Effects of the copper in the survival, growth and gill morphology of *Danio rerio* (Cypriniformes, Cyprinidae)

Efeitos do cobre na sobrevivência, crescimento e morfologia das brânquias de *Danio rerio* (Cypriniformes, Cyprinidae)

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Abstract: With the objective of evaluating the effects of copper on the survival, growth and gill morphology of juvenile zebrafish (*Danio rerio*), four partial chronic toxicity bioassays were conducted (seven days' duration) in a semi-static system with renewal every 24 hours. The results showed effects on both survival and growth, but the chronic value for survival (CV = 8.9 µg.Cu.L^{-1}) was nearly five times greater than that for growth (weight) (CV = 1.78 µg.Cu.L^{-1}). The negative effects on the survival and growth of the fish were not related to increased copper concentrations, but this relation was observed for the intensity of the gill lesions. Therefore, growth and gill morphology were a more suitable parameter than survival to evaluate the toxicity of copper, and thus should be considered in determining new concentration limits in environmental legislation for protection of aquatic life.

Keywords: copper, histopathology, toxicity tests, *Danio rerio*.

Resumo: Com o objetivo de avaliar os efeitos do cobre na sobrevivência, crescimento e morfologia das brânquias de indivíduos juvenis de *Danio rerio*, quatro testes de toxicidade crônica-parcial foram realizados (sete dias de duração) sob condições semi-estáticas com renovação a cada 24 horas. Os resultados mostraram efeitos tanto na sobrevivência quanto no crescimento dos organismos, sendo que o valor crônico encontrado para a sobrevivência (CV = 8.9 µg.Cu.L^{-1}) foi cerca de cinco vezes maior que aquele encontrado para o crescimento (peso) (CV = 1.78 µg.Cu.L^{-1}). Os efeitos negativos na sobrevivência e crescimento dos peixes não apresentaram relação com o aumento das concentrações de cobre. No entanto, esta relação foi observada na intensidade das lesões nas brânquias. Assim, o crescimento e a morfologia das brânquias foram variáveis mais sensíveis que a sobrevivência na avaliação da toxicidade do cobre e, portanto, devem ser considerados na determinação de novas concentrações-limite para a proteção da vida aquática em legislações ambientais.

Palavras-chave: cobre; histopatologia, testes de toxicidade, *Danio rerio*.

1. Introduction

Copper is an essential element for the entire biota. The lack of this nutrient can trigger enzymatic dysfunctions, but it can also be toxic to fish when present at high concentrations in the water (Pickering and Henderson, 1966).

In recent decades studies have shown that copper causes harmful effects that range from the molecular to structural levels in freshwater fish (Baker, 1969; Billiard et al.*,* 1981; Eddy, 1981; Bengtsson and Larsson, 1986; WHO, 1998; Kalay et al., 1999; Beaumont et al., 2000; Olsvik et al., 2000; Cerqueira and Fernandes, 2002; Mazon and

Fernandes, 2002; Van Heerden et al., 2004). Because of the vital functions of the gills (respiration, osmorregulation and excretion), they are in direct contact with the external environment, which facilitates interaction with toxic substances in the water. For this reason, they are considered excellent indicators of environmental quality (Wendelaar Bonga, 1997).

Over the past two decades, the histopathalogical and physiological changes in gills under acute and chronic exposure to heavy metals have been studied in many fish spe-

cies (Laurén and McDonald, 1987; Rajbanshi and Gupta, 1988; Benedetti et al., 1989; Matei and Komov, 1992; Lacroix et al., 1993; Sola et al., 1995; Galvez et al., 1998; Dang et al., 2000; Van Heerden et al., 2004). These studies have shown that the intensity of the lesions depends on the type of pollutant, concentration and time of exposure. These changes can impair the exchange of gases necessary to metabolism, ionic regulation and acid-base equilibrium (Mazon et al., 2002), thus compromising the survival of exposed individuals.

Despite the many studies of the toxicity of copper, there have been few reports of the combined use of various parameters to assess these effects. Therefore, the objective of this study is to evaluate the toxicity of the copper ion on the survival, growth and gill morphology of juvenile zebrafish (*Danio rerio*) exposed to sublethal concentrations in partial chronic bioassays. This investigation will increase the knowledge of the effects of copper on fish, which is important to serve as a basis for discussions of limitations on use of this substance. In this respect, this study can help in the formulation of new concentration limits for copper, to improve Brazilian environmental legislation.

2. Material and Methods

2.1. Acute toxicity tests

The *D. rerio* juveniles (total weight: 0.04 ± 0.02 g; standard length: 1.37 ± 0.22 cm) were obtained commercially and submitted to sensitivity tests with potassium dichromate for 96 hours, to determine the LC_{50} and evaluate their health. Then they were exposed to progressive concentrations of copper (20.0; 60.0; 120.0 and 360.0 $\mu g.Cu.L^{-1}$) in acute toxicity tests in reconstituted water (ABNT, 2004). These concentrations were established through six acute preliminary tests and subsequently they served like base to establish the sensitivity range and to define the sublethal concentrations to be used in the partial chronic bioassays. The chemical agent was used in the form of copper sulphate ($CuSO_4.5H_2O$) and expressed in $\mu g.L^{-1}$. All the tests were carried out in the Fish Reproduction Laboratory of the Department of Hydrobiology of the Federal University of São Carlos (São Carlos, São Paulo, Brazil) according to the mentioned standards. The bioassays were conducted with two repetitions containing a total of 12 juvenile fish (respecting the proportion 1g of fish per liter of solution) under controlled conditions of temperature (25 ± 1 °C) and photoperiod (12D:12L). The pH, conductivity, hardness and dissolved oxygen parameters were monitored throughout the experiments and the dead organisms removed and counted during the exposure period. The calculation of the 96 hours LC_{50} was done based on the trimmed Spearman-Karber computational program (Hamilton et al., 1977), and the sensitivity range was established according to the recommendations of the USEPA (1995).

2.2. Partial chronic toxicity bioassays

The concentrations used in the partial chronic experiments were based on the results of the acute bioassays. According to the recommendations of the SEMA (1988), selection of the test solutions is based on the smallest lethal concentration for organisms in acute toxicity tests, which in this study was 20.0 $\mu g.Cu.L^{-1}$. From this, we established four dilutions using 5 as factor of dilution (0.16; 0.8; 4.0 and 20.0 $\mu g.Cu.L^{-1}$). The bioassays were semi-static, lasting seven days with renovation every 24 hours, consisting of three repetitions with 10 specimens each (SEMA, 1988). The experiments were repeated four times to determine possible patterns, as well as the variability in the specimens' responses. The aeration was constant, the photoperiod (12D:12L) and temperature (25 ± 1 °C) were controlled, and every 24 hours the pH, hardness and conductivity of the water and fish mortality were recorded. The fish were not fed during the experiments, to keep the substances tested from binding to the food.

2.3. Weight and standard length measurements

At the start of the bioassays, 10% of the juvenile fish acquired were weighed and measured to assure their homogeneity. These fish were then discarded. The standard length and total weight of the surviving organisms in the tests were evaluated with a high precision scale and gage, respectively. The data were compared statistically to the measures obtained with the control organisms through the Mann-Whitney non-parametric test, allowing the detection of chronic effects on the growth and development of the test specimens. This assessment was not done in the bioassays that produced mortality above 90%, due to deficient data.

2.4. Histological assessment of the gills

The histological studies were carried out on the gills of the fish that survived the partial chronic tests with the copper concentrations. After the tests, the specimens were fixed in Bouin's fluid, dehydrated and embedded in paraffin, sliced into 4.0 to 6.0 μm seriated cuts using a MICRON HM 340E microtom and steel blade, stained with hematoxylin-eosin (HE), and then studied under an optical microscope. The type and degree of alterations were based on Poleksic and Mitrovic-Tutundisic (1994).
Evaluation of the toxicity results

The fish were removed and counted at the end of the bioassays. The Fisher's exact test based on the Bio Estat program (Ayres et al., 2001) was used to compare the survival of the fish exposed to the various copper concentrations versus that of the control group.

3. Results

3.1. Determination of the 96 hours LC_{50} and the sensitivity range

The results of the mortality of the fish exposed in the five acute (definitive) tests showed an average 96 hours LC_{50} of

73.83 µg.Cu.L^{-1} and a standard deviation of 29.73 µg.Cu.L^{-1} (Table 1). The lowest concentration that caused an effect in most of the bioassays was 20.0 µg.Cu.L^{-1}. By applying the model proposed by the USEPA (1995) (X ± 2SD), where the sensitivity range is between the mean (X) of the readings of the 96 hours LC$_{50}$ plus or minus twice the standard deviation (2 SD), we obtained a range of from 14.37 to 133.29 µg.Cu.L^{-1}.

3.2. Survival of the fishes in the partial chronic bioassays

Analysis of the four experiments revealed the variability in the specimens' responses. In two of the bioassays (1 and 4), the minimum survival percentage was 75% at the highest concentration (20.0 µg.Cu.L^{-1}). This result differed significantly from that of the control group. In the third test, the mortality percentage was around 80%, with a significant difference in relation to the control group, but this value occurred at a test concentration of 4.0 µg.Cu.L^{-1}. In the second bioassay there were no significant differences in relation to the control group, and the survival percentages were above 90% (Table 2). Based on the significant survival results in relation to the control, the lowest mean observed effect concentration (OEC) was 20.0 µg.Cu.L^{-1}, the highest non-observed effect concentration (NOEC) was 4.0 µg.Cu.L^{-1} and the mean chronic value (CV) was 8.9 µg.Cu.L^{-1} (Table 2). It was not possible to calculate these figures in the second and third bioassays.

The values of the pH, conductivity and hardness measured at the start and end of the bioassays varied among the four experiments, and no relationship was found between these parameters and the progressive increase in the copper concentrations.

3.3. Growth of the test organisms in the partial chronic bioassays

The homogeneity of the groups of fish obtained for the toxicity tests was satisfactory. There was a significant difference in standard length between the fish exposed to a concentration of 20.0 µg.Cu.L^{-1} and the control group

in bioassay 4 (B4). For weight, the significant differences were found between the concentration of 0.8 µg.Cu.L^{-1} and the control (B1); and between the 4.0 and 20.0 µg.Cu.L^{-1} concentrations and the control (B4). It was not possible to carry out the final biometry of the fish exposed in B2 because the control group was discarded accidentally. The mean OEC, NOEC and CV figures for the growth parameter (length) were respectively 20.0; 4.0 and 8.9 µg.Cu.L^{-1}, while for weight they were 0.8 (OEC); 4.0 (NOEC) and 1.78 (CV) µg.Cu.L^{-1}. Therefore, it was possible to observe that the CV for the growth parameter was nearly five times lower than the CV for survival, and weight (CV = 1.78) was more sensitive than growth (CV = 8.9) (Table 3).

3.4. Gill morphology

The fish in the control group had gills with normal morphology, i.e., development of the respiratory area with well-defined spaces between the secondary lamellae; secondary lamellae with distinguishable pillar cells and erythrocytes; normal development of the cartilaginous support in the respiratory area and placement of the stratified epithelium of the gill filament (Figure 1a). In general, the lesions found were proliferation of epithelial cells in the respiratory and non-respiratory region; reduction of the interlamellar spaces and the respiratory area; dilation of the blood capillaries in the secondary lamellae; dilation in the area of the central venous sinus and total fusion of some secondary lamellae (Figure 1). The lesions found on the gills of the fish exposed to copper became more accentuated with the progressive increase in the concentration, since at the three highest concentrations (0.8; 4.0 and 20.0 µg.Cu.L^{-1}) there were more intense proliferations and second-stage alterations (rupture of lamellae and aneurisms) (Figure 1c,d,e). Fusions of the walls of the blood vessels in the secondary lamellae were also observed at the concentration of 0.8 µg.Cu.L^{-1}.

4. Discussion

The mortality levels found in the acute toxicity bioassays were similar to those reported by Bertoletti (2000),

Table 1. Results of the definitive acute toxicity tests with copper showing the 96 hours LC$_{50}$ values and the limits of confidence (95%), according to the trimmed Spearman-Karber computational program.

Test Number	96 hours LC$_{50}$ µg.Cu.L^{-1}	Confidence limit (95%)	
		Lower	Upper
1	81.14	61.69	106.70
2	116.41	85.19	159.07
3	80.81	57.73	113.11
4	46.70	35.55	61.33
5	44.09	31.11	62.50
M	73.83	54.25	100.54
SD	29.73	21.85	40.63

M = mean; and SD = standard deviation.

Table 2. Survival of the test organisms in the four partial chronic bioassays and the OEC, NOEC and VC values and results of the Fisher test.

Concentrations of copper (µg.L^{-1})	Survival (%)			
	B1	B2	B3	B4
C	100	95	100	96
0.16	80	100	100	96
0.8	85	90	93	96
4.0	90	95	80*	96
20.0	75*	90	95	73*
OEC	20,0	-	-	20,0
NOEC	4,0	-	-	4,0
CV	8,9	-	-	8,9

C = control; B = bioassay; and * = significant in relation to the control.

Table 3. Comparison of the standard length and total weight parameters of *D. rerio* test organisms exposed to concentrations of copper in relation to the control group by means of the Mann-Whitney test, as well as, OEC, NOEC and CV values.

Copper (µg.L⁻¹)	Standard length (±SD) (cm)			Total weight (±SD) (g)		
	B 1	B 3	B 4	B 1	B 3	B 4
C	1.31 (±0.15)	1.19 (±0.08)	1.62 (±0.15)	0.0260 (±0.028)	0.0197 (±0.007)	0.0627 (±0.02)
0.16	1.23 (±0.15)	1.22 (± 0.09)	1.64 (±0.19)	0.0195 (± 0.011)	0.0219 (±0.007)	0.0603 (±0.02)
0.8	1.21 (±0.07)	1.23 (±0.12)	1.61 (±0.16)	0.0164* (±0.006)	0.0234 (±0.012)	0.0554 (±0.02)
4.0	1.21 (±0.09)	1.22 (±0.07)	1.60 (±0.16)	0.0204 (±0.010)	0.0215 (±0.006)	0.0507* (±0.02)
20.0	1.29 (±0.11)	1.17 (±0.09)	1.54* (±0.15)	0.0225 (±0.007)	0.0175 (±0.004)	0.0492* (±0.01)
OEC	-	-	20.0	0.8	-	0.8
NOEC	-	-	4.0	-	-	4.0
CV	-	-	8.9	-	-	1.78

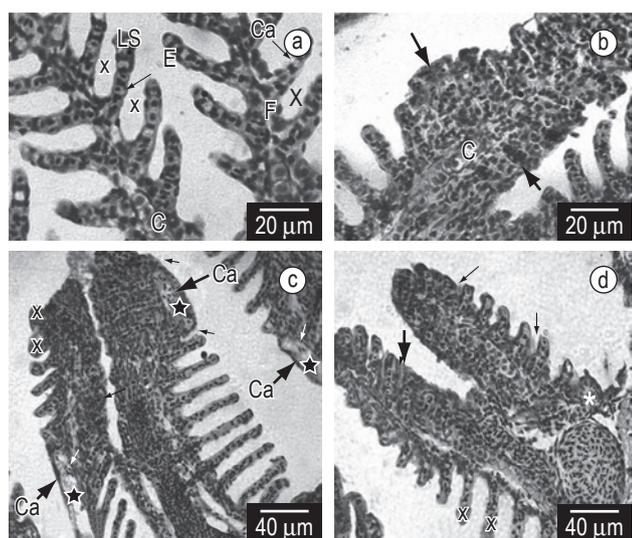C = control; B = bioassay; and * = significant in relation to the control.

Figure 1. Sagital sections of the gill filament of *D. rerio* individuals exposed to different concentrations of copper. a) Control. Observe the position of the secondary lamellae (LS) along the gill filament (F); stratified epithelium of the filament with well-defined interlamellar spaces (x); erythrocytes (E); cartilage (C) and blood capillaries (Ca). b) 0.16 µg.Cu.L⁻¹. Proliferation of epithelial cells and fusion of some lamellae (→). c) 0.8 µg.Cu.L⁻¹. More pronounced proliferation of cells between the secondary lamellae and fusion of various lamellae (→); dilation of blood capillaries in the secondary lamellae, hindering distinction between the pillar cells (x); fusion of the walls of the blood capillaries (➤ Ca) and rupture of secondary lamellae (★). and d) 20.0 µg.Cu.L⁻¹. Intense proliferation of cells between the secondary lamellae (➤); complete fusion of various lamella (→); dilation of blood capillaries in the secondary lamellae (x); aneurism (*) (HE stain).

confirming the LC₅₀ for this species under the same physical and chemical conditions of reconstituted water. Regarding sublethal effects, Kristensen (1994), in evaluating the sensitivity of survival and growth (weight and length) to various chemical compounds (organic and inorganic), found that in 90% of the tests the effect on growth was the most sensitive parameter at the juvenile stage, reflected in the OEC value. The author observed also that the concentrations that caused effects on growth were in the majority of cases three times lower than the concentrations found for survival. This situation was also verified by Pickering and Lazorchac (1995), in studying the survival and growth of larvae of *Pimephales promelas* exposed to sublethal concentrations of hexavalent chrome and bivalent copper. According to these authors, the OEC was nearly four to eight times below the values found for survival, except in the experiments with higher hardness. Similar results were obtained in the present study, where the OEC determined for survival was about five times higher, indicating greater sensitivity of the growth parameter (weight) to evaluate the toxicity of copper. Regarding the variability found in the responses of the organisms exposed in the different bioassays and the absence of a relationship between the effects observed and the copper concentrations, other recent works corroborate these results. Bertoletti (2000), in evaluating the chronic toxic effects of various chemical compounds at different initial phases of the life cycle of *D. rerio*, under the same experimental conditions as this study, found a NOEC of 92.0 µg.Cu.L⁻¹ for survival, i.e., a concentration roughly 23 times higher than the NOEC calculated in this study. The variability of the growth parameter can be attributed to several factors, such as: the sensitivity of the test organisms, which is reflected in their tolerance to toxic substances; the size of the organisms used in each bioassay (there was a difference of 26.5% in the length of individuals between the lots acquired); and intrinsic factors of each individual (competitive or aggressive behavior in the testing tank).

Sloman et al. (2000) found that the standard metabolic rate (SMR) of the subordinated fishes of the brown trout (*Salmo trutta*) was significantly greater than that of their respective dominant during a situation of social stress (caged). The authors concluded that the difference can offer disadvantage for these subordinated regarding the dominant

organisms. In this way, the caged during of the toxicity tests can have caused social stress in the organisms-tests, which possibly resulted in the variability of the results observed in the toxicity tests with copper in this work.

A work carried out with sublethal concentrations of copper (30.0 µg.L^{-1}) by Sloman et al., (2002) checked that, before a situation of caged, the juvenile subordinate organisms of the fish species *Onchorynchus mykiss* presented grater accumulation of copper in the gills and liver than that the organisms qualified like dominants. The authors verified that the subordinated fishes presented higher uptake of copper than dominant fish.

We also stress that at moderate concentrations, freshwater fish are able to regulate the essential elements in their bodies in relation to the outside environment (Nimmo, 1985), developing resistance to toxic substances (Lock et al., 1994). The degree of this capacity for tolerance can vary from species to species, and in the present study, it may have occurred from individual to individual of a single brood (Wlodék and Skóra, 1985). This demonstrates the importance of conducting repeated toxicity tests to obtain more accurate results of the effects of pollutants on the survival and growth of fish.

Rigolim-Sá (1998), evaluating the toxicity of the herbicide Roundup and the acaricide Omite on the initial ontogeny phases of the bagre fish (*Rhamdia hilarii*), besides finding that the lowest Roundup concentration did not have the smallest effect on growth (also using test fish from the same brood), found that similar individuals (from the same reproduction) did not have equal behaviors, and consequently did not have the same tolerance to toxic agents. This situation was clearly shown in the present study, mainly in the first bioassay, since the significant differences in the weight of the test and control fish occurred only at the concentration of 0.8 µg.Cu.L^{-1}. We should mention that at this concentration the survival rate was high, which also may have influenced their growth.

The negative effects on the survival and growth of the fish exposed to the copper ion in the present study were possibly affected by the gill lesions caused. All the exposed fish had some type of lesion. We observed a progressive increase in the proliferation of cells of the respiratory epithelium with increasing copper concentrations. Also, in some cases the intense hyperplasia led to complete fusion of some secondary lamellae. Similar responses have been observed of *Ictalurus nebulosus* at 48 hours and 40 days of exposure to concentrations of 5.0 µg.Cu.L^{-1} (Benedetti et al., 1989); of *Oncorhynchus mykiss* at 30 minutes of exposure to 6.4 µg.Cu.L^{-1} (Sola et al., 1995); of *Solea senegalensis* exposed for 7 days to 0.1 µg.Cu.L^{-1} (Arellano et al., 1999); and of *P. scrofa* exposed to 20.0; 25.0 and 29.0 µg.Cu.L^{-1} for 96 hours (Cerqueira and Fernandes, 2002; Mazon et al., 2002). These epithelial alterations generally are the most common responses in organisms exposed to toxic agents (Mallatt, 1985), and have also been reported in fish exposed to zinc (Galvez et al., 1998); cadmium (Oronsaye and Brafield, 1984; Sorensen, 1991); and aluminum (Matei and Komov, 1992).

According to Rajabanshi and Gupta (1988), lamellar fusion can lead to a pronounced reduction of the respiratory surface, because of the disappearance of the secondary lamellae, consequently hindering gas exchanges and other gill functions.

Lesions in the blood vessels have been observed in organisms exposed to higher concentrations of copper (4.0 and 20.0 µg.Cu.L^{-1}), among which aneurisms can be considered the most serious. Mazon et al. (2002), evaluating the effects of the copper ion on *P. scrofa* juveniles during 96 hours of exposure found aneurisms in the secondary lamellae of specimens exposed to 20.0; 25.0 and 29.0 µg.Cu.L^{-1}, and in some cases could observe rupture of the secondary lamellae and bleeding. These results corroborate those of the present study, where we found that copper can cause possibly irreversible effects at high concentrations. Nevertheless, the species we used was more sensitive, since the aneurisms were encountered at lower concentrations (4.0 and 20.0 µg.Cu.L^{-1}). This fact may be related to the exposure period, which was longer in the present study, and also to the different sensitivity of the species used.

In the natural environment, toxic substances are normally found at sublethal concentrations, and for this reason it is essential for effects that are not immediately visible (growth, morphological and physiological changes, etc.) to be evaluated in toxicity tests. Organisms submitted to chronic concentrations have reduced resistance, and instead of using energy for growth and reproduction, will divert it to detoxification of the pollutant. This can cause reduced size, reproduction and survival of juveniles, and consequently increase the vulnerability of the species itself, not to mention the balance of the ecosystem.

5. Conclusion

Despite copper's importance as a micronutrient in the metabolism of aquatic organisms, the work showed that even at very low concentrations this metal can compromise the survival and growth of *Danio rerio*. Among the parameters analyzed, weight and changes in the gills were the most sensitive to evaluate damages, with the last being the most suitable, since the severity of the lesions was directly related to the progressive increase in the copper concentrations. Further investigations of this nature are thus essential to formulate water quality standards that can effectively protect aquatic life.

Acknowledgements

References

ARELLANO, J.M., STORCH, V. and SARASQUETE, C. Histological changes and copper accumulation in liver and gills of Senegales sole, *Solea senegalensis. Ecotoxicol. Environ. Saf.*, 1999, vol. 44, no. 1, p. 62-72.

ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS (ABNT). NBR 15088. *Ecotoxicologia aquática:* Toxicidade aguda – Método de ensaio com peixes. Rio de Janeiro, 2004. 19 p.

AYRES, M., AYRES Jr., M., AYRES, D.L. and SANTOS, A.A. *Bio Estat 2.0.* Computacional Program. Belém: Sociedade Civil de Mamirauá, MCT-CNPQ, 2001.

BAKER, J.T.P. Histological and electron microscopical observations on copper poisonings in the winter flounder (*Pseudopleuronects americanus*). *J. Fish. Res. Bd. Can.*, 1969, vol. 26, p. 2785-2793.

BEAUMONT, M.W., BUTLER, P.J. and TAYLOR, E.W. Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. *Aquat. Toxicol.*, 2000, vol. 51, no. 2, p. 259-272.

BENEDETTI, I., ALBANO, A.G. and MOLA, L. Histomorphological changes in some organs of the brown bullhead, *Ictalurus nebulosos* Le Sueur, following short and long-term exposure to copper. *J. Fish. Biol.*, 1989, vol. 34, no. 2, p. 273-280.

BENGTSSON, B. and LARSSON, A. Vertebral deformities and physiological effects in fourhorn sculpin (*Myxocephalus quadricornis*) after long-term exposure to a simulated heavy metals containing effluent. *Aquat. Toxicol.*, 1986, vol. 9, no. 4-5, p. 215-229.

BERTOLETTI, E. *Estimativa de efeitos tóxicos crônicos com Danio rerio (Pisces, Cyprinidae)*. São Paulo: Faculdade de Saúde Pública, Universidade de São Paulo – USP, 2000. [Master thesis in Public health].

BILLIARD, R., BRY, C. and GILLET, C. Stress, environment and reproduction in teleost fish. In PICKERING, AD. (Ed.). *Stress and Fish*. London: Academic Press, 1981. p. 185-207.

CERQUEIRA, C.C.C. and FERNANDES, M.N. Gill tissue recovery copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa. Ecotoxicol. Environ. Saf.*, 2002, vol. 52, no. 2, p. 83-91.

DANG, ZC., LOCK, RAC., FLIK, G. and WENDELAAR BONGA, SE. Na^1/K^1-ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to copper. *J. Exp. Biol.*, 2000, vol. 203, no. 2, p. 379-387.

EDDY, F.B. Effects of stress on osmotic and ionic regulation in fish. In PICKERING, AD. (Ed.). *Stress and Fish*. London: Academic Press, 1981. p. 77-102.

GALVEZ, F., WEBB, N., HOGSTRAND, C. and WOOD, C.M. Zinc binding to the gills of rainbow trout: The effect of long-term exposure to sublethal zinc. *J. Fish. Biol.*, 1998, vol 52, no. 6, p. 1089-1104.

HAMILTON, MA., RUSSO, R.C. and THURSTON, R.U. Trimmed Sperman-Karber method for estimating median lethal concentration in toxicity bioassays. *Environ. Sci. Technol.*, 1977, vol. 11, no. 7, p. 714-719.

KALAY, M., AY, Ö. and CANLI, M. Heavy metal concentrations in fish tissues from the northeast Mediterranean sea. *Bull. Environ. Contam. Toxicol.*, 1999, vol. 63, no. 5, p. 673-681.

KRISTENSEN, P. Sensitivity of embryos and larvae in relation to other stages in the life cycle of fish: a literature review. In MÜLLER, R. and LLOYD, R. (Eds.). *Sublethal and chronic effects of pollutants on freshwater fish.* United Nation: Fishing News Books, 1994. p. 155-174.

LACROIX, G.L., RICHARD, H.P., BELFRY, C.S. and MARTIN-ROBICHAUD, D.J. Aluminium dynamics on gills of Atlantic salmon fry in the presence of citrate and effects on integrity of gill structures. *Aquat. Toxicol.*, 1993, vol. 27, no. 3-4, p. 373-402.

LAURÉN, D.J. and MCDONALD, D.G. Effects of copper on branchial ion regulation in raibow trout, *Salmo gairdneri*, Richardson modulation by water hardness and pH. *J. Comp. Physiol.*, 1985, vol. 155, no. 5, p. 635-644.

LOCK, R.A.C., BALM, P.H.M. and WENDELAAR BONGA, S.E. Adaptation of freshwater fish to toxicants: stress mechanisms induced by branchial malfunctioning. In MULLER R. and LLOYD, R. (Eds.). *Sublethal and Chronic effects of Pollutants on freshwater fish*. United Nation: Fishing News Books, 1994. p. 214-134.

MALLATT, J. Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Can. J. Fish. Aquat. Sci.*, 1985, vol. 42, no. 4, p. 630-648.

MATEI, V.E. and KOMOV, V.T. The effect of aluminum and low pH of water on gill ultrastructure and electrolyte content in blood plasma of juvenile *Salmo salar. J. Evol. Biochem. Physiol.*, 1992, vol. 28, no. 5, p. 453-460.

MAZON, A.F., CERQUEIRA, C.C.C. and FERNANDES, M.N. Gill cellular changes induced by copper exposure in the South American tropical fish *Prochilodus scrofa. Environ. Res. Section.*, 2002, vol. 88, no. 1, p.52-63.

NIMMO, D.R. Pesticides. In RAND, G.M. and PETROCELLI, S.R. (Eds.). *Fundamentals of aquatic toxicology:* methods and applications. New York: Hemisphere, 1985. p. 335-373.

OLSVIK, P.A., PÅL, G., ANDERSEN, R.A. and ZACHARIASSEN, K.E. Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. *Aquat. Toxicol.*, 2000, vol. 50, no. 4, p. 301-316.

ORONSAYE, J.A.O. and BRAFIELD, A.E. The effect of dissolved cadmium on the chloride cells of the gills of the stickleback. *Gasterosteus acule*atus L. *J. Fish Biol.*, 1984, vol. 25, no. 2, p. 253-258.

PICKERING, Q.H. and HENDERSON, P. The acute toxicity of some heavy metals to different species of warm water fishes. *Air Water Pollut. Int. J.*, 1966, vol. 10, no. 6, p. 453-463.

PICKERING, Q.H. and LAZORCHAK, J.M. Evaluation of the robustness of the Fathead minnow, *Pimephales promelas*, larval

survival and growth test, U.S.EPA Method 1000.0. *Environ. Toxicol. Chem.*, 1995, vol. 14, no. 4, p. 653-659.

POLEKSIC, V. and MITROVIC-TUTUNDIZIC, V. Fish gills as monitor of sublethal and chronic effects of pollution. In: MULLER, R. and LLOYD, R. (Eds.). *Sublethal and Chronic effects of Pollutants on freshwater fish*. United Nation: Fishing News Books, 1994. p. 339-352.

RAJBANSHI, V.K. and GUPTA, A.K. Alterations in the architecture of gill surface produced by water-borne copper in *Hepteroneusters fossilis* (Bloch). *Acta Hydrochim. Hydrobiol.*, 1988, vol. 16, no. 3, p. 325-332.

RIGOLIM-SÁ, O. *Toxicidade do herbicida Roundap (glifosato) e do acaricida Omite (propargito) nas fases iniciais da ontogenia do bagre, Rhandia hilari (VALENCIENNES, 1840) (Pimelodidae, Siluriforme)*. São Carlos: Universidade Federal de São Carlos – UFSCar, 1998. [Master thesis in Ecology and Natural Resources].

SECRETARIA ESTATUAL DO MEIO AMBIENTE (SEMA). Avaliação da toxicidade para peixes (D.3). In: *Manual de testes para avaliação da ecotoxicidade de agentes químicos*. Brasília, 1988. 10 p.

SLOMAN, KA., MOTHERWELL, G., O'CONNOR, KI., and TAYLOR, AC. The effect of social stress on the Standard Metabolic Rate (SMR) of brown trout, *Salmo trutta. Fish Physiology an Biochemistry.*, 2000, vol. 23, no. 1, p. 49-53.

SLOMAN, K.A., BAKER, D.W., WOOD, C.M. and MCDONALD, G. Social interactios affect physiological consequences of sublethal copper exposure in Rainbow trout, *Onchorynchus mykiss. Environ. Toxicol. Chem.*, 2002, vol. 21, no. 6, p. 1255-1263.

SOLA, F., ISAIA, J. and MANSONI, A. Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss. J. Appl. Toxicol.*, 1995, vol. 15, no. 5, p. 391-98.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY (USEPA). *Quality criteria for water*. Washington, D C., 1995.

VAN HEERDEN, D., VOSLOO, A. and NIKINMAA, M. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor α (HIF - 1α) levels in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.*, 2004, vol. 69, no. 3, p. 271-280.

WENDELAAR BONGA, S.R. The stress response in fish. *Physiology Reviews*, 1997, vol. 77, p. 591-625.

WENDELAAR ONGA, S.E. and LOCK, R.A.C. Toxicants and osmoregulation in fish. *Netherlands Journal of Zoology*, 1991, vol. 42, no. 2-3, p. 478-493.

WLÓDEK, J.M. and SKÓRA, S. Redression investigation of the length onweight dependence in seven freshwater fish. *Acta Hydrobiol.Krakov*, 1985, vol. 25, no. 26, p. 497-506.

WORLD HEALTH ORGANIZATION. *Copper*. Geneva: WHO, 1998.