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Acute copper toxicity in juvenile fat snook *Centropomus parallelus* (Teleostei: Centropomidae) in sea water

Bruno L. Oliveira[1], Luiz F. Loureiro Fernandes[1], Adalto Bianchini[2], Adriana R. Chippari-Gomes[3], Bruno F. Silva[3], Geisamanda P. Brandão[1] and Levy C. Gomes[3]

Three experiments were designed to assess the accumulation and acute toxicity of copper (Cu) in juvenile fat snook *Centropomus parallelus*. The first experiment was performed to determine the 96-h lethal concentration (LC_{50}) of Cu. The second experiment was designed to assess the effects of sublethal concentrations of Cu (0.47 and 0.94 mg/L), while the third one allowed us to test the recovery capacity of fish exposed to the sublethal concentrations Cu and kept in sea water without Cu addition. The LC_{50} value for Cu was found to be 1.88 mg/L Cu. Fish exposed to the sublethal concentrations of Cu showed a significant accumulation of Cu in gills at 96 h respect to the control ones (0.43 µg/g Cu). No significant difference was observed in the accumulation of Cu in gills between fish exposed to 0.47 mg/L (1.09 µg/g Cu) and 0.94 mg/L (1.26 µg/g Cu). Exposure (24 and 96 h) to the sublethal concentrations of Cu tested induced DNA damage in the erythrocytes. The results show that acute exposure to sublethal concentrations induces Cu accumulation and DNA damage in fish, these effects being recovered after 240 h in sea water without Cu addition.

Três experimentos foram realizados para avaliar o acúmulo e toxicidade aguda do cobre (Cu) em juvenis de robalo-peva *Centropomus parallelus*. O primeiro experimento foi realizado para determinar a concentração letal (96h-CL_{50}) de Cu. O segundo experimento foi realizado para avaliar os efeitos de concentrações subletais de Cu (0,47 e 0,94 mg/L), enquanto o terceiro permitiu testar a capacidade de recuperação dos peixes expostos a concentrações subletais do Cu e posteriormente mantidos em água do mar sem acréscimo de Cu. O valor de LC_{50} encontrado para o Cu foi de 1,88 mg/L. Os peixes expostos as concentrações subletais de Cu mostraram um acúmulo significativo nas brânquias em relação ao controle em 96 h de exposição (0,43 µg/g Cu). Nenhuma diferença significativa foi observada entre os peixes expostos a 0,47 mg/L de Cu (1,09 µg/g) e 0,94 mg/L de Cu (1,26 µg/g). A exposição (24 e 96 h) para as concentrações subletais de Cu induziram danos no DNA. Os resultados mostram que a exposição aguda a concentrações subletais induz o acúmulo de Cu e danos ao DNA nas brânquias dos peixes, onde estes efeitos são recuperados após 240 h em água do mar sem adição de Cu.

Key words: Aquatic toxicology, Comet assay, Gills, Lethal toxicity, Micronucleus test.

Introduction

Aquatic animals are naturally exposed to various metals, and both geochemical processes and human activities govern the chemical form and concentration of these chemical contaminants in water (Sousa *et al.*, 2007). Mining, smelting, and other industrial activities generate soluble or particulate material residues that are further released via the atmospheric or industrial wastes. These residues are destined to end up in most estuaries, seas, and oceans as toxic metals and compounds (Lokhande *et al.*, 2011).

Copper (Cu) is an important metal employed in many industrial processes, being considered as highly toxic to biota, even at low concentrations (Who, 1998). Moreover is an essential trace element that plays a critical role in the biochemistry of aerobic organisms. In man, this metal is

[1]Universidade Federal do Espírito Santo - Base Oceanográfica. Rodovia ES-010 km 16, 29199-970 Aracruz, ES, Brazil. brunodlo@yahoo.com.br (BLO); luiz.ufes@gmail.com (LFLF); geisamanda@gmail.com (GPB)
[2]Universidade Federal do Rio Grande - FURG, Instituto de Ciências Biológicas. Avenida Itália km 8, 96203-900 Rio Grande, RS, Brazil. adaltobianchini@furg.br
[3]Universidade Vila Velha, UVV. Comissário José Dantas de Melo, 21, 29102-770 Vila Velha, ES, Brazil. adriana.chippari@uvv.br (ARCG); bferreiradasilva@gmail.com (BFS); levy.gomes@uvv.br (LCG)

utilized by a diverse but limited number of enzymes to enable electron transfer reactions in key metabolic pathways (Culotta & Gitlin, 2001).

Several methods have been employed to determine Cu contamination in aquatic organisms (Lemos, 2005; Moller, 2006; Jorge *et al.*, 2013). Many authors suggest the use of genotoxic analyses, such as the comet assay and micronucleus test (Avishai *et al.*, 2002; Matsumoto *et al.*, 2006). These methods have some advantages, since they can be used for simultaneous evaluation of the degree of DNA lesions in many tissues of the tested individual, thus enabling comparisons among tissue responses under identical treatment conditions. Therefore, these methods can be used together to screen chemical substances and further investigate associations with DNA damage and recovery of sentinel species, such as fish (Ali *et al.*, 2009).

The fat snook, *Centropomus parallelus*, inhabits shallow coastal and estuarine waters from the Southern Florida to the Brazilian coast (tropical and subtropical waters), and does not undergo long migrations (Rivas, 1962; Martin & King, 1991). *C. parallelus* are top predators, relying on fish and shellfish as their most important sources of food (Chaves, 1963). Analysis of the sublethal effects of Cu on key prey species, such as the fat snook, is of paramount importance to evaluate the negative impacts on the population fitness, as well as to assess a possible metal biomagnification throughout the food chain. Exposure of *C. parallelus* to Cu has been already tested in fresh water (Oss *et al.*, 2013). However, the degree of Cu accumulation and its consequent toxicity are highly dependent on the water chemistry. In the present study, experiments were performed using sea water, where Cu toxicity to aquatic animals is shown to be reduced (Martins & Bianchini, 2008). Experiments were performed to assess the lethal concentration of Cu to juveniles of the *C. parallelus*, as well as the DNA damage induced by Cu accumulation of fish exposed to sublethal concentrations. Finally, was also observed, the fish ability to recover after acute exposure to sublethal concentrations of Cu.

Material and Methods

Experimental water and fish

The sea water used in the experiments was collected at the estuarine area of the Piraqueaçu River (Espírito Santo State, southeastern Brazil). After collection, sea water was filtered using a sequence of Cuno filters (20, 10, and 5 µm) and sterilized using an ultraviolet (UV) filter system. Physicochemical parameters (temperature, salinity, pH, and conductivity) of the sea water were determined over the experimental period.

Juveniles (mean value ± standard error; body mass: 3.5 ± 1.7 g; total body length: 6.8 ± 1.3 cm; n = 288) of the *Centropomus parallelus* were bought from a fish farm and acclimated to sea water in two 1000 L tanks for 25 days. Fish were fed daily with a commercial feed containing 60% protein (Inve, Belgium).

Acute Cu toxicity

After acclimation, fish were randomly transferred to eighteen 30 L tanks (10 fish per tank). The tanks were equipped with constant aeration, and oxygen levels maintained closed to the saturation. Five concentrations of Cu (as $CuSO_4.5H_2O$) were then tested: 0.4, 0.8, 1.6, 3.2, and 6.0 mg/L Cu. One control test (no Cu addition to water) was also run. The tested concentrations were determined in a preliminary test. All tests were performed in triplicate. Fish mortality was determined every 24 h for 96 h. Death criteria adopted was the lack of movement and no reaction to tactile stimuli. The lethal concentration of Cu for 50% of the individuals tested after 96 h (96-h LC_{50}) was calculated based on the cumulative mortality data using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977).

Exposure to sublethal Cu concentrations

Based on the 96-h LC_{50} value determined as described above, another experiment was conducted to determine the sublethal effects of Cu on juvenile fat snook. Combinations of three Cu concentrations (nominal = 0, 0.47 and 0.94 mg/L; measured = 0.03±0.01, 0.53±0.07, and 1.03±0.14 mg/L Cu) and two exposure periods (24 and 96 h) were tested. Cu concentrations tested corresponded to around 0, 25% and 50% of the 96-h LC_{50} value determined for Cu. Ten fish were tested in each combination of Cu concentration and exposure period, totalizing 60 experimental units. Fish were individually exposed to Cu in 2 L tanks. After exposure, fish were collected, anesthetized (benzocaine; 100 mg/L), and had their blood sampled by puncture of the caudal vein using a heparinized syringe. Blood samples were placed in Eppendorf tubes for comet assay and micronucleus test. Gills were then dissected and immediately rinsed in an EDTA (1 mM) solution to remove the loosely bound Cu. Samples were used for measurement of gill Cu content. Samples of the experimental media were collected at 2, 24 and 96 h of exposure and acidified (1% HNO_3; Suprapur, Merck) for Cu concentration measurement.

Fish recovery experiment

The third experiment was performed with 48 juvenile fat snook exposed to a concentration corresponding to 50% of the 96 h LC_{50} value of Cu (0.94 mg/L Cu) for 96 h. Fish were divided into eight 30 L plastic tanks (six fish per tank). Four control (no Cu addition into the sea water) and four Cu (0.94 mg/L Cu) tanks were tested. After exposure, fish from each tank were transferred to a new 30 L plastic tank containing control sea water (no Cu addition) to test the fish ability to recover from the pre-exposure to Cu. After 96 and 240 h of the recovery period, three fish were collected from each tank, anesthetized (benzocaine, 100 mg/L) and

had their blood sampled for comet assay and micronucleus test, as described in the previous item. Gills were then dissected and immediately rinsed in an EDTA (1 mM) solution to remove the loosely bound Cu. Samples were further used for measurement of gill Cu content. Also, samples of the experimental water media were collected at 0 and 96 h of the exposure period to Cu, and at 96 and 240 h of the recovery period. Water samples were acidified (1% HNO_3; Suprapur, Merck) and further used for Cu concentration analysis.

Analytical procedures

Water Cu concentration and gill Cu content measurements

Cu concentration in water samples were determined by atomic absorption spectrophotometry (detection limit = 10 µg/L, ë = 324.7 nm, GBC Avanta 932, IL, USA) following procedures described by Pinho & Bianchini (2010) and Monteiro *et al.* (2013).

Gill samples were weighed, dried at 80ºC, and digested with 10% HNO_3 (Suprapur; Merck) for 24 h. Completely digested samples were diluted using MilliQ water. Tissue Cu content was determined by atomic absorption spectrophotometry (GBC Avanta 932, IL, USA), following procedures previously described by Carvalho *et al.* (2013). Results were expressed as µg/g Cu.

Comet assay

DNA fragment analysis was evaluated in fish erythrocytes using the alkaline comet assay and silver nitrate staining as described by Tice *et al.* (2000). Two glass slides for each fish were previously coated with 1.5% agarose (1.80 g agarose and 120 mL alkaline phosphate buffer; pH 7.4). Blood samples were 20 times diluted with a phosphate buffer solution. An aliquot (10 µL) of the diluted blood was mixed with 100 µL of low melting-point agarose (0.15 g agarose in 20 mL alkaline phosphate buffer, pH 7.4) at 40ºC. Slides were covered with coverslips to spread the gel and left stand until its solidification. After removing the coverslips, slides were placed into a lyses solution prepared with 146.1 g NaCl (2.5 M), 37.2 g EDTA (100 mM), 1.2 g Tris (10 mM), 1 L distilled water. The pH of the solution was adjusted to 13 with NaOH.Triton X-100 (1%) and DMSO (10%) were added at the time of use. Slides were left in a refrigerator and protected from light for at least 2 h. After cell lyses, slides were placed in an electrophoretic apparatus and submerged into an electrophoresis buffer solution (300 mM NaOH and 1 mM EDTA, pH>13) for 20 min. The electrophoretic run was performed at 25V and 300mA for 15 min. Slides were then neutralized with a Tris buffer solution, washed with distilled water, dried at 37ºC for 2 h, fixed in fixing solution (15% trichloroacetic acid, 5% zinc sulfate heptahydrate, 5% glycerol, and 1 L distilled water) for 10 min, washed three times with distilled water, and dried at 37ºC for 2 h. After drying, slides were hydrated with distilled water for 5 min and stained with a silver staining solution prepared with 66 mL of a calcium carbonate solution (12.5 g calcium carbonate dissolved in 250 mL distilled water) and 34 mL of a silver nitrate solution (50 mg of silver nitrate, 50 mg of ammonium nitrate, 250 mg tungostosilicic acid dissolved in 125 µL formaldehyde). Slides were stained at 37ºC for 15 min.

Cell DNA damage was assessed in 100 cells using an optical microscope (Nikon, Model 50i) and subsequently classified into four damage classes according to the Comet tail length: class 0 - cell without a Comet tail; class 1 - cell showing a Comet tail length shorter than the nucleus diameter; class 2 - cells with a Comet tail length 1- to 2-fold larger than the nucleus diameter; class 3 - cell showing a Comet tail length more than 2-fold larger than the nucleus diameter (Grazeffe *et al.*, 2008). The damage index (DI) was calculated based on the sum of the number of damaged cells multiplied by the respective damage class (0-3), according to Heuser *et al.* (2008).

Micronucleus test

Blood was dropped onto a glass slide and smeared. The blood smear was left to dry overnight, fixed in a 100% methanol bath for 30 min, stained with a 5% Giemsa solution for 40 min, and observed under an optical microscope (Nikon, Model 50i). The frequence of micronucleated cells was determined based on the analysis of 1,000 erythrocytes per slide (Campana *et al.*, 1999; Grisolia, 2002). The cytogenetic analysis was conducted using the optical microscope at 1000 x magnification. Micronuclei were identified following the criteria proposed by Fenech *et al.* (2003). For all treatments, two slides per fish were assessed.

Data presentation and statistical analysis

Data were expressed as mean ± standard deviation. Data on gill Cu content and frequence of micronucleated cells for Cu-exposed fish were compared with those for control fish (no Cu addition into the water) using the Kruskal-Wallis analysis of variance followed by the Dunn's test. Difference in DNA damage indices between Cu-exposed and control fish was determined using analysis of variance followed by the Dunnett's test. In all cases, the significance level adopted was 95% ($p<0.05$).

Results

Physicochemical parameters of sea water

Mean values of the physicochemical parameters of the sea water employed in the experiments were: temperature = 24.6 ± 0.6ºC; salinity = 32.2 ± 1.1; pH = 7.58 ± 0.38; and conductivity = 47.46 ±0.20 mS/cm.

Acute Cu toxicity

Based on total measured Cu concentration, the 96-h LC_{50} value (95% confidence interval) was 1.88 (1.67-2.11) mg/L Cu.

Sublethal exposure to Cu

No fish mortality was observed over the 96 h period of exposure.

Gill Cu content

After an exposure of 96 h, the Cu content in gill of fish kept under control condition (no Cu addition into the water) was 0.43 ± 0.03 µg/g wet tissue. In fish exposed to 0.47 and 0.94 mg/L Cu, it was 1.09 ± 0.26 and 1.26 ± 0.19 µg/g wet tissue, respectively. There was a significant increase in gill Cu content of fish from both Cu exposure treatments relative to the control. However, there was no significant difference in gill Cu content between fish from the two Cu treatments.

Comet assay

After 24 h of Cu exposure, the frequency of damaged erythrocytes was significantly lower in control fish than in those exposed to the highest Cu concentration (0.94 mg/L) (Table 1). In turn, the DNA damage index was significantly greater on fish exposed to 0.47 and 0.94 mg/L Cu than in control fish (Fig. 1A). After 96 h exposure, there was a significant increase in the frequency of damaged erythrocytes from fish exposed to 0.47 and 0.94 mg/L Cu (Table 1). Also, the same difference was observed for the damage index (Fig. 1A).

Micronucleus test

Micronucleus frequency was significantly higher in erythrocytes of fish exposed to 0.47 and 0.94 mg/L Cu than in control fish in both periods (Fig. 1B).

Recovery

Water Cu concentration

Cu concentration in the water during the 96 h exposure period was 1.03 mg/L Cu, a value within the expected limits of the desired concentration (50% of the LC_{50}; 0.94 mg/L). During the recovery period (240 h), Cu concentration in sea water employed to maintain control and Cu-exposed fish was always <0.009 mg/L.

Table 1. Frequency (%) of classes of DNA damage in erythrocytes of the *Centropomus paralellus* exposed to Cu (0.47 and 0.94 mg/L) for 24 and 96 h. Data are mean ± standard deviation. *Denotes significant different mean values between control and Cu-exposed fish ($p \leq 0.05$).

Copper concentration (mg/L)	Damage classes			
	0	1	2	3
	24 h of exposure			
0	22.4±11.9	29.9±8.2	20.3±7.8	27.4±12.6
0.47	5.1±4.8	12.6±5.5	24.8±10.8	57.5±17.2
0.94	3.2±3.9*	10.1±6.1*	31.5±11.1	55.2±16.8*
	96 h of exposure			
0	13.2±3.8	19.8±4.5	24.8±6.6	42.2±9.2
0.47	9.1±7.3*	15.8±7.8*	24.1±7.4	51.0±18.7*
0.94	3.8±3.5*	9.6±5.0*	26.4±9.7	60.2±16.4*

Gill Cu content

During the recovery period, there was no significant difference in gill Cu content between control and Cu-exposed fish. Also, there was no significant change in gill Cu content over time (96 and 240 h). The Cu content in the gill of control fish was 3.48 ± 0.81 µg/g at 96 h and 3.04 ± 0.51 µg/g wet tissue at 240 h. In fish recovered in 96 and 240 h, it was 4.03 ± 1.51 and 3.12 ± 0.85 µg/g wet tissue, respectively.

Comet assay

After 96h recovery period, Cu-exposed fish showed a significant decrease in the frequency of damaged erythrocytes (Table 2). There was no decrease of the frequency of damaged erythrocytes after 240 h of recovery in Cu-exposed fish. Also, the damage index was not significantly different between control and Cu-exposed fish after 96 h and 240 h of the recovery (Fig. 2A).

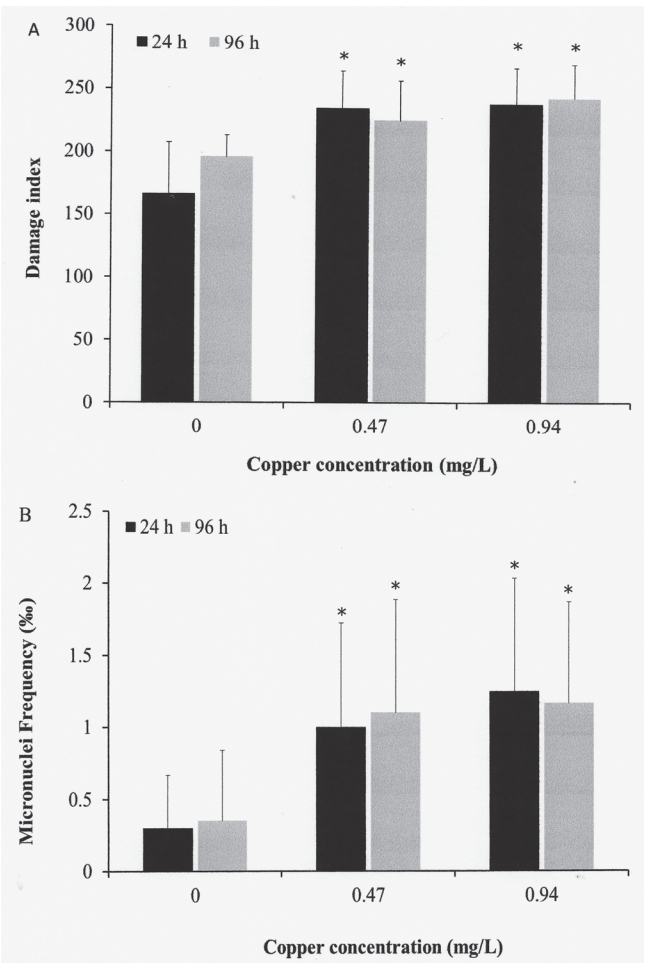

Fig. 1. DNA damage (A) and frequency of micronuclei (B) in erythrocytes of the *Centropomus paralellus* maintained under control condition or exposed to 0.47 and 0.94 mg/L of Cu for 24 and 96 h. *Denotes significant different mean values between control and Cu-exposed fish within the same Cu concentration and exposure time ($p \leq 0.05$). No significant difference was observed between exposure times within the same Cu concentration ($p > 0.05$).

Micronucleus test

The number of micronuclei was significantly higher in erythrocytes of fish exposed to 0.94 mg/L Cu than in those kept under control condition at 96 h after the start of the recovery period. At the end of the recovery period (240 h), there was no significant difference between control and Cu-exposed fish (Fig. 2B).

Discussion

The LC_{50} for fat snook is two-fold lower than that obtained by El-naga et al. (2005) with another saltwater fish *Mugil seheli* (=*Moolgarda seheli*) (5.36 mg/L). The variation in the LC_{50} values for the same metal may be due to species type, chemical structure of metal compound, and the conditions of the experiment (water temperature, salinity, oxygen content and pH) (El-naga *et al.*, 2005). The LC_{50} for sea water fish is higher than for some freshwater fish like *Prochilodus scrofa* (= *Prochilodus lineatus*) (0.014 mg/L Cu at pH 8) (Carvalho & Fernandes, 2006), *Tanichthys albonubes* (0.027 mg/L) (Jing *et al.*, 2013). The main reason for this difference is the variation in the Cu speciation between fresh and sea water, as there is a greater complexation of the metal in sea water consequently lower bioavailability for absorption by fish.

Cu content data indicated that the waterborne metal is readily accumulated in the gills of the *Centropomus parallelus*. In fact, fish exposed to 0.47 and 0.94 mg/L Cu showed similar gill Cu content at the end of exposure. This finding indicates that maximum Cu accumulation occurred, suggesting that gill binding sites were saturated even at the lower concentration of Cu tested. Similar results were reported by Martins & Bianchini (2008) for the pompano *Trachinotus marginatus* exposed to a lower concentration of Cu (0.369 mg/L) for 6 h.

Fish gills have shown to be an important biotic ligand for Cu and have been described as a primary site for accumulation of Cu from the dissolved phase (Romeo *et al*., 1994; Zia & McDonald, 1994; Martins & Bianchini, 2008). According to Santore *et al*. (2001), Cu toxicity in fish occurs when the accumulation of this metal at the gill tissue exceeds the saturation capacity of binding sites, especially those associated with the Na^+ and Ca^{2+} channels. Despite the fact that a saturation of Cu binding sites at the gills of *C. parallelus* was observed, there was no fish mortality over the experimental period. This finding suggests that other biotic ligand(s) than gills would be also involved in Cu toxicity in the *C. parallelus* in sea water.

The comet assay allows for differentiation between natural variability and genotoxicity, which is the variation induced in the genetic material by stress associated with contaminants (Lemos, 2005). There are a limited number of studies using the comet assay with marine fish when compared to those performed in freshwater species (Frenzilli *et al.*, 2009). Di Paolo (2006) applied the comet assay in *C.*

Table 2. Frequency (%) of classes of DNA damage in erythrocytes of the *Centropomus paralellus* maintained under control condition (no Cu addition into the sea water) for the whole experimental period or pre-exposed (96 h) to Cu (0.94 mg/L), transferred to control sea water and maintained under this condition for recovery during 96 and 240 h. Data are expressed as mean ± standard deviation. *Denotes significant different mean values between control and fish pre-exposed to Cu ($p \leq 0.05$).

Cu exposition concentration (mg/L)	Damage classes			
	0	1	2	3
	96 h of recovery			
0	15.4±9.0	18.8±6.8	27.1±9.0	38.7±12.3
0.94	8.1±6.5*	11.4±8.6*	25.7±8.6	54.7±13.9*
	240 h of recovery			
0	9.6±7.2	10.1±8.0	23.7±10.7	56.5±18.4
0.94	8.4±6.8	8.4±6.5	16.3±8.9	66.9±18.5

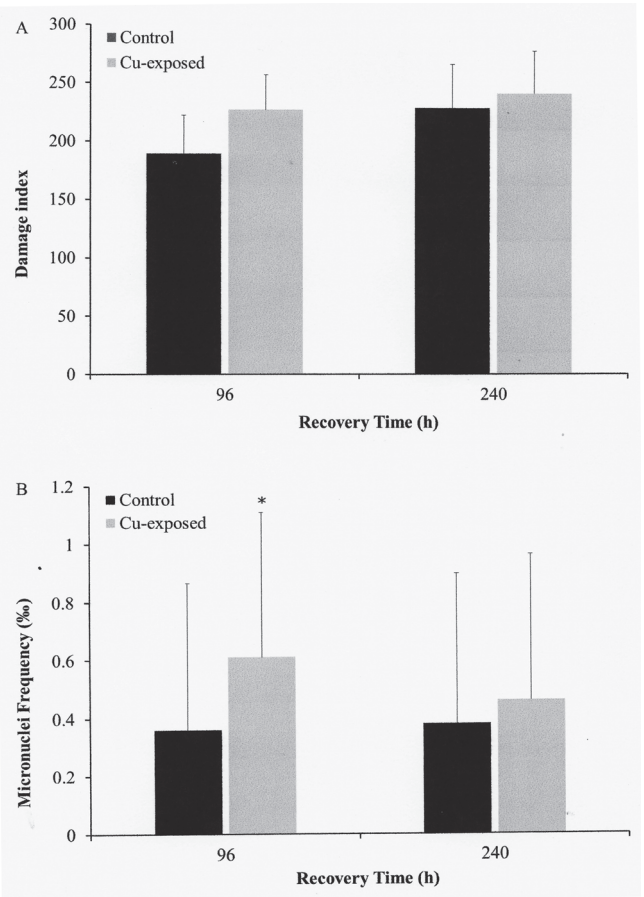

Fig. 2. DNA damage (A) and frequency of micronuclei (B) in erythrocytes in the *Centropomus paralellus* exposed (96 h) to Cu (0.94 mg/L), transferred to control sea water and maintained under this condition for 96 and 240 h. *Denotes significant different mean values between control and Cu-exposed fish ($p \leq 0.05$). No significant difference was observed between exposure times ($p > 0.05$).

parallelus exposed to other xenobiotic (ß-naphthoflavone) and found the method to be very sensitive. For fat snook, DNA damage was significantly higher in Cu-exposed than in control fish, however the comet assay did not detect a significant difference between fish exposed to the two Cu concentrations tested. The fact that damage was similar in fish exposed to 0.47 and 0.94 mg/L Cu even at 24 h of exposure indicates that Cu affects DNA as soon it reaches and accumulates in the fish blood.

Micronuclei were observed in most studies of short-term exposure (24- 96 h) and are considered sensitive biomarkers of genotoxicity (Cavas *et al.*, 2005). Kirschbaum *et al.* (2009) compared the micronucleus frequency in erythrocytes of *C. parallelus* from a polluted (São Vicente) and a protected estuary (Cananéia) at São Paulo State (southeastern Brazil). They reported values up to 10-fold higher in fish from the polluted site respect to those from the protected site, indicating that the micronucleus assay is reliable for monitoring the health of marine and estuarine ecosystems. In this study, micronucleus frequency was found to be 3-fold higher in fish exposed to Cu (0.47 and 0.94 mg/L) than in those kept under control conditions. Despite the effect observed in erythrocytes of *C. parallelus*, Cu genotoxicity seemed to be lower than that observed in freshwater species. For example, Cavas *et al.* (2005) subjected *Cyprinus carpio* to 0.01 and 0.25 mg/L Cu and the results showed micronucleus frequency almost 4-fold higher than that observed in *C. parallelus*.

According to Grazeffe *et al*. (2008), increased concentrations of contaminants induce additional damage to the genetic material. However, once the contaminant is removed the level of damage repair is expected to increase. Analysis of the gill Cu content over the recovery period indicated a depuration of Cu from the gill tissue of the fat snook. In fact, no significant difference in gill Cu content was observed between control and Cu-exposed fish after the recovering period. A very similar response was reported by Boock & Machado-Neto (2000), who reported almost complete depuration of Cu in fish subjected to a 144 h period of recovery in "clean" water after being exposed to Cu oxychloride for 96 h. At this point, it is interesting to note that lesions detected through the comet assay, are amenable to correction (Tice *et al.*, 2000), and therefore considered as being pre-mutagenic (Kammann *et al.*, 2001). In the present study, the higher damage index detected through the comet assay after 96 h of exposure to Cu was not seen anymore after the 240 h depuration period. This finding indicates a repair of injuries to the genetic material caused by the acute exposure to Cu.

Although micronuclei are often reported as genotoxicity biomarkers in aquatic animals, studies on fish exposed to pollutants have indicated that the micronucleus frequency in erythrocytes tends to decrease over time (Campana *et al.*, 1999; Cavas & Ergene-Gozukara, 2005). In the present study, a reduced number of erythrocytes with micronuclei were also observed in fish exposed to Cu and subjected to the recovery period respect to those kept only under control conditions. However, this response was only seen after 240 h of the beginning of the recovery period, but not after 96 h. Oss *et al.* (2013) showed that micronucleus frequency in erythrocytes did not decrease in *C. parallelus* 30 days after subchronic exposure to 25 µg/L Cu in fresh water. The low micronuclei frequency observed in *C. parallelus* in sea water could be explained considering a higher rate of erythrocyte renewal in Cu-exposed fish than in control ones. During blood cell kinetics, erythrocytes are continuously renewed and the damaged erythrocytes tend to be eliminated from the body more quickly than the undamaged cells (Flora *et al.*, 1993).

As pointed by Hagger *et al.* (2006), *C. parallelus* can be considered a relevant species in monitoring programs using biomarkers. Findings of this study are in complete agreement with this idea. In fact, the combination of the two assays used showed to be adequate and useful in evaluating Cu genotoxicity in *C.parallelus* induced by sublethal exposure to Cu in sea water.

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