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**SUB-LETHAL EFFECTS OF WATERBORNE COPPER IN EARLY DEVELOPMENTAL
STAGES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)**

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ABSTRACT

The aim of this work was to study the impact of copper during a sub-chronic exposure to environmental concentrations in the early life stages of rainbow trout (*Oncorhynchus mykiss*). Eyed-stage embryos of rainbow trout, at 265 °D, were exposed in semi-static conditions to sub-lethal concentrations of CuSO₄ up to the larval stage (528 °D) under laboratory-controlled conditions. During 3 weeks, they were exposed to the environmentally-realistic concentration of 2 µg/L Cu and to a 10-fold higher concentration, 20 µg/L Cu. Several biological (survival, hatching success, malformation, growth) and behavioral (swimming activity) and molecular endpoints (genotoxicity and gene transcription) were studied. Exposure to 20 µg/L Cu had an inhibitory effect on hatching and increased half-hatched embryos (25%). At the end of the exposure, no significant differences were observed in growth of the larvae exposed to the highest Cu concentration. However, larvae exposed to 2 µg/L Cu exhibited increased growth in comparison with non-exposed larvae. The percentage of malformed larvae was significantly higher for both copper conditions, with skeletal malformations being the most observed. Expression of several genes was evaluated in whole larvae using quantitative real-time PCR. Genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest (*p53*) were significantly repressed in both copper conditions when compared to control. In addition, potential genotoxic effects on larvae were investigated by the comet assay on blood cells, but this test did not demonstrate any significant DNA damage on larvae exposed to copper. This study confirms the adverse effects of copper on early life stages of rainbow trout even at the lowest environmentally relevant tested concentration.

Keywords: copper, *Oncorhynchus mykiss*, early life-stage, developmental toxicity, genotoxicity, gene transcription.

1. Introduction

Copper is one of the few metals that occurs naturally in ecosystems, as well as being one of the first used by humans since 8000 B.C. Over 95 % of all copper in circulation today has been extracted since 1900. It is an essential trace element involved in numerous biological processes, such as photosynthesis in plants. It also plays an essential role in certain enzyme functions in animals, including copper-zinc superoxide dismutase and cytochrome c-oxidase (Eisler, 1998). Copper is widely used in industrial, agricultural and urban activities, and consequently is one of the priority substances listed in the Water Framework Directive (WFD). With its significant anti-microbial and anti-fungal properties, copper has been used extensively in vineyards, resulting in high levels of transfer to soils and aquatic ecosystems (Devez et al., 2005; El Azzi et al., 2013; Komárek et al., 2010; Ruyters et al., 2013). Recent reports have shown that Cu-containing fungicides and boat antifouling paints appear to be the main anthropogenic sources of Cu in aquatic ecosystems (Babcsányi et al., 2014; Devier et al., 2005; Petit et al., 2013; Singh & Turner, 2009). Monitoring this element in the coastal marine environment by the Chemical Contamination Observation Network (ROCCH, IFREMER) using the Pacific oyster (*Magallana gigas*) as a sentinel species showed an increase in its concentration at certain points along the French coast, such as Arcachon Bay (Claisse and Alzieu, 1993; Gamain et al., 2016).

Pollution of surface water and groundwater is one of the biggest environmental issues. The background copper concentrations in unpolluted water bodies varied between 0.2 to 6 µg/L (Flemming and Trevors, 1989; Willis and Bishop, 2016). Levels of copper can increase from anthropogenic pollution as a result of urban and agricultural runoffs (Davis et al., 2001; Devez et al., 2005). Copper concentration in Beysehir lake (Turkey) was up to 100 µg/L in spring 2004 (Tekin-Özan, 2008). The river Lot (southern France), with a large history of metal pollution, concentrations of copper in water column was 17 µg/L in 1987; but, this concentration decreased in 2006 to 3.2 µg/L (Shinn et al, 2009). In several Asturians rivers (Spain), copper

concentrations were between 2.5 and 4.9 µg/L (Linde et al., 1998), and in Boulder river (Montana, USA) was ranged from 1.4 to 23 µg/L (Farag et al., 2007).

High concentrations of copper in ecosystems may be harmful to aquatic organisms. Indeed, although essential, Cu induces toxic effects during chronic exposure and over-accumulates in living organisms (Marr et al., 1996; Kamunde et al., 2002; Vardy et al., 2013). Since copper can participate in Fenton and Haber-Weiss reactions, it can produce reactive oxygen species (ROS), which react with H₂O₂ and cause cellular damage to lipids, nucleic acids and proteins that can lead to cell death (Manzl et al., 2004; Prousek, 2007; Bopp et al., 2008). Many studies have highlighted the toxic effects of copper on fish and mollusks (Vardy et al., 2013; Gamain et al., 2016; Boukadida et al., 2017). Sensitivity to copper can vary greatly from one fish species to another. For example, Vardy et al. (2013) determined the lethal concentrations (LC₅₀) at 96 h of Cu exposure at different life stages of fish, which for swim-up larvae of white sturgeon and rainbow trout was 10 and 21 µg/L of Cu, respectively. McKim et al. (1978) examined embryo mortality of eight different fish species after Cu exposure and observed significant mortality between 37 and 555 µg/L. Besides, this sensitivity to metals could also differ according to the development stage (McKim et al., 1978; Vardy et al., 2013) and water chemistry parameters like hardness, pH, organic carbon and alkalinity (Brix et al., 2017; Crémazy et al., 2017; Eisler, 1998).

Several reports have revealed that early life stages of fish are a great deal more vulnerable to pollutants than juveniles and adults (Eaton et al., 1978; Hutchinson et al., 1998; Van Leeuwen et al., 1985; Vardy et al., 2013). Embryos and larvae organs, such as liver, gills and kidney, are not entirely developed, which can result into a higher sensitivity to toxicants (Mohammed, 2013). Additionally, larvae have a permeable skin that could allow the uptake of pollutants (Mohammed, 2013). Because fish embryos and larvae have an important role in the trophic chain and, in the persistence of population as well, the use of these stages are relevant for the assessment of toxicity in the aquatic environment. Several reports have studied copper toxicity

in early life stages of fishes (Barjhoux et al., 2012; El-Fiky, 2001; Kazlauskienė, 2002; Stasiūnaitė, 2005; Witeska et al., 2010).

Early life stages of rainbow trout (*Oncorhynchus mykiss*) can be easily raised under laboratory conditions, and it is considered, along with other species, as a model animal in ecotoxicology (OECD, 2013). Due to its slow embryo-larval development, toxicity tests allow longer sub-chronic exposures to toxicants. This species has been extensively studied and, because of its low tolerance to poor water quality, is considered as one of the most commonly used sentinel species for aquatic ecotoxicology (De Boeck et al., 2004; Hansen et al., 2002; Kamunde et al., 2002; Olsson et al., 1990).

The purpose of the present work was to evaluate and to characterize the sub-lethal effects of environmental concentrations of copper to early developmental stage of rainbow trout. At the end of the 3-week Cu exposure, several end-points like viability, hatching rate, developmental abnormalities, larval swimming behavior, genotoxicity (DNA damage), lipid and protein peroxidation, and gene expression were analyzed.

2. Materials and methods

2.1. Chemicals

Copper sulfate ($\text{CuSO}_4 \cdot \text{H}_2\text{O}$, CAS 7758-99-8, 99.99%) and low melting point agarose (LMPA) were purchased from Sigma Aldrich (Lyon, FR). Phosphate-buffered saline (PBS), nitric acid 65% and normal melting point agarose (NMPA) were purchased from Fluka Chemie (Buchs, CH).

2.2. Exposure condition

Rainbow trout embryos (*Oncorhynchus mykiss*) at eyed stage were provided by INRA-PEIMA (Sizun, FFR). They were incubated in total darkness under a temperature of 12°C. Embryos

were exposed to copper from 265 DD (degrees days) up to the larval stage of 528 DD for a total of 23 days of exposure. Two concentrations of exposure for copper were chosen: 2 (environmental concentration) and 20 µg/L. These solutions ~~Copper solutions (2 and 20 µg/L Cu)~~ were prepared using CuSO₄·5H₂O and the required concentrations were calculated according to the quantity of copper ions (Cu²⁺). Each studied condition, and a control without Cu, involved 3 replicates with 100 embryos in 1 L aquaria. Exposure water was prepared in three 5 L tanks of Saint-Etienne spring water (63 mg/L Ca, 10.2 mg/L Mg, 1.4 mg/L Na, 0.4 mg/L K, 173.2 mg/L HCO₃⁻, 51.3 mg/L SO₄²⁻, 2 mg/L NO₃⁻, pH 7.6, <1 mg/L Cl⁻, <1.5 mg/L DOC). A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous flow rate of water (9 mL/min) into the incubation chambers. To keep the require copper concentration in aquaria, water was renewed every 48 h. At the end of the experiment, larvae were sampled per replicate as follow: 10 larvae to record for biometrics and morphological anomalies, 6 larvae for the study of swimming behavior, 6 larvae for blood sampling for genotoxicity test. The remaining larvae were pooled and stored at -80°C for biochemical analysis and gene transcription.

2.3. Analysis of copper in water and larvae

Fish and water samples were analyzed to determine Cu concentrations. Fish samples (2 pools of 5 larvae from each replicate) were lyophilized and digested by nitric acid (0.3 mL of HNO₃ 67 %). Four tubes containing the international certified reference material, Dolt-5 samples (Fish liver), were prepared in order to verify the accuracy of the method. Approximately 80 mg of this material was digested by 3 mL of nitric acid in polypropylene tubes. Then, this mixture was heated by Hot Block for 3 h at 100°C, and after cooling 1.5 mL and 15 mL of ultrapure water (Milli-Q®) was respectively added to sample larvae and certified reference. Finally, 3 blank samples were also prepared in the same conditions (3 mL of HNO₃ before heating and 15 mL of Milli-Q water added after cooling). 40 mL of water sample of each condition were collected in 3 random days through the experiment, and acidified with 5 % of nitric acid (Nitric

acid 65 %, Fluka). Copper concentrations in water of condition “20 µg/L” and fish samples were analyzed by inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent Technologies), whereas copper concentrations in water of controls and “2 µg/L” conditions were analyzed by atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, USA). Detection limits (DL) for ICP-OES was 2.26 µg/L Cu²⁺ and for atomic absorption was 0.5 µg/L Cu²⁺.

2.4. Phenotypic effects

Dead embryos and larvae were recorded daily and removed immediately to prevent water alterations. Individuals were considered half-hatched when at least a part of the body was inside the chorion. Embryonic and larval mortality refer to the number of dead individual compared to the total number of embryos at the start of the experiment or total number of totally hatched larvae. Half-hatched mortality was only considered when at least a part of the body was inside the chorion. Hatching time refers to the number of degree days (DD: number of days x temperature) from fertilization to hatching. Hatching rate is calculated by dividing the number of half-hatched embryos by the total number of embryos at the beginning of the experiment. The appearance of anomalies was microscopically examined in 10 larvae per replicate randomly chosen. The larvae were placed in a Petri dish with carbonated water and ice to sedate them. Photos were taken for each larva with a stereomicroscope (MZ7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, we measured total body and head length for each larva using Leica Microsystems software v3.8 (Nanterre, France). Larvae were also observed to locate possible malformations such as edemas, spinal and craniofacial deformities, cardiac anomalies, hemorrhages and yolks sac resorption.

2.5. Genotoxicity test

The comet assay was performed according to the Singh et al. (1988) protocol, adapted by [Le Bihanic et al. \(2014\)](#). Blood sampling were performed in 6 larvae per replicate by decapitation using a heparinized pipette. Samples were stored in a microtube with 200 μ L of cryopreservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and immediately frozen in liquid nitrogen until analysis. These storage conditions have already been described by Barillet et al. (2005) and Bony et al. (2008), this protocol has been validated for rainbow trout blood cells in our laboratory. 50 μ L of cell suspension was added to 100 μ L of LMPA (Low Melting Point Agarose). Two aliquots of 50 μ L of the suspension were placed onto slides previously covered with NMPA (Normal Melting Point Agarose, 1 %, w/w) and a coverslip (18x18 mm) was added. The gels were left to harden for 15 min at 4°C and then the coverslips were removed from each slice and a second layer of LMPA was placed on the gels with new coverslips and hardened at 4°C for 15 minutes. The slices were put in a lysis solution (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1% Triton x-100; 10% DMSO; pH adjusted to 10 with NaOH) at 4°C for 90 minutes. Slices were placed horizontally on a tray with electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH> 13) for 20 minutes to allow the DNA to unwind. Electrophoresis was performed with a voltage of 25 V and 290 mA for 20 minutes. After electrophoresis, slices were rinsed 3 times with a neutralizing solution (0.4 M Tris, pH 7.5) for 5 minutes at 4°C. Then, slices were soaked with absolute ethanol for 20 minutes and dried at a room temperature for at least 24 hours. Slices were stained with 20 μ g/mL of ethidium bromide solution. Comet analysis was performed using an epifluorescence microscope, and DNA damage was measured with Comet Assay IV software on 100 cells randomly selected. Results are expressed as percentage of degradation of DNA tail. Heavily degraded cells, or inexistent head with diffuse tail, were not counted in the tail DNA measurements as proposed by Kumaravel et al. (2009). However, they were counted as hedgehogs on a total of 100 cells per gel.

2.6. Behavioral analysis: swimming test

The swimming behavior of 528 DD larvae was studied using the Daniovision Image analysis system version 10.0 Noldus. Six larvae per replicate were placed in 6-well microplates containing 8 mL of exposure water. The microplates were placed in the recording chamber in the dark at 12 °C for about 30 min to acclimate the larvae. Then the analysis was started. An infrared camera in the chamber recorded the movements of each larvae focusing on their center of gravity. The analysis takes 30 min with dark/light/dark cycle of 10 min each. This cycle allows to analyze the behavior of larvae in response to light stimulation. Ethovision software allows to record the average speed of each larvae every 30 seconds. For each larva, the swimming performance was assessed by determining the total distance moved (cm) in 10-minute period, and time of mobility accumulated (s) on larvae. Mobility is defined as the period of time where the area of total larva is modified albeit central point of larvae had not changed. If more than 60 % of the area was changed, it was considered as highly mobile. Otherwise, less than 60 %, it was considered only as mobile.

2.7. Biochemical analysis

Preparation of supernatant

Pools of 5 larvae were frozen in liquid nitrogen and then stored at -80°C until analysis. At the moment of analysis, yolk sac from larvae were removed and then larvae (approximately 250 mg) were homogenized in a chilled phosphate buffer (0.1 M; pH 7.5) using an UltraTurrax® tissue homogenizer fitted with a potter at 3,000 rpm (4°C). The supernatant was centrifuged at 9,000 g for 25 min at 4°C to obtain the supernatant S9 fraction. This fraction contains soluble proteins from cytosol and microsomes (Parmentier et al., 2007). Total protein, TBARS levels and protein carbonyls content were analyzed using the S9 fraction.

Total protein

The total protein concentration was measured on S9 fraction by the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as a standard and Folin's reagent. Measurements were done using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation

Lipid peroxidation was determined using the adapted technique of Buege and Aust (1978) adapted to a microplate reader. 500 μ L of a solution containing 20 % of trichloroacetic acid (TCA) and 20 % butylated hydroxytoluene (BHT) was added to 500 μ L of S9 fraction and then mixture was centrifuged for 10 min at 9,000 g. Afterwards, 600 μ L of supernatant with 480 μ L of TRISbase (25 mM)-TBA (thiobarbituric acid-100 mM) and 120 μ L of 0.6 N HCl heated at 80°C for 15 min. The tubes were then cooled and mixed. TBARS levels were read using a UV-spectrophotometer (Biotek Synergy HT) in a microplate at 530 nm. Results were expressed as nmoles of thiobarbituric acid reactive substance (TBARS) equivalents per milligram of protein.

Protein Carbonyl analysis

Protein carbonyl content was assessed using the spectrophotometry method described in Augustyniak et al., (2015). 50 μ L of a solution of 11 % streptomycin sulfate – phosphate buffer (100 mM pH 7.4) were added to 500 μ L of S9 fraction, then it was mixed and incubated for 15 min at room temperature. Afterwards, mixtures were centrifuged for 10 min at 6,000 g. Then 200 μ L supernatant was added to 800 μ L of DNPH (2,4-dinitrophenylhydrazine 10 mM) used as a sample tube, and to 800 μ L of HCl 2.5 M used as a control tube; then, incubated for 1 h at room temperature with vortexing every 15 min. Proteins were precipitated with 1 mL of 20 % TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were rinsed with 1 mL of ethanol-ethyl acetate (v:v), vortexed and centrifuged three times. Then pellets were solubilized with 500 μ L of 6 M guanidine HCl and centrifuged at 10,000 g for 10 min. The carbonyl content was measured using a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results were expressed as nanomoles of DNPH incorporated/mg protein.

2.8. Gene expression

6 larvae per replicate were collected in a storage buffer (RNA later, Qiagen). Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

RNA extraction

Total RNA extraction from whole larvae was performed with the kit “SV Total RNA Isolation system” (Promega) following the supplier’s recommendations. Larvae were homogenized using the MP fastprep®-24 (Biorad, 6 m/s, 40s) with 14.4 mm diameter ceramics beads (MP Biomedicals, Lyssing Matrix D Bulk).

Retro-transcription of total RNA into cDNA

The retro-transcription of total purified RNA was carried out with the kit “GoScript Reverse Transcription System” (Promega). To execute the Reverse transcription, 1 µL of oligodT (1 µM) and 1 µL of hexanucleotides (1 µM) have been added to 10 µL of total purified RNA (1 µg). Then, the mix was heated at 70°C for 5 minutes with a thermocycler (Eppendorf Mastercycler) followed by 5 minutes at 4°C to allow primer annealing. Subsequently, 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of MgCl₂ (25 mM), 1 µL of reverse transcriptase (1 U/µL) and 0.5 µL of RNAsine were added. The reverse transcription reaction was performed for 1 h at 42°C in a thermocycler. The cDNA thus obtained were stored at -20°C pending analysis by quantitative real-time PCR reaction.

Quantitative real-time PCR

Thirteen genes were investigated and specific primer-pairs designed with primer3plus software (Table 1). All the primer-pairs used in this study have an efficiency upper than 95%. Real-time qPCR was carried out using GoTaq® qPCR Master Mix kit (Promega). PCR reactions contained 17 µL of a mixture of Nuclease-Free Water and GoTaq® qPCR Master Mix containing the SyberGreen fluorescent dye, 2 µL of specific primer pairs mix (200 µM each) and 1 µL of cDNA. Real-time quantitative PCR reactions were performed in a Mx3000P® qPCR System (Stratagene). The amplification program consisted in one cycle at 95°C for 10

min, then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity was determined for each reaction from the dissociation curve of the PCR products. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95°C.

Cycle thresholds (Ct) were obtained from MxPro™ qPCR software for each gene. Two different reference genes were investigated (Rpl7 and EF1α) and were found to be stable in our conditions. Consequently, relative quantification of each gene expression level was normalized according to the mean Ct value of these two reference genes according to the $2\Delta Ct$ methods described by Livak and Schmittgen (2001). The ratio of transcription level to exposed individuals with the control ones gives the induction factor (>2) or repression factor (<0.5) of studied genes.

2.9. Statistics

Each studied condition was triplicated identically and each replicate was considered as an independent sample. All data are represented by the mean \pm SE (Standard Error). Statistical analyzes were carried out using R (<http://cran.r-projet.org/>). The Normality of data distribution was verified on the residues by the Shapiro-Wilk test ($p < 0.01$) and the homogeneity of variances was evaluated by the Levene test ($p < 0.05$). In the case of normal distribution, a one-way ANOVA analysis was used ($p < 0.05$) followed by a Tukey post-hoc test. In the case that data was not normal, data was evaluated by non-parametric tests of Kruskal-Wallis ($p < 0.05$).

3. Results

3.1. Test condition

Dissolved oxygen in water varied between 81.4 and 95.7 % throughout embryonic and larval exposure. Table 2 shows Cu concentrations determined in the exposure water. Water samples were collected in 3 random days. In the control group, Cu concentrations were below the detection limit. Cu concentrations of the treatment at 20 µg/L were close to the nominal concentration. However, results from 2 samples of the treatment at 2 µg/L were below the detection limit (0.5 µg/L Cu).

3.2. Embryonic and larval survival

No significant differences in embryonic and larval survival were observed between the control group and the condition at 2 µg Cu /L (Table 3). Regarding the condition of 20 µg/L Cu, a significant decrease of embryonic survival was observed compared to control. Mortality of half-hatched embryos was significantly higher for those exposed to 20 µg/L Cu compared to other conditions. In addition, Figure 1 and Table 3 show a significant lower hatchability for this condition in comparison to control and 2 µg/L Cu. On the other hand, the hatching time (DD) did not show any significant differences between the control and the two Cu exposure conditions (Table 3).

3.3. Larvae copper accumulation

After 3 weeks of exposure, larvae exposed to 20 µg/L Cu showed a significantly higher accumulation of Cu ($p < 0.01$) compared to control and larvae exposed to 2 µg/L Cu (Table 3). No significant difference in Cu accumulation was observed in larvae exposed to 2 µg/L Cu comparatively to control.

3.4. Biometrics

Exposure to the higher concentration of Cu (20 µg/L) did not promote significant changes on larvae biometrics compared to control (Table 3). However, larvae exposed to 2 µg/L Cu were significantly longer compared to control larvae. However, this difference is small representing only 4.14 % of increase between the two conditions. No significant differences were observed between the length of head and the ratio between the size of head and the total length for the two studied Cu conditions and the control (Table 3).

3.5. Malformations

Both copper conditions impaired normal development of larvae, where total malformations were significantly higher when compared to control condition (Table 3). Spinal anomalies (kyphosis, lordosis and c-shaped curvature) were the most frequently observed malformation, but its incidence was much higher in the Cu exposed groups than in control. The incidence of all the other recorded abnormalities (edemas, yolk sac malabsorption and craniofacial malformation) tended to increase with increasing Cu concentration in the exposure water, although no significant differences were observed among groups (Table 3).

3.6. Behavior

Responses to light stimulation of larvae are presented in Figure 2. The results represent the average distance traveled by larvae (Figure 2-A) and the cumulative time of high mobility (Figure 2-B) and mobility (Figure 2-C) of larvae. Over the light period, larvae from all conditions showed similar responses to light stimulation and all larvae increased their distance traveled and their mobility compared to the first dark period. However, no significant differences were observed between conditions on all studied parameters.

3.7. Genotoxicity

Results from the comet assay did not reveal any significant DNA damage in both Cu studied conditions when compared to control larvae (Figure 3-A). Nevertheless, the percentage of severely damage cells or ghost cells ("hedgehog") was significantly increased in larvae exposed to 20 µg/L Cu in comparison to control (Figure 3-B).

3.8. Lipid and protein oxidation

Larvae exposed to 2 and 20 µg/L of Cu exhibited no significant changes on the content of thiobarbituric acid (Figure 3-D) and protein carbonylation (Figure 3-C) comparatively to non-exposed larvae.

3.9. Gene expression

Cu effects on gene transcription were pronounced on both larvae exposed to 2 and 20 µg/L Cu (Table 4). Indeed, genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest and apoptosis (*p53*) were significantly repressed compared to control larvae. In contrast, gene transcription related to DNA repair system (*ogg1* and *rad51*) was not significantly altered at the end of 3-weeks Cu exposure. Also, no effects were observed on *cat* and *12s* genes for both studied conditions. In the case of larvae exposed to 2 µg/L Cu, the transcription of *sod* was significantly decreased when compared to control larvae.

4. Discussion

Copper is a ubiquitous metal that is naturally present in the environment. Although copper is an important trace element for the proper functioning of the organism, strong concentrations may be harmful to aquatic organisms (Flemming and Trevors, 1989; McKim et al., 1978). In the present study, exposure to 2 µg/L Cu did not increase embryonic and larval survival and

hatching rate of rainbow trout when compared to control. Regarding the exposure to 20 µg/L Cu, a significant decreased of embryonic viability was observed. However, the embryonic viability was superior to the survival criteria given by OECD (2013) at 75 % for control condition of rainbow trout. On the other hand, our results from this condition show a strong mortality during hatching of rainbow trout. Regarding the literature, our results are comparable to those found by other authors. McKim et al., (1978) demonstrated an almost complete embryo mortality on rainbow trout when exposed to 37 µg/L Cu. Compared to other species, rainbow trout showed greater sensitivity to Cu. For example, for a complete embryo mortality of brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*) and herring (*Coregonus artedii*) about 555 µg/L of Cu were needed (McKim et al., 1978). Vardy et al. (2013) determined that the LC₅₀ at 96 h for yolk-sac stages of white sturgeon (*Acipenser transmontanus*), rainbow trout and fathead minnows (*Pimephales promelas*) was 22, 40 and 102 µg/L of Cu respectively. It was also observed that later larval stages of white sturgeon and rainbow trout were even more sensitive to the effects of Cu than yolk sac stages (9 and 22 µg/L of Cu respectively). Another study was performed on two different stages of rainbow trout, embryo and swim-up stages, where lethal responses to Cu were observed from 22 µg/L for both stages after a sub-chronic exposure (Besser et al., 2005). Cu lethality not only depends on the sensitivity of the fish species and stage, but also on the water chemistry parameters such as pH, concentration of DOM (dissolved organic matter), and hardness (Brix et al., 2017; Crémazy et al., 2017). Crémazy et al. (2017) observed that tolerance of juvenile rainbow trout to Cu was augmented when pH, hardness and concentration of DOM were increased.

Mortality of half-hatched embryos exposed to 20 µg/L of Cu was significant during hatching time; hence, the hatchability was significantly reduced compared to the control group. Hatching can be a very critical step for fishes. Throughout the embryonic stage, the chorion offers protection to embryos in a contaminated environment. However, during hatching this protection is no longer present and the larvae are in direct contact with the contaminated medium (Arukwe et al., 1997). One hypothesis is that copper may have an inhibitory effect on the activity of the

chorionase enzyme, which degrades the inner area of the chorion to facilitate the hatching by mechanical and osmotic process (Johnson et al., 2007; Jezierska et al., 2009; Barjhoux *et al.*, 2012).

At the end of exposure, control larvae had accumulated $5.66 \pm 0.13 \mu\text{g/g}$ Cu (d.w.). According to Shearer (1984), Cu accumulation in unexposed larvae of rainbow trout can vary between 2 and $7 \mu\text{g/g}$ Cu d.w. Results of Cu accumulation on unexposed larvae from Shearer (1984) and Hansen et al. (2002) are comparable to our results. Marr et al. (1996) revealed that swim-up fry rainbow trout exposed to $9 \mu\text{g/L}$ Cu accumulated $9.89 \mu\text{g/g}$ Cu after 20 days exposure and continue to accumulate up to $17.91 \mu\text{g/g}$ after 60 days exposure. This accumulation is higher than that obtained in our study after 3-weeks of exposure at $20 \mu\text{g/L}$ Cu. Several authors have suggested that copper accumulation is regulated by the depuration and the slow exchange capacity of metals with the surrounding medium (Carbonell and Tarazona, 1994; Marr et al., 1996). Despite the lethal effects observed, no significant changes on larvae biometry was reported at the end of exposure to $20 \mu\text{g/L}$ Cu. A decrease on total size of larvae was expected, since the process of metabolism and homeostasis consume energy and the growing rate of fish may be affected as explained by Marr et al. (1996). Several studies have shown a relationship between the metal accumulation and the total size of larvae when they are exposed to metals (Guo et al., 2016; Marr et al., 1996). However, our results seem to be consistent with the low Cu accumulation in larvae exposed to this condition. Regarding Vardy et al (2013) results about the differences in tolerance according to the larval stage, we might presume that the lack of Cu accumulation in our larvae is probably link to the fact that, at the end of the 3-weeks Cu exposure, our larvae were still absorbing their yolk-sac and probably just starting to change to exogenous feeding. Swim-up rainbow trout larvae used in Marr et al. (1996) were fed and were more active leading to a larger exposure to Cu since more water is pumped through the gills. Since our larvae were not fed, there was no contamination or Cu accumulation by trophic way.

For larvae in control conditions, the most commonly observed malformations were edema, vertebral abnormalities and craniofacial anomalies. These kinds of malformations in control condition have already been noticed by several authors (Barjhoux et al., 2012; Jezierska et al., 2009; Witeska et al., 2010), and can be attributed to parental factors or laboratory culture conditions. In this study, exposure to 2 and 20 µg/L Cu showed a significant increase on malformed larvae compared to control larvae. Vertebral abnormalities (kyphosis, lordosis, scoliosis and C-shape form) were the most recorded among all observed deformities. In the literature, vertebral deformations are also the most observed malformations (Barjhoux et al., 2012; Jezierska et al., 2009; Witeska et al., 2010). For instance, 80% of malformations recorded on barbel larvae (*Barbus barbus*), after exposure to different concentrations of copper and lead were vertebral abnormalities (Ługowska and Kubik, 2011). Japanese medaka larvae (*Oryzias latipes*) showed 35 and 59 % of spinal deformities following an embryonic exposure to copper and cadmium spiked-sediment (Barjhoux et al., 2012). These defects can be caused by disturbances induced by metal exposure that may occur at early stages of development or difficulties encountered during a laborious hatching (Jezierska et al., 2009).

One aim of this work was to study the impact of copper on swimming behavior of larvae. Exposed larvae covered similar distances as control larvae and no significant difference was observed on travelled distance. The study of Haverroth et al. (2015) showed significant reduction on total distance traveled and restricted mobility on zebrafish (*Danio rerio*) exposed to 6 µg/L Cu after 24 hours. This can suggest that locomotor patterns were weakened (Haverroth et al., 2015). Atchinson et al. (1980) described the different ways by which metals can alter the locomotion on fish, either by the influence on the attraction or the evasion to a contaminated area, the impaired sensory perception on hyper or hypoactivity, or reducing the swimming performance.

The comet assay did not reveal any significant induction of DNA damage promoted by Cu exposure. However, several studies have reported DNA damage upon exposure to Cu or other metals (Barjhoux et al., 2012; Bopp et al., 2008; Canalejo et al., 2016; Oss et al., 2013; Santos

et al., 2010). Barjhoux et al. (2012) demonstrated the genotoxic potential of Cu and Cd on *Japanese medaka* larvae after embryonic exposure to environmental sediments from contaminated sites of the Lot-Garonne-Gironde continuum. Oss et al. (2013) observed an increase of micronuclei levels after 30-days exposure to environmental concentrations of Cu (0, 25, 50 µg/L) on juvenile fat snook (*Centropomus parallelus*), and these differences did not change after 30-days of recovery. In one study, a significant increase of micronuclei levels was also observed on European seabass (*Dicentrarchus labrax*) exposed to Cu (0.01-1 mg/L) for 96 h (Canalejo et al., 2016). However, at 24 h exposure, differences were only observed on fish exposed to the strongest Cu concentration (1 mg/L). Canalejo et al. (2016) also observed that micronuclei frequency was even more important for fish exposed to Cu than those exposed to As or Pb at the same concentrations. Environmental concentrations of Cu (3.2 to 128 µg/L) induced DNA strand breaks in a concentration-dependent manner in blood cells of mature male stickleback after exposure for 4-days (Santos et al., 2010). Oss et al. (2013) observed an increase of micronuclei levels after 30-days exposure to environmental concentrations of Cu (0, 25, 50 µg/L) on juvenile fat snook (*Centropomus parallelus*), and these differences did not change after 30-days of recovery. In our study comet assays were performed after 3-weeks exposure which could allow DNA repair system activation and efficient DNA damage repair. However, this is not in accordance to Oss et al. (2013) which did not observed recovery of micronuclei. On the other hand, we found an increase of very damaged cells counted as “hedgehog” in the group of 20 µg/L Cu. An increase of these cells may be explained by the generation of DNA strand breaks through the production of reactive oxygen species by Cu. These “hedgehog cells” were currently considered as apoptotic or necrotic cells (Barjhoux et al., 2012). However, some studies have evidenced that these hedgehog cells could be repaired and should not be considered as apoptotic cells (Lorenzo et al., 2013; Meintières et al., 2003). To determine the real nature of these cells, other analytical techniques would be required such as flow cytometry. In our study case, an increase of these cells may be explained by the generation of DNA strand breaks through the production of reactive oxygen species by Cu. To

confirm this hypothesis, it would be necessary to evaluate ROS production in cells and/or to determine the real nature of these cells by flow cytometry.

One way Cu exerts its toxicity is by the formation of reactive oxygen species (ROS), which at important levels can damage the cell through lipid peroxidation, protein denaturation and DNA damage (Romeó et al., 2000; Sevcikova et al., 2011; Vutukuru et al., 2006). Catalase (*cat*) and superoxide dismutase (*sod*) are two of the enzymes that protect cells from the oxidative damage caused by ROS. *Sod* catalyzes the dismutation of the superoxide anion radical into oxygen and hydrogen peroxide, which could be further decomposed by the *cat* into water and oxygen. In our study, after a sub-chronic exposure to Cu, RT-PCR results for *cat* expression did not reveal any significant modulation on rainbow trout larvae. Woo et al. (2009) observed an important induction of *cat* gene in liver when Javanese medaka (*Oryzias javanicus*) was exposed to different concentrations of Cu, Cd, and Zn for 48 h. In the study of Casanova et al. (2013) *cat* expression on tambaqui fish liver increased after 1h of exposure to copper and decreased to basal levels at 3 h of exposure. The expression level of cytoplasmic *sod* was decreased on larvae exposed to 2 µg/L of Cu when it was compared to control group. No significant induction of *sod* expression was observed on liver of Javanese medaka (Woo et al., 2009) and zebrafish (Craig et al., 2007) after exposure to Cu. However, Craig et al. (2007) stated an important induction on *sod* and *cat* genes expression on liver when zebrafish was under protective treatment of 10 mM Na⁺ plus Cu. Activation and deactivation of the antioxidant defense system are determined by the duration and the intensity of the stress applied to the organism (Casanova et al., 2013).

Larvae exposed to copper conditions exhibited a significant repression of genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest and apoptosis (*p53* and *bax*). Regarding detoxification genes, it was surprising since, after 21 days of Cu exposure, it was expected an induction, in particular of those genes encoding the metallothioneins (*mt1* and *mt2*). Metallothionein (MTs) proteins have the capacity to bind xenobiotic heavy metals through their thiol groups providing protection against metal toxicity and oxidative stress. Indeed,

506 previous studies have demonstrated an induction of *mt1* and *mt2* genes when exposing fish to
507 metals including Cu (Gonzalez et al., 2006; Olsson et al., 1989; Sheader et al., 2006; Woo et
508 al., 2006). The study of Doering et al., (2015) also observed a down-regulation response of *mt*
509 on liver explants from white sturgeon exposed for 24 h to Cu. Glutathione-s-transferase (*gst*)
510 has an important role in detoxification of xenobiotic compounds, catalyzing the conjugation of
511 reduced glutathione (GSH) to a variety of endogenous and exogenous electrophilic functional
512 groups. Induction of *gst* gene expression has been reported in Javanese medaka liver after
513 being exposed to different heavy metals (Cu, Ag, Cd, Ni, and Cr) and in European flounder
514 (*Platichthys flesus*) liver exposed to Cd after 1 and 3 days of exposure (Sheader et al., 2006;
515 Woo et al., 2009). However, we studied gene expression in whole trout larvae and not in a
516 specific fish organ like mostly previous studies refer. Gene repressions of *mt1*, *mt2* and *gst*
517 could be explained by different hypotheses. The first one, it is the high sensitivity that rainbow
518 trout larvae have to Cu could lead to a significant repression of these genes after 3-weeks
519 exposure at these concentrations of Cu. Certain studies had already observed the differential
520 regulation of *mt* gene expression in fish exposed to metals like Cu and Cd (Minghetti et al.,
521 2008; Tiwari et al., 2010). Differential expression of *mt* gene was observed in intestine, gill,
522 kidney and liver of sea bream (*Sparus aurata*) after exposure to dietary (0.3-130 mg/kg) or
523 waterborne (300 µg/L) copper for 30 days (Minghetti et al., 2008). Tiwari et al. (2010) studied
524 the kinetics of *mt* expression on freshwater murrel (*Channa punctata*) after exposure to
525 3.74 mg/L of Cd indicating that the expression varied according to the time of exposure. At this
526 study, Tiwari et al. (2010) observed induction of *mt* expression on liver and repressions on
527 kidney and gill within the first 8 h of exposure, and induction on kidney and repressions of *mt*
528 expression on liver and gill after 14 days of exposure. Both studies revealed that *mt* expression
529 was tissue specific. Besides, rainbow trout larvae could also be more sensitive during the
530 transition from endogenous yolk-sac reserve to the exogenous feeding as hypothesized by
531 Vardy et al. (2013). Indeed, Vardy et al. (2013) found that later larval life stages of white
532 sturgeon and rainbow trout were more sensitive to Cu than at yolk-sac stage. In our study, at
533 the end of these 3-weeks of Cu exposure, rainbow trout larvae were probably starting to turn

to exogenous feeding, and since their biological activity was increased more water passed through the gills increasing their exposure to Cu (Vardy et al., 2013). Another hypothesis could be that larvae may have implemented other mechanisms of detoxification. Indeed, other proteins like ABC transporters have been previously described to be efficient for trace metal detoxification (Gonzalez et al, 2006).

Cell cycle arrest is one of the principal functions of p53 when it recognizes DNA damage (Verhaegh et al., 1997). Having a defective *p53* gene could lead to tumorigenesis and cancer. In our study, transcription of *p53* was repressed in both Cu conditions. According to several studies (Verhaegh et al., 1997; Sandrini et al., 2009) *p53* gene expression could be regulated by the activation of p53 protein. Therefore, a possible inactivation of p53 protein, after Cu exposure, could lead to the inactivation of *p53* gene. Additionally, the study of Verhaegh et al. (1997) reported the inactivation of p53 protein after direct interaction with copper on human cell line. Regarding the expression of *bax* gene, only a slight non-significant repression ($p=0.0656$) was observed in larvae exposed to 20 µg/L of Cu. Interestingly, Sandrini et al. (2009) observed that *bax* gene was induced and *p53* gene was repressed on zebrafish liver cell line after 24 h of exposure to Cu. Concerning the DNA repair-related genes *ogg1* and *rad51*, we did not see any significant modulation. But since we did not observe genotoxicity on blood cells with the comet assay, a possible explanation is that DNA strand breaks may have been repaired prior the comet analysis at 21 days of Cu exposure.

Malondialdehyde (MDA), measured as TBARS (thiobarbituric acid reactive substances), is one of the end-products of lipid peroxidation and it is a biomarker of oxidative stress. ROS degrade polyunsaturated lipids forming MDA, which appears to be potentially mutagenic since it reacts with deoxyadenosine and deoxyguanosine, arising DNA adducts (Janero, 1990). In our study, both Cu concentrations did not increase MDA levels in larvae. However, several authors have reported the effects of high concentrations of metals and other xenobiotics on lipid peroxidation in aquatic organisms (Baker et al., 1998; Doyotte et al., 1997; Radi and Matkovics, 1988; Romeó et al., 2000; Thomas and Wofford, 1993; Vutukuru et al., 2006). For example, Vutukuru

et al. (2006) showed an increase of lipid peroxidation in the viscera of *Esomus danricus* exposed to 5.5 mg/L of Cu. Baker et al. (1998) fed juvenile grey mullet (*Chelon labrosus*) with a high-Cu diet for 4 weeks and found a rise of 3-fold in hepatic MDA. Pandey et al., (2001) observed that *Channa punctatus* exposed to 10 mg/L of Cu for 4 weeks did not induced lipid peroxidation in liver, kidney and gill. Interestingly, Cu showed to have a protective role on lipid peroxidation in liver when fish were exposed to endosulfan for 24 h (Pandey et al., 2001).

Regarding the carbonyl protein content, non-significant increase was observed on larvae after 3 weeks of copper exposure. Protein carbonyl content is one of the most used biomarker of protein oxidation (Stadtman and Berlett, 1998) because of its irreversible alteration. Craig et al. (2007) observed an induction of protein carbonyl levels in liver and gills after 48 h of exposure to 15 µg/L of Cu on zebrafish. Besides, Craig et al. (2007) also remarked the protective actions of Ca^{2+} and Na^{2+} ions in generation of protein carbonyl levels in gills and liver when they were added during Cu-treatment. Ransberry et al., (2015) alluded tissue-specific responses, since exposure of 50 and 200 µg/L of Cu on zebrafish induced protein carbonyls in intestines, but not in the gills and livers.

Cu is known to cause oxidative damage in lipids and proteins, with a potential to induce genotoxic effects. However, in our study no significant alterations were observed on lipid peroxidation, protein contents and DNA damage. We could hypothesize that the concentrations of Cu used in this study were not enough to cause oxidative alteration in lipid, protein and DNA of rainbow trout.

5. Conclusion

Copper is an essential trace element important for the metabolism of the body. However, at high concentrations it can interfere with certain processes such as protein synthesis and cellular damage. This study provides an extensive evaluation of Cu impact on early developmental stages of rainbow trout that includes phenotypic, behavioral, genetics and

587 biochemical endpoints. Results revealed sub-lethal effects of copper on development mainly
588 on embryos exposed to the higher copper concentration. Defects included increase of half-
589 hatched embryos, spinal deformities, genotoxicity and deregulation of genes involved in
590 detoxification and cell cycle arrest. Mortality was higher at hatching but larvae appeared to
591 adapt to the contaminated condition. However, alteration on gene expression, primarily those
592 involved in detoxification (gst, mt1 and mt2) could compromise the survival of this species
593 under natural conditions, since environmentally realistic concentration of copper were used in
594 this study.

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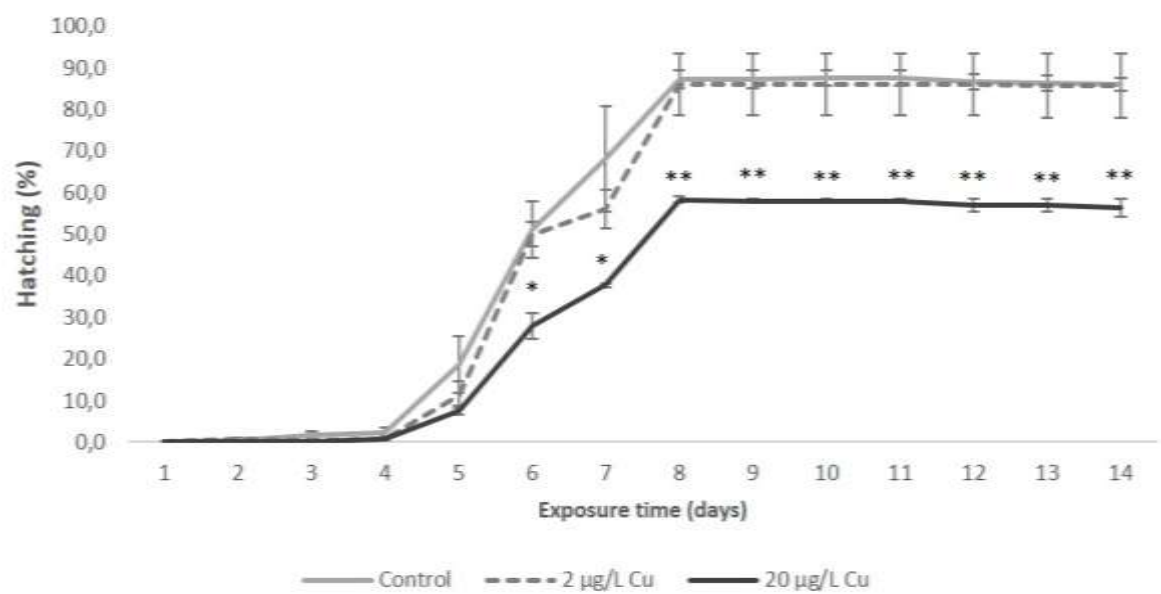
FIGURE CAPTIONS

Figure 1. Hatching rate for each studied condition. Asterisks indicate significant differences compared to control condition. Values are Mean \pm SE. N=3, ANOVA, *p<0.05, **p<0.01

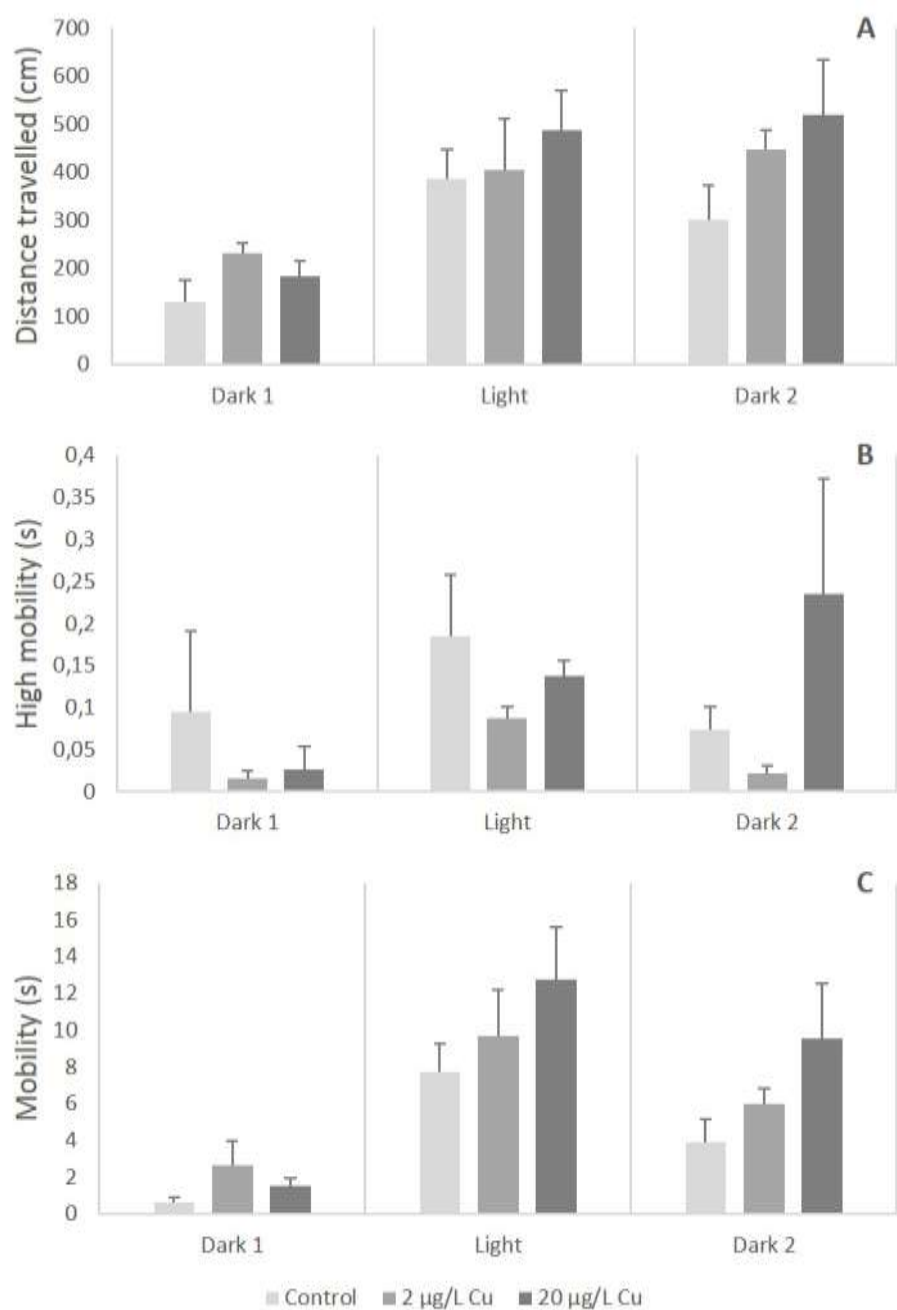
Figure 2. Total distance travelled by rainbow trout larvae (A), and cumulative time of high mobility (B) and mobility (C) of larvae. Locomotion was recorded in 30 minutes video analysis. Values represent average over 10 minutes interval. Values are Mean \pm SD, N=3, ANOVA.

Figure 3. Percentage of DNA tail intensity (A) and hedgehog (B) from blood cells of rainbow trout larvae assessed with the comet assay. Protein carbonyl content (C) and lipid peroxidation expressed as TBARS (D) of whole-body rainbow trout larvae. Protein carbonyl is expressed as nanomoles of carbonyl/mg of protein, and lipid peroxidation is expressed as nanomoles of TBARS/mg of protein. All values are expressed as Mean \pm SE, N=3, ANOVA.

Figure 1.



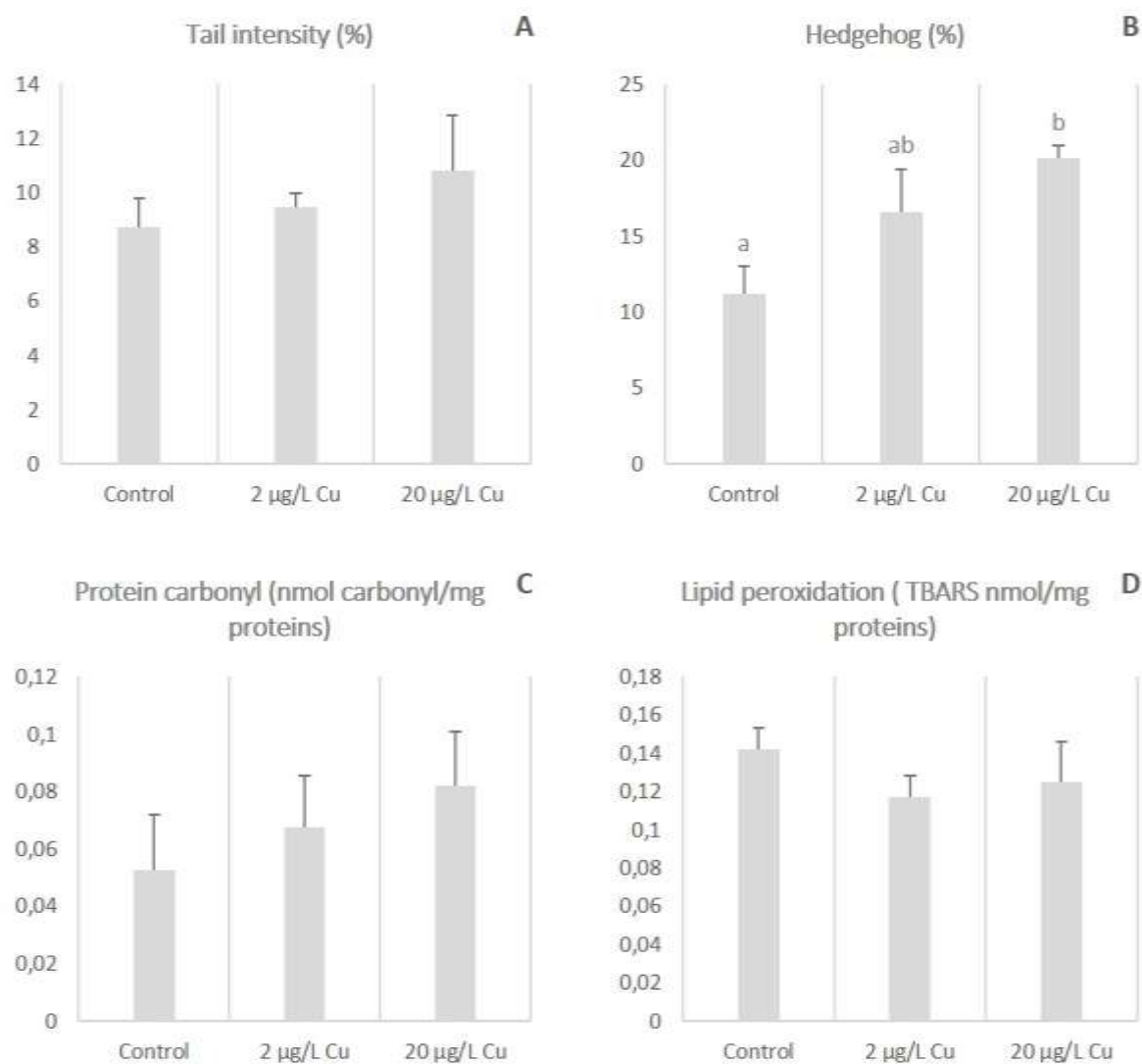
929 Figure 2.



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932 Figure 3.



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Table 1: Accession number and specific primer pairs for *Oncorhynchus mykiss* used in our study.

Gene	Accession number	Primer (5' – 3')
<i>Rpl7</i>	NM_001160672.2	GGTCGCTCTCACAGACAACA ^a TTATGTCCGTCCTCTGGGT ^b
<i>EF1α</i>	NM_001124339.1	ATGGGCTGGTTCAAGGGATG ^a GATCATACCGGCCTTCAGGG ^b
<i>Cat</i>	FJ226382.1	CAGGTGTCTTTCTTGTTTCAG GTCCAGGATGGGAAGTTGC
<i>Sod</i>	NM_001124329.1	TGATTGGGGAGATCTCGGGT ^a CGGGTCCAGTGAGAGTCAAC ^b
<i>Gst</i>	BT073173.1	ATTTTGGGACGGGCTGACA CCTGGTGCTCTGCTCCAGT
<i>Mt1</i>	M18104.1	GTGGATCCTGCAAGTGCTCA ^a GTAATGCACCAGGCCTCACT ^b
<i>Mt2</i>	XM_021597409.1	ATCTTGCAACTGCGGTGGAT ^a CCTCACTGACAACAGCTGGT ^b
<i>Cox</i>	KP013084.1	TCGTTTGAGCCGTGCTAGTT ^a CTTCTGGGTGGCCGAAGAAT ^b
<i>12S</i>	KY798500.1	GCGCCAGCTTAAAACCCAAA ^a GCCCATTTCTTCCCACCTCA ^b
<i>Ogg1</i>	XR_002474791.1	CTGATGGACAAGGCCAGTGT ^a GTAAGGACCCCATGGCTGTC ^b
<i>Rad51</i>	XM_021612309.1	AGGCTGGAGGAGGACATCAT ^a GTATTTGAGGGTGGCAGCCT ^b
<i>Bax</i>	BT074328.1	CAGAAAACCCAGGGAGGCAT ^a AGAACACATCCTGGGCACAG ^b
<i>p53</i>	NM_001124692.1	CCTACCAATGAGTAAGACAT ^a CCCGAAACATCCCGCT ^b

^aForward primer^bReverse primer

937 **Table 2:** Mean copper concentrations measured in exposure chambers (µg/L).

	Nominal Cu concentration (µg/L)		
	<i>0.0 (Control)</i>	<i>2</i>	<i>20</i>
T1	<0.5	<0.5	20.34
T2	<0.5	<0.5	18.73
T3	<0.5	1.45	18.71

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939

Table 3: Developmental endpoints in embryos and larvae of rainbow trout following exposure to waterborne Cu. Values are Means \pm SE. Different letters in the same row indicate significant differences between conditions. N=3, ANOVA/Kruskall-Wallis, $p < 0.05$.

	Control	2 $\mu\text{g/L}$ Cu	20 $\mu\text{g/L}$
<i>Acute toxicity</i>			
Embryo survival (%)	97.3 \pm 1.7^a	97.3 \pm 1.7^a	88.3 \pm 1.8^b
Larval survival (%)	92.9 \pm 0.6	94.7 \pm 1.1	90.3 \pm 3.8
Half-hatched mortality (%)	7.0 \pm 1.5^a	9.7 \pm 5.7^{a, b}	25.0 \pm 4.0^b
<i>Sub-lethal toxicity</i>			
Hatching rate (%)	90.3 \pm 3.2^a	87.7 \pm 7.3^a	63.3 \pm 3.2^b
Hatching time (DD)	332.8 \pm 3.3	334.9 \pm 2.1	336.8 \pm 1.2
Total length (mm)	17.6 \pm 0.3^a	18.36 \pm 0.02^b	17.9 \pm 0.3^{ab}
Head length (mm)	3.8 \pm 0.1	4.1 \pm 0.02	4.0 \pm 0.1
Ratio of head/body length (%)	21.8 \pm 0.4	22.5 \pm 0.1	22.2 \pm 0.3
Whole body copper concentration ($\mu\text{g/g}$ d.w.)	5.66 \pm 0.08^a	5.71 \pm 0.02^a	6.03 \pm 0.004^b
Abnormalities (%)			
Total deformed larvae	23.3 \pm 3.3^a	58.4 \pm 2.7^b	67.8 \pm 13.2^b
Edemas	6.7 \pm 6.7	7.8 \pm 4.2	17.8 \pm 9.0
Yolk-sac malabsorption	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Spinal	20.0 \pm 5.8^a	52.4 \pm 3.5^b	65.1 \pm 11.8^b
Craniofacial	6.7 \pm 3.3	10.8 \pm 1.7	14.7 \pm 10.8
Cardio-vascular	3.3 \pm 3.3	10.8 \pm 5.5	15.5 \pm 7.8
Haemorrhages	0.0 \pm 0.0	3.0 \pm 3.0	15.5 \pm 7.8

945 **Table 4:** Transcription of genes involved in antioxidant defense (*cat*, *sod*), DNA repair (*ogg1* and *rad51*),
 946 detoxification (*gst*, *mt1* and *mt2*), mitochondria metabolism (*cox*, *12S*), apoptosis (*bax*) and cell cycle arrest (*p53*)
 947 in whole-body rainbow trout larvae. Results are expressed as fold changes relative to control condition (1/4 is
 948 corresponded to a 4-fold decrease). Asterisk indicate differences with the control condition (N = 3, ANOVA/Kruskal-
 949 Wallis, *p < 0.05; **p<0.01).

	<i>cat</i>	<i>sod</i>	<i>gst</i>	<i>mt1</i>	<i>mt2</i>	<i>cox</i>	<i>12s</i>	<i>ogg1</i>	<i>rad51</i>	<i>bax</i>	<i>p53</i>
2 µg/L Cu	-	1/6**	1/4*	1/4*	1/7*	-	-	-	-	-	1/7*
20 µg/L Cu	-	-	1/7**	1/7**	1/7*	1/3	-	-	-	1/3	1/8*

950