50} of copper for *P. scrofa* is shown in Table 1 and, Fig. 1 shows the cumulative mortality during the 96 h exposure to copper. Copper toxicity was higher in water with pH 8.0. Although copper, as cation free (Cu^{2+}), is more bioavailable in water pH below 5.0 (Tao et al. 2000), for which reason is expected copper toxicity to be higher in low pH, our results revealed an inverse correlation between copper concentration in water and toxicity in this species.

Table 1. Copper concentration (LC_{50} values) for 96-h static-acute toxicity tests of *Prochilodus scrofa* conducted in water pH 4.5 and 8.0 at 25°C

Water pH	Control survival rate (%)	96h-LC_{50} (μgCu L^{-1})	95% confidence limits (LC_{50})	
			Lower LC_{50}	Upper LC_{50}
4.5	100	200	180	250
8.0	100	15	13	19

Our findings are supported by the 96h-LC_{50} for copper (29 ± 3 μgCu L^{-1}) in water pH 7.3, hardness 24 mg L^{-1} as $CaCO_3$ and 25°C for *P. scrofa* reported by Mazon and Fernandes (1999). A similar correlation was also found for *Oncorhynchus mykiss*, in which the 96h-LC_{50} was 66.0, 4.2 and 2.8 μgCu L^{-1}, respectively, in 1986). The competition of H^+ and Cu^{2+} ions for the same sites in the gill

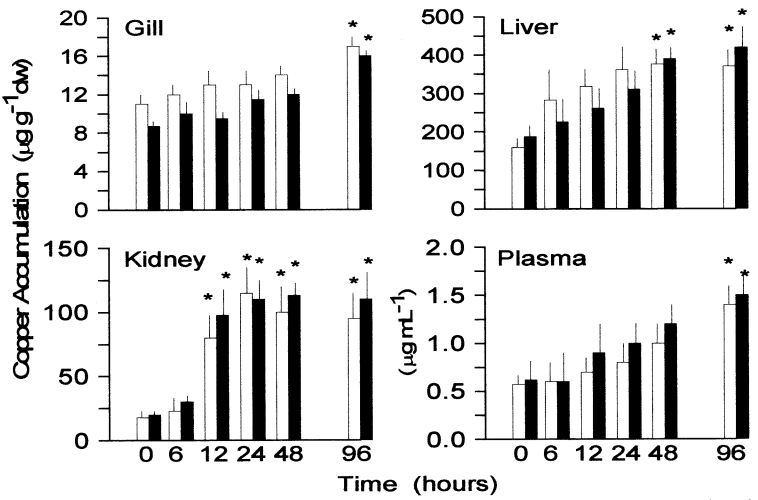

Figure 2. Copper accumulation in the gills, liver, kidney and plasma of *Prochilodus scrofa* during 96h-LC_{50} exposure to copper in water with pH 4.5 (200 $\mu gCuL^{-1}$)(open bars) and pH 8.0 (15 $\mu gCuL^{-1}$)(black bars). The bar values are mean ± SEM. (*) indicates significant differences from the controls (Time: 0 h)

epithelium (Laurén and McDonald 1985), which is the main body surface for copper water-blood diffusion distance (Mazon et al. 1998, 2002) may explain the lower toxicity of copper in low water pH in *P. scrofa*. In contrast, the reduced concentration of H^+ and the low levels of Ca^{2+} in ion-poor and soft waters may favor Cu^{2+} binding to the gill surface membrane, increasing the uptake of copper and, hence, its toxicity in high water pH (Meador, 1991).

Copper accumulation increased in the gills, liver, kidney and plasma during the 96h-LC_{50} of copper exposure (96 h-LC_{50} = 200 and 15 $\mu gCuL^{-1}$ respectively in water with pH 4.5 and 8.0)(Fig. 2), displaying a time-dependence pattern which was positively correlated with the time of exposure. The accumulation of copper in the gills and plasma was described by a linear equation $Y_{Accumulation} = A + B X_{ExpTime}$ (gills: $Y=10.09+0.94X$, $r^2 = 0.95$, $p < 0.05$ and $Y=7.94+1.16X$, $r^2 = 0.91$, $p < 0.05$, respectively, at water pH 4.5 and 8.0; plasma: $Y=0.49+0.14X$, $r^2 = 0.94$, $p <0.05$ and $Y=0.51+0.16X$, $r^2 = 0.97$, $p < 0.05$, respectively, with water pH 4.5 and 8.0) while the copper accumulation in the liver and kidneys was described by a potential equation $Y_{Accumulation} = AX_{ExpTime}^{B}$ (liver: $Y=293.71X^{0.15}$, $r^2 = 0.99$, $p < 0.05$ and $Y=244.07X^{0.28}$, $r^2 = 0.93$, $p < 0.05$, respectively, with water pH 4.5 and 8.0; kidney: $Y=37.36X^{0.74}$, $r^2 = 0.73$, $p < 0.05$ and $Y=43.39X^{0.63}$, $r^2 = 0.96$, $p < 0.05$, respectively, with water pH 4.5 and 8.0). Such potential pattern of copper accumulation in the liver and kidney may be related to the detoxification and excretion function of these organs which may favor faster but limited accumulation of copper. In both water pH levels, copper accumulation was significantly higher in the kidneys and liver respectively, after 12 and 48h of exposure to copper, and in the gill tissue and plasma after 96h. No copper

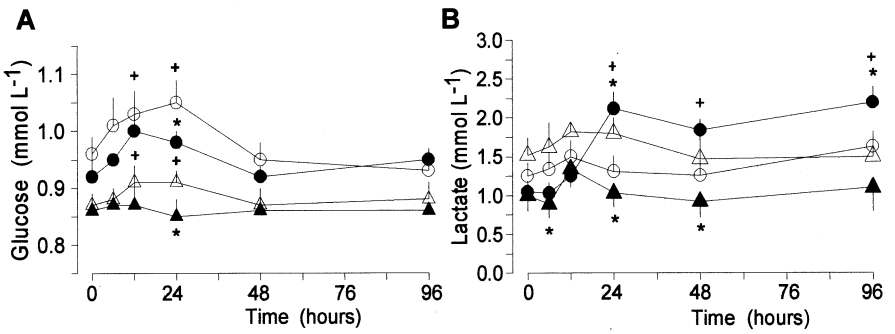

Figure 3. Plasma glucose (A) and lactate (B) concentration of *Prochilodus scrofa* from the control groups (○ △) and groups exposed to 96h-LC_{50} for copper (● ▲) in water with pH 4.5 (200 μgCuL^{-1}) (○ ●) and 8.0 (15 μgCuL^{-1})(△ ▲). The points indicate mean values and the bars indicate ± 1 SEM. (*) indicates significant difference from the controls and (+) indicates significant difference from the value of fish exposed to copper with pH 4.5 at time 0.

accumulation in the muscle tissue was found in both water pHs (data not shown). At the end of experiments, the amount of copper accumulated in the liver, kidneys and plasma was about 250% higher than in those of the controls and, in the gills was only about 160% higher. The copper uptake by the gills, followed by transportation via the blood stream to other organs, may explain the lower accumulation in the gills than in liver and kidneys. The copper accumulated in plasma, however, suggests that the capacity of liver and kidneys to remove copper from the organism in the concentrations produced by the 96h-LC_{50} exposure was exceeded.

It is important to note that, although copper accumulation in the tissues and plasma was similar in both water pHs, the copper concentration (96h-LC_{50}) in water with pH 8.0 (96h-LC_{50}=15 μgCuL^{-1}) was significantly lower than with pH 4.5 (96h-LC_{50}=200 μgCuL^{-1}), indicating a higher copper uptake in water with high pH. The production of large quantities of mucus has been reported in fish exposed to low water pH and in fish exposed to numerous toxic chemicals (Tao et al., 2000) and is generally accepted to partially account for the lower toxicity of copper in low water pH as copper-binding mucus is continuously washed out from the gills by flowing water during respiration. However, larger amounts of mucus were produced and excreted by the gills and skin of *P. scrofa* kept in water with pH 8.0 but not in pH 4.5, which would be expected to bind the copper ions and reduce toxicity in water with pH 8.0. Nevertheless, copper toxicity was higher in water with pH 8.0, suggesting a rapid copper-gill membrane interaction of $Cu(OH)_2$, the main copper species in ion-poor and soft water with a high pH.

Figures 3 to 5 depict the changes in glucose and lactate in plasma, and glycogen and lactate in the liver and white muscle of control and copper-exposed fish kept in water with pH 4.5 (200 μgCuL^{-1}) and 8.0 (15 μgCuL^{-1}) during the 96h period.

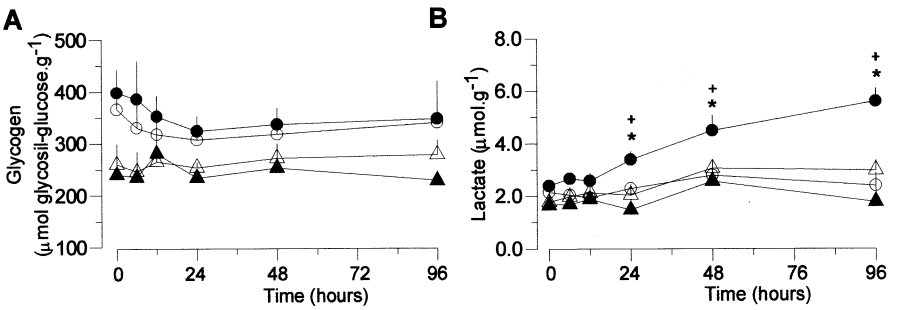

Figure 4. Glycogen (A) and lactate (B) concentration in the liver of *Prochilodus scrofa* from the control groups (○ △) and groups exposed to 96h-LC_{50} for copper (● ▲) in water with pH 4.5 (200 µgCuL^{-1}) (○ ●) and 8.0 (15 µgCuL^{-1}) (△ ▲). The points indicate the mean values while the bars indicate ± 1 SEM. (*) indicates significant difference from the controls and (+) indicates significant difference from the values of pH 8.0 at time 0.

All glucose values from control and copper exposed fish kept in water with pH 4.5 were significantly different from those of fish kept in water with pH 8.0 ($p<0.05$). The changes in plasma glucose occurred in the first 24h under each experimental condition (Fig. 3A) may reflect an increase of energy demand to maintain homeostasis. Circulatory glucose comes from liver glycogenolysis and gluconeogenesis and it must be mobilized at rates to match the animal's immediate energy needs. Glycogen concentration in the liver of *P. scrofa* kept in water pH 4.5 (controls and copper exposed groups) was significantly different in fish kept in water pH 8.0 ($p<0.05$). Since glycogen concentration showed only a slightly decrease (nonsignificant, $p > 0.05$) in the liver of fish kept in water with pH 4.5 (controls and copper exposed groups) and in the controls kept in water with pH 8.0 (Fig 4A) during the 96h experimental period, gluconeogenesis seems to be an important way to provide substrate for energy metabolism. Nevertheless, individual glycogen levels vary substantially, making it difficult to detect changes.

The increase in lactate concentration in the plasma of fish exposed to copper in water with pH 4.5 after 24 h of exposure (Fig. 3B) indicates the use of anaerobic glycolysis by the tissues to supply energy requirements. Anaerobic glycolysis may result from heightened tissue activity concomitant to low oxygen availability. Changes in the gill tissue, such as hypertrophy of epithelial cells of lamellae and hyperplasia of filament epithelium, reduce the gill surface area and increase the water-blood diffusion distance (Mazon et al, 2002), thereby reducing the amount of oxygen uptake by the gills to supply oxygen (Wilson and Taylor 1993, Sakuragui et al 2003) for the oxidative metabolism of the fish's tissues. However, the possible low oxygen availability due to changes in the gill tissue did not explain the plasma lactate levels of fish exposed to copper in water with pH 8.0 as similar gill tissue changes in fish exposed to copper in water with pH 4.5 and 8.0 were reported by Takasusuki (2000). Further investigation is needed to clarify this point.

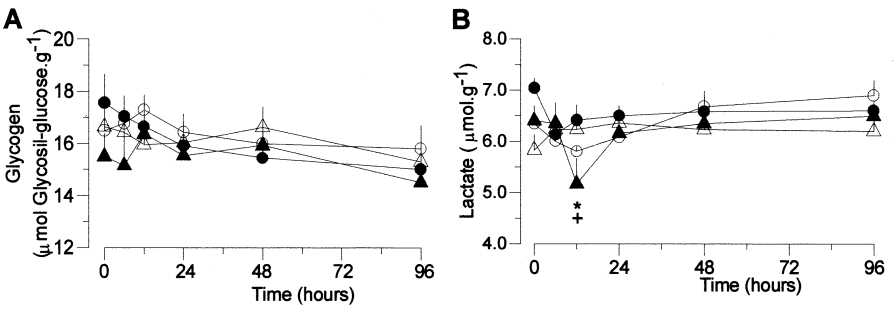

Figure 5. Glycogen (A) and lactate (B) concentration in the white muscle of *Prochilodus scrofa* from the control groups (○ △) and groups exposed to 96h- LC_{50} for copper (● ▲) in water with pH 4.5 (200 µgCuL^{-1})(○ ●) and 8.0 (△ ▲).The points indicate the mean values while the bars indicate ± 1 SEM. (*) indicates significant difference from the controls and ($^{+}$) indicates significant difference from the values of pH 8.0).

Liver is a vital organ for animal metabolism and the main detoxification organ. The liver's activity is expected to increase during copper exposure. Therefore, the increase of lactate level in the liver of *P. scrofa* exposed to copper in water with pH 4.5 (Fig. 4B) may be ascribed to the increase of anaerobic metabolism in the organ and/or to the accumulation of exogenous lactate for restoration of the glycolytic cycle, since plasma lactate is destined to the liver to be metabolized.

The concentration of glycogen and lactate in the white muscle showed no significant changes under any of the experimental conditions (Fig. 5A, B). It suggests that the amounts of carbohydrate supplied to muscle cells support its energy metabolism or replenish intracellular stores mainly in fish exposed to copper that remained 90% of time resting on aquarium bottom.

In conclusion, although *P. scrofa* can live in water with pH ranging from 4.5 to 8.0, the water pH 4.5 is more stressful than pH 8.0, as shown by the more evident changes observed in the plasma glucose concentration of the control fish kept in water with pH 4.5. However, copper toxicity was higher in fish kept in water with pH 8.0 (96h-LC_{50} = 15 µgCuL^{-1}) than in water with pH 4.5 (96h-LC_{50} = 200 µgCuL^{-1}) suggesting faster copper absorption in high water pH which was evidenced by similar copper accumulation in plasma and tissues in both water pH. As pointed out by McDonald and Wood (1993) and Takasusuki (2000) and evidenced in the present study most physiological changes occur during the first 24h of acute exposure to pollutants, the nominated "shock phase", and the surviving fish show partial or total recovery of some physiological variables.

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