TOXICITY AND EFFECTS OF COPPER SULFATE ON PARASITIC CONTROL AND HEMATOLOGICAL RESPONSE OF TAMBAQUI *Colossoma macropomum*

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ABSTRACT

The purpose of this work was to determine the mean lethal concentration (LC_{50}-96h) of copper sulfate ($CuSO_4$) for juveniles *Colossoma macropomum* (Characidae), to evaluate the effectiveness of different concentrations of $CuSO_4$ on elimination of parasites, as well as to study the effects of $CuSO_4$ on blood parameters after short-term exposure. After determination of the LC_{50}-96h from 17.5 mg L^{-1} of $CuSO_4$, it was investigated the effects of exposure for 48 h to 1.75, 4.37 and 8.75 mg L^{-1} of $CuSO_4$, on parasites and blood parameters. In gills and skin, concentrations of 4.37 and 8.75 mg L^{-1} of $CuSO_4$ eliminated Monogenoidea *Anacanthorus spathulatus*. In fish exposed to different concentrations of $CuSO_4$, plasma total protein, chloride, potassium, glucose and copper levels, hemoglobin and hematocrit were similar to controls, whereas sodium levels presented decrease in fish exposed to 1.75 and 4.37 mg L^{-1}. Red blood cell counts decreased in fish exposed to different three concentrations of $CuSO_4$, while the Mean Corpuscular Volume (MCV) increased. However, exposure to 8.75 mg L^{-1} of $CuSO_4$ caused decrease on total leukocytes, lymphocytes, neutrophils and PAS-positive granular leukocytes (PAS-GL) number when compared to control fish.

Key words: Blood; freshwater fish; leukocytes; parasites; Monogenoidea

TOXICIDADE E EFEITOS DO SULFATO DE COBRE NO CONTROLE PARASITÁRIO E NA RESPOSTA HEMATOLÓGICA DE TAMBAQUI *Colossoma macropomum*

RESUMO

O presente trabalho determinou a concentração letal média (CL_{50}-96h) do sulfato de cobre ($CuSO_4$) para juvenis de tambaqui *Colossoma macropomum* (Characidae), avaliou a eficácia de diferentes concentrações de $CuSO_4$ na eliminação de parasitos e também estudou os efeitos do $CuSO_4$ nos parâmetros sanguíneos após curta exposição. Após determinada a CL_{50}-96h de $CuSO_4$ em 17,5 mg L^{-1}, foi investigado os efeitos da exposição de 48 h com 1,75, 4,37 e 8,75 mg L^{-1} de $CuSO_4$ no tratamento para parasitos e nos parâmetros sanguíneos. Nas brânquias e pele, concentrações de 4,37 e 8,75 mg L^{-1} eliminaram *Anacanthorus spathulatus* (Monogenoidea). Nos peixes expostos a 1,75, 4,37 e 8,75 mg L^{-1} de $CuSO_4$ os níveis plasmáticos de proteína total, cloreto, potássio, glucose e cobre e a hemoglobina e hematócrito foram similares aos controles, porém os níveis plasmáticos de sódio decresceram com exposição a 1,75 e 4,37 mg L^{-1}. Nos peixes expostos às três diferentes concentrações de $CuSO_4$, a contagem de eritrócitos diminuiu enquanto o Volume Corpuscular Médio (VCM) aumentou. Porém, a exposição a 8,75 mg L^{-1} de $CuSO_4$ diminuiu o número total de leucócitos, linfócitos, neutrófilos e leucócitos granular PAS-positivo (LG-PAS) quando comparado aos peixes controle.

Palavras chave: Sangue; peixe de água doce; leucócitos; parasitos; Monogenoidea

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INTRODUCTION

In recent times, there has been tremendous increase in the development of fish farming attributable to the existence of Brazilian native species. An important fish for Brazil freshwater aquaculture has been the tambaqui *Colossoma macropomum* Cuvier, 1818 (Characidae). The culture of tambaqui is increasing in the several regions from Brazil, and its production was from 46,454.2 t in 2009 (MPA, 2010). Consequently, parasitic infections and diseases are some of the factors hindering high productivity in tambaqui aquaculture (TAVARES-DIAS *et al.*, 2006; MORAIS *et al.*, 2009).

Copper sulfate ($CuSO_4$) has been considered one of the most effective chemical against several parasitic infections and diseases. Thus, it has been widely used in treatment of infections caused by protozoan (SCHLENK *et al.*, 1998; CARNEIRO *et al.*, 2005) and monogenoideans (THONEY, 1990; TAVARES-DIAS *et al.*, 2002). In contrast, studies on effects of $CuSO_4$ on parasites and blood parameters of *C. macropomum* are scarce. However, MATSUO *et al.* (2005) investigated the effects of copper on ion transport and gill metal binding in *C. macropomum* reared in soft water.

Large concentrations of $CuSO_4$ damage the gill epithelium, hematopoietic tissues, kidney, spleen and liver of fish (NUSSEY *et al.*, 1995a; MAZON *et al.*, 2002; FIGUEIREDO-FERNANDES *et al.*, 2007). Consequently, they alter the blood parameters (NUSSEY *et al.*, 1995a; MAZON *et al.*, 2002; TAVARES-DIAS *et al.*, 2002; CARVALHO and FERNANDES, 2006) and osmoregulation (NUSSEY *et al.*, 1995a; MAZON *et al.*, 2002; CARVALHO and FERNANDES, 2006; SINGH *et al.*, 2008). Immunosuppression can also be observed (MAZON *et al.*, 2002; TAVARES-DIAS *et al.*, 2002), due to the fact that monocytes and neutrophils are sensitive to heavy metals (WITESKA and WAKULSKA, 2007). Blood assessment constitutes an important tool to evaluate the fish health and may vary according to toxicants (NUSSEY *et al.*, 1995a, b, c; MAZON *et al.*, 2002; SINGH *et al.*, 2008).

Copper (Cu^{++}) toxicity is influenced by water alkalinity and hardness; hence, when used in water with low concentrations of $CaCO_3$, the ion Cu^{++} may cause physiological changes in fish. The cupric ion interferes in the linking of ionic regulatory proteins by obstructing their regulatory function (ADHIKARI, 2003). In fish, Cu^{++} is an essential trace metal for metabolic functions. However, it is potentially toxic when the internal available concentration exceeds the capacity of physiological detoxification processes (FIGUEIREDO-FERNANDES *et al.*, 2007).

The aim of this study was to determine the lethal concentration (LC_{50}-96h) of copper sulfate ($CuSO_4$) and evaluate the effects of this chemical product on elimination of parasites and biochemical and hematological parameters from tambaqui *C. macropomum*.

MATERIALS AND METHODS

Test animals and acclimatization

Juveniles *Colossoma macropomum* were obtained from a tank at the Amazonian Research Institute/INPA (Manaus, Brazil), acclimatized during 15 days in aquariums of 100 L with artificial aeration and fed *ad libitum* for this study.

Determination of lethal copper sulfate concentration (LC_{50})

After acclimatization, hundred five fish selected at random were kept in a static system of water with artificial aeration. These fish had their food suspended one day before the exposure to $CuSO_4$ and were not fed throughout the study. This experimental design had seven treatments with three replicates by treatment and five fish for each replicate. These juveniles of tambaqui *Colossoma macropomum* (13.7 ± 0.5 cm length and 40.4 ± 6.3 g) were exposed to 0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 mg L^{-1} of copper sulfate ($CuSO_4{\cdot}5H_2O$) for determination of lethal concentration (LC_{50}). The Trimmed Spearmann-Karber method (HAMILTON *et al.*, 1977) was used for LC_{50}-96h determination.

Blood parameters measurements after sublethal exposure of copper sulfate

Sixty juveniles *C. macropomum* (13.9 ± 0.7 cm length and 40.8 ± 7.1 g) were selected at random and distributed in aquariums with 100 L capacity, in a static system with artificial aeration. These fish had their food suspended one day before the

exposure to $CuSO_4$ and were not fed throughout the study. The fish were exposed to 0, 1.75, 4.37 and 8.75 mg L^{-1} of $CuSO_4$ for 48 h, concentrations that correspond to 10%, 25% and 50% of LC_{50}-96h, for evaluation of blood parameters and evaluation of this chemotherapeutic effectiveness. This experimental design had also seven treatments with three replicates by treatment and five fish for each replicate.

After 48 h of exposure to $CuSO_4$ blood sample of each fish was collected from the caudal vessel using syringes containing a drop of ethylenediaminetetraacetic acid (10%) and separated in two aliquots: one to obtain the plasma and the other for blood parameters determination. The blood was centrifuged for seven min at 750 g to separate the plasma and then frozen at -80 °C until the analysis. Plasma glucose was determined by the glucose oxidase method; total plasma protein concentration, by burette reaction; plasma sodium and potassium concentrations, in a flame emission photometer (Zeiss M4Q2); chloride was measured by mercury thiocynate method, using a commercial kit (Sigma 461), and plasma copper levels, in an Atomic Absorption spectrophotometer (Perkin Elmer - Mod. 1100b).

The total blood sample was used to determine the total erythrocytes in a hemocytometer, hematocrit (Hct) by microhematocrit method and hemoglobin concentration ([Hb]) with Drabkin's reagent at 540 nm absorbance. The mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood smears were stained in May Grünwald-Giemsa-Wright (TAVARES-DIAS and MORAES, 2003) to obtain total white blood cells (WBC) and thrombocytes, according to the method previously described (TAVARES-DIAS and MORAES, 2007). Differential leukocytes count was performed by identifying 200 leukocytes from the smears, and these cells were identified according to TAVARES-DIAS *et al*. (1999) for tambaqui *C. macropomum*.

Antiparasitic treatment with sublethal copper sulfate

The fish exposed to 0, 1.75, 4.37 and 8.75 mg L^{-1} of $CuSO_4$ for 48 h as described above, were also used for evaluation this chemotherapeutic effectiveness on parasites. The mucus from the fish body surface was examined for presence of parasites, and gills of 15 fish were removed for examination under common light microscopy, fixation, quantification (TAVARES-DIAS *et al*., 2001a, b) and identification of the parasites (KRITSKY *et al*., 1979; MOLNÁR and BÉKÉSI, 1993) for the evaluation of this chemotherapeutic effectiveness.

Water quality

Dissolved oxygen, electric conductivity and water temperature were measured with digital oxymeter (YSI-55/12) and digital meter (WTW-D-812), respectively. Alkalinity, hardness and nitrite followed the recommendations of BOYD and TUCKER (1992). Total ammonia (NH_3+ NH_4^+) was determined by the colorimetric method in spectrophotometer (Amersham Pharmacia Biotech, mod. Novaspec II). The levels of the cupric ion, magnesium and calcium were measured in an atomic absorption spectrophotometer (Atomic Absorption Spectrometer, Perkin Elmer, Model 1100b). The mean values of water quality are presented in Table 1.

Statistical analysis

The values were expressed as mean ± deviation standard. Analysis of variance was performed to investigate differences among mean for the treatments, and when these differences were significant the mean values were compared by Tukey test at $P<0.05$ (ZAR, 1999).

RESULTS

Lethal concentration (LC_{50}) of copper sulfate

There were no registers of death among the fish of the control group (0 mg L^{-1} of $CuSO_4$) during the determination of LC_{50}-96h, as expected. Initially, with to CuSO4 exposure, fish became agitated, looked for the water surface and showed an increase in accelerated opercula beatings.

The analysis of the water physical-chemical parameters of the aquariums did not show alterations, except by the hardness, which increased when compared to the control without $CuSO_4$. Determination of copper ions (Cu^{2+}),

calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the water showed that the majority of the treatments presented a significant increase of them ($P<0.05$), except for the 20.0 mg L^{-1} and 25.0 mg L^{-1} concentrations of $CuSO_4$, in which they were similar to the control (Table 1).

The toxicity test for the determination of the LC_{50} yielded a concentration of 17.5 mg L^{-1} of $CuSO_4$ (equivalent to 4.3 mg L^{-1} of copper), with 95% CI = 16.3-18.7 mg L^{-1}. The seven different concentrations used in this assay, and the percentage of fish mortality are shown on Figure 1.

Table 1. Water quality parameters from the aquarium of *C. macropomum* exposed to lethal $CuSO_4$ for the determination of the LC_{50}-96h. Mean values ± standard deviation (n = 3). Values followed by the same letter do not differ by Tukey test ($P<0.05$)

Parameters	0.0 mg L^{-1}	12.5 mg L^{-1}	15.0 mg L^{-1}	17.5 mg L^{-1}	20.0 mg L^{-1}	22.5 mg L^{-1}	25.0 mg L^{-1}
O_2 (mg L^{-1})	5.7 ± 0.3a	7.0 ± 0.6a	6.9 ± 0.7a	6.9 ± 0.7a	5.2 ± 0.9a	7.1 ± 0.8a	4.6 ± 1.0a
pH	7.9 ± 0.1a	7.7 ± 0.4a	7.7 ± 0.3a	7.7 ± 0.3a	7.6 ± 0.3a	7.8 ± 0.3a	7.5 ± 0.3a
Temperature (°C)	27.9 ± 0.5a	27.0 ± 1.2a	26.8 ± 1.0a	26.7 ± 0.9a	27.9 ± 0.4 a	26.0 ± 0.9a	28.2 ± 0.4a
Electric conductivity (µS cm^{-1})	90.1 ± 2.7a	124.7 ± 7.9a	132.0 ± 7.4a	121.3 ± 4.7a	107.4 ± 4.1a	117.5± 2.8 a	107.9 ± 4.2a
Alkalinity (mg L^{-1})	61.6 ± 1.9a	62.0 ± 1.9 a	61.1 ± 3.4a	63.9 ± 3.8a	62.4 ± 4.9a	59.9 ± 3.0a	60.9 ± 3.2a
Hardness (mg L^{-1})	4.1 ± 1.5a	64.3 ± 8.3b	52.6 ± 11.7b	64.9 ± 15.2b	41.2 ± 14.2b	60.3 ± 19.8b	36.6 ± 11.0b
Ammonia (mg L^{-1})	1.0 ± 0.60a	1.5 ± 0.1a	1.1 ± 0.04a	1.5 ± 0.6a	0.9 ± 0.1a	1.5 ± 0.1a	1.0 ± 0.1a
Nitrite (mg L^{-1})	0.1 ± 0.1a	0.1 ± 0.1a	0.98 ± 045a	0.1 ± 0.1a	0.8 ± 0.1a	0.9 ± 0.4a	0.9 ± 0.1a
Cu^{2+} (mg L^{-1})	0.0 ± 0.0a	1.10 ± 0.26b	1.08 ± 0.21b	1.10 ± 0.25b	0.61 ± 0.46a,b	0.95 ± 0.44b	0.44 ± 0.09a,b
Mg^{2+} (mg L^{-1})	0.11 ± 0.01a	0.16 ± 0.00b,c,d	0.18 ± 0.01b,c	0.17± 0.01b,c	0.14 ± 0.15a,d	0.17 ± 0.01b,c	0.14 ± 0.05a,d
Ca^{2+} (mg L^{-1})	0.03 ± 0.06a	0.23 ± 0.04b	0.25 ± 0.02b	0.24 ± 0.04b	0.04 ± 0.03a	0.28 ± 0.08b	0.02 ± 0.02a

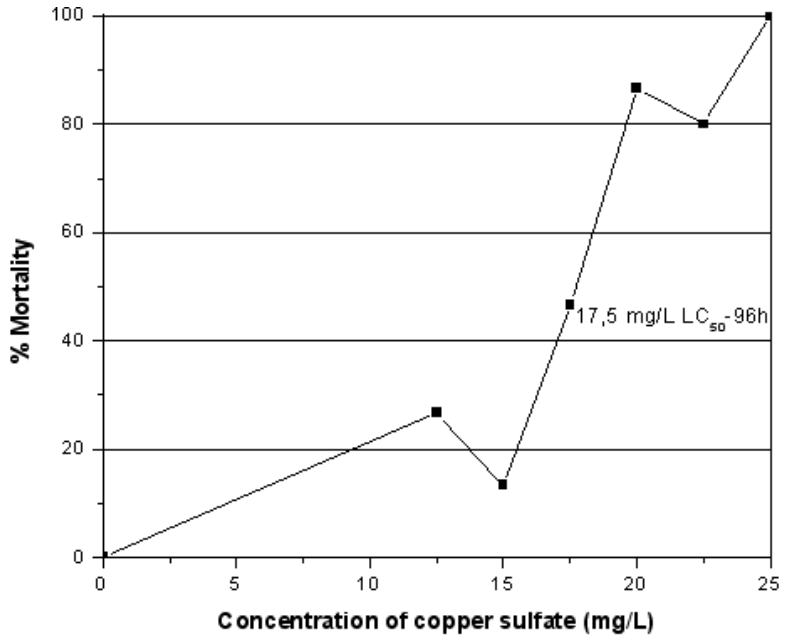

Figure 1. Graphical estimation of copper sulfate LC_{50} for juveniles *C. macropomum* during 96h of exposure

Antiparasitic treatment with sublethal copper sulfate

Analyses of the water physical-chemical parameters are presented in Table 2. No alteration occurred in these parameters, except by the copper ions (Cu^{2+}), which were higher in fish exposed to $CuSO_4$ when compared with the control group.

Table 2. Water quality parameters from the aquarium of *C. macropomum* exposed to concentrations of sublethal $CuSO_4$, during 48h. Mean values ± standard deviation (n = 15). Values followed by the same letter do not differ by Tukey test (P<0.05)

Parameters	0.0 mg L^{-1}	1.75 mg L^{-1}	4.37 mg L^{-1}	8.75 mg L^{-1}
Dissolved oxygen (mg L^{-1})	6.7 ± 0.3a	6.8 ± 0.4a	6.9 ± 0.4 a	6.8 ± 0.4a
pH	7.5 ± 0.1a	7.6 ± 0.1a	7.6 ± 0.2 a	7.7 ± 0.1a
Temperature (^oC)	26.0 ± 0.1a	26.0 ± 0.1a	25.9 ± 0.1a	25.9 ± 0.1a
Electric conductivity (μS cm^{-1})	108.1 ± 16.0a	99.0 ± 5.7a	115.5 ± 3.2a	125.0 ± 2.0a
Alkalinity (mg L^{-1})	63.4 ± 2.1a	63.6 ± 2.9a	63.4 ± 2.0a	63.5 ± 2.3a
Hardness (mg L^{-1})	4.3 ±1.5a	72.7 ± 16.7a	71.9 ± 14.6a	72.9 ±16.4a
Ammonia (mg L^{-1})	1.0 ± 0.3a	1.1 ± 0.3a	1.16 ± 0.2a	1.01 ± 0.2a
Nitrite (mg L^{-1})	0.13 ± 0.03a	0.16 ± 0.04a	0.26 ± 0.7a	0.29 ± 0.02a
Cu^{2+} (mg L^{-1})	0.00 ± 0.00a	0.13 ± 0.03b	0.41 ± 0.01b	0.85 ± 0.07b
Mg^{2+} (mg L^{-1})	0.14 ± 0.02a	0.15 ± 0.01a	0.16 ± 0.01a	0.15 ± 0.01a
Ca^{2+} (mg L^{-1})	0.56 ± 0.19a	0.52 ± 0.03a	0.59 ± 0.13a	0.50 ± 0.12 a

Following exposure of 48 h to $CuSO_4$ for the evaluation of effectiveness of chemical product, there were neither changes in controls fish behavior nor death, as expected. However, after 48 h of treatment, 100% of the fish in the control group were parasitized by *Anacanthorus spathulatus* Kritsky, Thatcher & Kayton, 1979 (Monogenoidea: Dactylogyridae), but in groups exposed to different concentrations of $CuSO_4$ a reduction in the number of these parasites or a complete elimination was found (Table 3). There was no significant difference (P>0.05) in the effectiveness of this chemotherapic among the three concentrations used here.

Table 3. Infection levels by *A. spathulatus* in juveniles *C. macropomum* skin and gills after 48h of exposure to concentrations of sublethal $CuSO_4$. Values ± standard deviation; n = 15. Values followed by the same letter do not differ by Tukey test (P<0.05)

Sublethal $CuSO_4$	Gills (n = 15)	Skin
0.0 mg L^{-1}	243.9 ± 95.5a	14.7 ± 11.5a
1.75 mg L^{-1}	8.9 ± 10.9b	0.0 ± 0.0b
4.37 mg L^{-1}	1.8 ± 2.4b	0.0 ± 0.0b
8.75 mg L^{-1}	0.0 ± 0.0b	0.0 ± 0.0b

Infection by *Ichthyophthirius multifiliis* Fouquet, 1876 (intensity of zero to 10.0 parasites/field) and *Myxobolus colossomatis* spores Molnár and Békési, 1993 (intensity of zero to 40.0 parasites/field from microscopy) was found in controls fish. In fish exposed to 1.75 mg L^{-1} of $CuSO_4$ showed gills and skin parasitized by *I. multifiliis* (intensity of 0 to 10.0 parasites/field from microscopy) and *M. colossomatis* spores (intensity of 0 to 44 parasites/field from microscopy). In the group exposed to the 4.37 mg L^{-1} concentration, the intensity of *I. multifiliis* varied from zero to 69.0 parasites/field from microscopy and the intensity of *M. colossomatis*, from zero to 23.0 parasites/field from microscopy, but in the group exposed to an 8.75 mg L^{-1} concentration it varied from zero to 30.0 and zero to 35.0 parasites/field from microscopy, respectively. However, these levels of infection on fish treated with 1.75, 4.37 and 8.75 mg L^{-1} of $CuSO_4$ were similar to controls fish.

Blood parameters measurements after sublethal exposure of copper sulfate

Biochemical analysis showed that plasma glucose, total protein, potassium, and chloride concentration did not differ among control and fish exposed to $CuSO_4$. Nevertheless, total protein levels in fish exposed to 1.75 and 8.75 mg L^{-1} showed increase (P<0.05) than those exposed to 4.37 mg L^{-1}. Fish exposed to 1.75 and 4.37 mg L^{-1} of $CuSO_4$ showed a significant decrease (P<0.05) in plasma potassium when compared to those exposed to 8.75 mg L^{-1}. Decreased plasma sodium concentration in the plasma in fish exposed to 1.75 and 4.37 mg L^{-1} of $CuSO_4$ was also observed when compared with the control group and with the groups exposed to 8.75 mg L^{-1} (Table 4).

Red blood cells count decreased (P<0.05) in fish exposed to $CuSO_4$ when compared to unexposed fish while MCV increased (P<0.05). Hematocrit, hemoglobin and MCHC did not differ between fish exposed to $CuSO_4$ and unexposed ones. However, significant increase (P<0.05) of hematocrit in fish submitted to 8.75 mg L^{-1} compared to fish submitted to 1.75 and 4.37 mg L^{-1} was found (Table 4).

Table 4. Biochemical and red blood cell parameters in juveniles *C. macropomum* exposed to concentrations of subletal $CuSO_4$, during 48h. Mean values ± standard deviation (n = 15). Values followed by the same letter do not differ by Tukey test (P<0.05)

Blood parameters	0.0 mg L^{-1}	1.75 mg L^{-1}	4.37 mg L^{-1}	8.75 mg L^{-1}
Glucose (mg dL^{-1})	31.3 ± 7.8a	29.3 ± 5.0a	37.2 ± 11.1a	38.4 ± 8.2a
Total protein (g dL^{-1})	1.5 ± 0.2a, b,c	1.7 ± 0.3a,b	1.4 ± 0.2c	1.8 ± 0.3a
Sodium (mmol L-1)	164.5 ± 22.9b	142.7 ± 9.0a	144.7 ± 15.7a	160.0 ± 23.1b
Potassium (mmol L^{-1})	11.9 ± 2.1ab	9.8 ± 1.8a	10.8 ± 2.7a	16.0 ± 6.3b
Chloride (mmol L^{-1})	76.9 ± 5.6a	80.15 ± 5.9a	76.2 ± 5.6a	79.9 ± 3.4a
Potassium (mmol L^{-1})	11.9 ± 2.1ab	9.8 ± 1.8a	10.7 ± 2.7a	15.9 ± 6.3b
Copper (mmol L^{-1})	0.006 ± 0.006a	0.013 ± 0.021a	0.013 ± 0.012a	0.014 ± 0.008a
Erythrocytes (x 10^6 µL $^{-1}$)	1.185 ± 0.333b	0.699 ± 0.185a	0,683 ± 0.296a	0.746 ± 0.35a
Hematocrit (%)	21.4 ± 2.5ab	18.8 ± 3.3a	20.1 ± 2.9a	23.8 ± 3.4b
Hemoglobin (g dL^{-1})	6.4 ± 1.1a	6.5 ± 0.9a	6.7 ± 1.5a	7.2 ± 1.1a
MCV (fL)	192.6 ± 50.9a	287.2 ± 92.6b	351.3 ± 161.5c	351.2 ± 121.1c
MCHC (g dL^{-1})	30.3 ± 6.4a	35.0 ± 6.2a	34.3 ± 9.9a	30.9 ± 5.1a

The total number of thrombocytes in fish exposed to $CuSO_4$ was similar to controls, but in fish exposed to 8.75 mg L^{-1} was found a decrease number when compared to fish exposed to 1.75 mg L^{-1}. Reduction (P<0.05) in total number of leukocytes was also found in fish exposed to 8.75 mg L^{-1} in comparison to the unexposed ones (Table 5).

Regarding the differential count of leukocytes, the number of lymphocytes, monocytes, eosinophils and PAS-GL did not differ between the animals exposed to 1.75 and 4.37 mg L^{-1} and the unexposed ones. On the contrary, when they were exposed to 8.75 mg L^{-1}, number of lymphocytes and PAS-GL decreased significantly when compared with the control group. The fish treated with 1.75 and 8.75 mg L^{-1} presented a reduced number of neutrophils in comparison to the untreated ones. The number of eosinophils in fish exposed to 1.75 mg L^{-1} was higher than that observed in 8.75 mg L^{-1} (Table 5).

Table 5. Thrombocytes and leukocytes count in juveniles *C. macropomum* exposed to subletal concentrations of copper sulfate. Mean values ± standard deviation (n = 15). Values followed by the same letter do not differ by Tukey test (P<0.05). PAS-GL = PAS-positive granular leukocytes

Parameters	0.0 mg L^{-1}	1.75 mg L^{-1}	4.37 mg L^{-1}	8.75 mg L^{-1}
Thrombocytes (µL)	21,125 ± 4,066ab	29,183 ± 8,015b	26,059 ± 12,415ab	18,193 ± 7,052a
Leukocytes (µL)	18,357 ± 4,856b	18,058 ± 4,963ab	17,196 ± 9,032ab	12,996 ± 4,873a
Lymphocytes (µL)	11,221 ± 2,975b	11,289 ± 3,911ab	9,941 ± 5,428ab	7,725 ± 2,577a
Monocytes (µL)	2,400 ± 1,082a	3,009 ± 1,791a	2,854 ± 1,735a	2,294 ± 1,042a
Neutrophils (µL)	3,297 ± 951b	2,292 ± 1,058a	2,727 ± 1,815ab	2,045 ± 1,856a
PAS-GL (µL)	1,105 ± 560a	872 ± 585ab	1,032 ± 826ab	675 ± 383b
PAS-GL (µL)	1,105 ± 560b	872 ± 585ab	1,032 ± 826ab	675 ± 383a
Eosinophils (µL)	334 ± 324ab	596 ± 348b	642 ± 507ab	257 ± 103a

DISCUSSION

Lethal concentration (LC_{50}) of copper sulfate

Fish tolerance to $CuSO_4$ may suffer interference from the water physical-chemical parameters (SCHLENK *et al.*, 1998; TAVARES-DIAS *et al.*, 2002; ADHIKARI, 2003). Juveniles C. *macropomum* proved to be highly tolerable to $CuSO_4$ in waters with medium hardness and alkalinity, since a LC_{50}-96h = 17.5 mg L^{-1} was

found here. This LC_{50}-96h in *C. macropomum* was similar to the one reported for tilapia in high alkalinity water (MUKHOPADHYAY and KOPNAR, 1984). However, it was higher than the ones reported for *Channa punctactus* and *Labeo rohita* (ADHIKARI, 2003) kept in high alkalinity water and for *Oreochromis niloticus* in medium alkalinity water (VERA and POCSIDIO, 1998). The $CuSO_4$ toxicity may also vary significantly among fish species due to other factors such as fish size, species unique mechanisms for the metabolism of copper ion (DE BOECK *et al.*, 2004) and physiological conditions of the individuals.

Antiparasitic effects of sublethal copper sulfate

Copper $CuSO_4$ has been considered one of the most effective and flexible weapon against several parasitic infections. This product has been used for controlling and treating infections caused by *I. multiliis* (SCHLENK *et al*., 1998; CARNEIRO *et al*., 2005). However, due to *I. multiliis* complex life cycle, treatments must be carried out during several days until control is achieved (SCHLENK *et al*., 1998). In juveniles *C. macropomum,* high concentrations of $CuSO_4$ were not effective for eliminating infections caused by *I. multiliis* and *M. colossomatis*, probably because these concentrations were too low for the elimination of these parasites. Similarly, in *Piaractus mesopotamicus*, treatments with 0.5 and 1.0 mg L^{-1} of $CuSO_4$ during three consecutive days have not been effective in the elimination of *Henneguya piaractus* (TAVARES-DIAS *et al*., 2002). Therefore, an effective treatment does not seem to be based in chemotherapy with $CuSO_4$, which has also not affected the spores of *M. colossomatis* after 48 h of exposure. Myxosporean spores have long living; therefore, 48 h of treatment with $CuSO_4$ was short-time to eliminate the spores as well as to reduce their production.

Anacanthorus spathulatus is a pathogenic monogenoidean and hence can cause decrease on the respiratory capacity of host fish (KRITSKY *et al*., 1979; MORAIS *et al*., 2009). For juveniles *C. macropomum*, 4.37 and 8.75 mg L^{-1} of $CuSO_4$ reduced the number of *A. spathulatus* on the gills and skin after 48 h of exposure. Similarly, a low concentration of $CuSO_4$ (0.25 mg L^{-1}) used during 85 days, removed the monogenoidean *Neodermophthirius harkemai* from the skin of lemon shark *Negaprion brevirostriscontrast* (POYNTON *et al*., 1997). In contrast, treatments with 0.5 and 1.0 mg L^{-1} of $CuSO_4$ during three consecutive days had a limited efficacy in elimination of monogenoidean *Anacanthorus penilabiatus* in gills of teleost *P. mesopotamicus* (TAVARES-DIAS *et al*., 2002). The $CuSO_4$ also showed little efficacy on the control of monogenoidean *Benedeniella posterocolpa* on *Rhinoptera bonasus* rays since it acts more on oncomiracidium than on adult forms (THONEY, 1990). These negative results can be also due to the different concentrations and times used in the treatments, as wells as to the water physical-chemical parameters and the monogenean species sensibility to $CuSO_4$ (TAVARES-DIAS *et al*., 2002). Therefore, for the use effective of CuSO4, besides all the above-mentioned knowledge, it is also essential to know the life cycle of the monogenean species, as well as the lethal concentration for each fish species; since a low concentration may not have the desired effect in the eradication of these parasites and high doses may be extremely toxic and consequently lethal to fish.

Blood parameters measurements after sublethal exposure of $CuSO_4$

Copper may be extremely toxic to fish and causes tissue damages in gills and hematopoietic organs. Gills are the primary target organ for the toxic action of copper (MAZON *et al*., 2002, FIGUEIREDO-FERNANDES *et al*., 2007). Therefore, these changes in both tissues lead to biochemical and hematological disturbances (NUSSEY *et al*., 1995a, b, c; MAZON *et al*., 2002; CARVALHO and FERNANDES, 2006). Copper inhibits the excretion of ammonia through the gills; increases cortisol levels, stimulates the protein catabolism and increases levels of blood ammonia. Consequently, an inhibitory effect on the Na^+/K^+ATPase in the gills is observed (GROSSELL *et al*., 2002). Thus, alterations on the chloride plasma levels have been reported as a tool to evaluate sublethal concentration of $CuSO_4$ (GRIFFIN *et al*., 1999; GROSSELL *et al*., 2002).

In freshwater fishes, a reduction on chloride, sodium and potassium levels can indicate damage in gill cells by compromising the osmoregulation (NUSSEY *et al*., 1995b; GRIFFIN *et al*., 1999). In this assay, an increase in $CuSO_4$ concentration did

not change the levels of plasma chloride and potassium. On the other hand, the concentrations of 1.75 and 4.37 mg L^{-1} of $CuSO_4$ decreased plasma sodium levels and indicated influx of this ion in the tissues. Nevertheless, MATSUO *et al.* (2005) have not observed sodium alterations in fingerlings *C. macropomum* exposed to 50-400 µg L^{-1} copper. Low concentrations of copper (20, 25 and 29 µg L^{-1}), for 96 h, caused a decrease in plasma sodium and chloride, but an increase in potassium in *Prochilodus lineatus* stimulating the ion influx (MAZON *et al*., 2002). However, toxicity of copper to fish varies with physical and chemical water parameters and it must be remarked that in those studies the water alkalinity was different.

In this work, any concentration of copper used does not influence in total protein levels, and also not caused stress in juveniles *C. macropomum*, because the blood glucose levels remained unaffected. *Ictalurus punctatus* exposed to 1.70 mg L^{-1} copper showed no damage in the liver and gills due to an increase in total protein levels (GRIFFIN *et al*., 1999). Hyperglycemia can take place as a stress response to release of catecholamine and corticosteroids (TAVARES-DIAS *et al*., 2002; WITESKA, 2005). This occurs after exposure to toxicants (WITESKA, 2005) or copper (GRIFFIN *et al*., 1999; TAVARES-DIAS *et al*., 2002).

In common-carp *Cyprinus carpio* (WITESKA, 2005) and *Prochilodus lineatus* (CARVALHO and FERNANDES, 2006) the exposure to copper induces blood alterations, characterized by an increase on the hemoglobin concentration, hematocrit and red blood cells count. This fact may be attributed to a compensatory effect in response to oxygen transport capacity (MAZON *et al*., 2002). In *C. macropomum*, it was observed that the number of red blood cells decreased after treatment. However, MCV values increased in fish exposed to 4.37 and 8.75 mg L^{-1} of $CuSO_4$, while, for the hematocrit in fish exposed to 1.75 and 4.37 mg L^{-1}, a decrease was found in relation to those treated with 8.75 mg L^{-1}.

In *Channa punctatus*, 0.36 mg L^{-1} copper sulfate also reduced the number of red blood cells and hematocrit (GRIFFIN *et al*., 1999). This fact is explained because erythrocyte production by the hematopoietic organs decreases due to the destruction of circulating cells (SINGH *et al*., 2008). On the other hand, WILLIAMS & WOOTTEN (1981) related an increase in hematocrit of rainbow trout (*Oncorhynchus mykiss*) treated with $CuSO_4$ for 24 h, corroborating our results with the highest concentration (8.75 mg L^{-1}).

The increase in MCV observed in this assay might be related to the larger erythrocyte volume caused by hypoxia situation, as reported by TAVARES-DIAS *et al.* (2002) in *P. mesopotamicus*. According to NUSSEY *et al.* (1995a, b) and MAZON *et al.* (2002), these alterations are attributed to the damage that copper causes in gills and hematopoietic organs. Although copper is an essential element to fish, it needs to be carefully used for treatment or prophylaxis. When the copper concentration exceeds the tolerating level, fish may be acutely or chronically affected.

Thrombocytes in fish are also cells involved in natural and acquired immunity (PASSANTINO *et al*., 2005; TAVARES-DIAS and MORAES, 2007). These cells are extremely important to homeostasis and coagulation (NUSSEY *et al*., 1995b; WITESKA, 2005). In tilapia, *Oreochromis mossambicus*, exposure to copper caused hemophilia and thrombocytopenia (NUSSEY *et al*., 1995b). This study showed that sublethal concentrations of $CuSO_4$ did not influenced in hemostasis. However, WITESKA (2005) reported accelerated coagulation under stress conditions and it is not necessarily accompanied by an increase in thrombocytes number. This number might be also affected by cortisol levels by destructing these cells as reported in thrombocytopenia. In this study, such fact was observed in fish exposed to 8.75 mg L^{-1} of copper.

Leukocytes are the primary line of immune defense. One of the most elementary ways to assess the immune system is to explore changes in the white blood cell count and its types (TAVARES-DIAS and MORAES, 2007). Therefore, the immune system response to copper sulfate appears to be related to modulation of the immune system. In *C. macropomum* examined in this study leukocytes features were similar to described by, TAVARES-DIAS *et al.* (1999) for this same species. However, some neutrophils and eosinophils here were found with toxic granulations that indicate high production of its

contents. WITESKA and WAKULSKA (2007) reported that phagocytes (neutrophils and monocytes) are sensitive to heavy metal intoxication.

A high CuSO4 content causes immunosuppression, as reported by MAZON *et al*. (2002); TAVARES-DIAS *et al*. (2002) and WITESKA and WAKULSKA (2007), due to the cortisol effect that induces apoptosis of B lymphocytes (WITESKA, 2005; WITESKA and WAKULSKA, 2007). In this assay using juveniles *C. macropomum*, increased $CuSO_4$ concentrations caused leucopenia, characterized by a low number of lymphocytes, neutrophils and PAS-GL. Similarly, in *P. mesopotamicus* exposed to low concentration of copper sulfate, reduced number of lymphocytes and PAS-GL was reported by TAVARES-DIAS *et al.* (2002). Stress response is known by to cause changes on the immune system. On this point of view, in a situation of monocytopenia and neutropenia in *O. mossambicus*, the migration and phagocytic activity in the gills, liver and kidney are disrupted by exposure to copper (NUSSEY *et al*., 1995c). On the contrary, leukocytosis with increase in the lymphocytes and eosinophils numbers was followed by decrease in the numbers of monocytes and basophils in *C. punctatus* (SINGH *et al*., 2008). Leukocytosis has been attributed to an increase in leukocyte to protect the organism against infections in copper-damaged tissue (MAZON *et al*., 2002).

CONCLUSIONS

In juveniles *C. macropomum*, since 4.37 mg L^{-1} of CuSO4 showed 99.3% of efficacy in the treatment against monogenoideans *A. spathulatus*, therefore concentrations between 5.0-6.0 mg L^{-1} may be sufficient for eliminating these parasites, in the trial conditions used here. Short-term exposure to sublethal $CuSO_4$ causes physiological alterations affecting the osmotic imbalance. Furthermore, higher $CuSO_4$ concentration also causes severe immunosuppression in fish, which may make the organism susceptible to diseases. Blood parameters of *C. macropomum* may be used safely as a tool in field for monitoring the contamination caused by this heavy metal. The concentrations of $CuSO_4$ used in aquaculture are lower or higher when compared to this study, however these causes toxicity to *C. macropomum*. Therefore, this chemical product must be used with parsimony for treating fish's parasitic infection, because the use of $CuSO_4$ in fish farm is quite complex and depends on the interplay of several environmental factors.

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