Elsevier Editorial System(tm) for Marine Pollution Bulletin

Manuscript Draft

Manuscript Number: MPB-D-17-00147R1

Title: Biomarker responses of Cu-induced toxicity in European seabass Dicentrarchus labrax: assessing oxidative stress and histopathological alterations

Article Type: Research Paper

Keywords: Biomarkers; Copper; Dicentrarchus labrax; Oxidative stress; Histopathological alterations

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Abstract: A comprehensive approach to chemical accumulation and biological effects of short-term Cu-exposure in juveniles of European seabass (Dicentrarchus labrax) has been achieved. Fish were exposed to 0.01-10 mgL-1 nominal Cu concentrations for 24-96 hours. Metal concentrations in water and gills, liver, muscle and brain tissues were studied along with oxidative stress biomarkers (superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation). Induction of oxidative damage was observed in all the organs with differential antioxidant responses; gills appearing as the most sensitive from low environmentally water Cu concentrations as 0.01 mgL-1. Histopathological alterations were also observed in liver and gills, even without a significant Cu accumulation.
The results shows that the combination of oxidative stress parameters, particularly lipid peroxidation and glutathione peroxidase activities, and histopathological alterations provides a good model fish and reliable early biomarkers for monitoring Cu pollution in seawater and might call for the protection agencies to revise Cu environmental standards.

HIGHLIGHTS

An approach to chemical accumulation and biological effects of Cu-exposure in seabass

Induction of oxidative damage has been observed in organs of seabass by Cu-exposure

Histological alterations are produced in liver and gills of seabass after Cu-exposure

Oxidative stress and histological alterations as reliable biomarkers for Cu pollution

Biomarker responses of Cu-induced toxicity in European seabass *Dicentrarchus labrax:* assessing oxidative stress and histopathological alterations

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ABSTRACT

A comprehensive approach to chemical accumulation and biological effects of short-term Cu exposure in juveniles of European seabass (*Dicentrarchus labrax*) has been achieved. Fish were exposed to 0.01-10 mg L^{-1} nominal Cu concentrations for 24-96 hours. Metal concentrations in water and gills, liver, muscle and brain tissues were studied along with oxidative stress biomarkers (superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation). Induction of oxidative damage was observed in all the organs with differential antioxidant responses; gills appearing as the most sensitive from low environmentally water Cu concentrations as 0.01 mg L^{-1}. Histopathological alterations were also observed in liver and gills, even without a significant Cu accumulation.

The results shows that the combination of oxidative stress parameters, particularly lipid peroxidation and glutathione peroxidase activities, and histopathological alterations provides a good model fish and reliable early biomarkers for monitoring Cu pollution in seawater and might call for the protection agencies to revise the Cu environmental standards.

Keywords:

Biomarkers; Copper; *Dicentrarchus labrax*; Oxidative stress; Histopathological alterations

1. INTRODUCTION

Due to the environmental transporting systems (such as rivers, sewage effluents, drainage canals...) many of pollutants discharged into natural water end up in seawater-related ecosystems. Among them, trace metals including Cu are considered a matter of concern due to their persistence, non-degradability and their tendency to accumulate in aquatic organisms [Begum et al., 2005]. They can be discharged into the marine environment and remain in solution or in suspension, precipitate to the bottom, or be assimilated by organisms, creating a potential source of trace metal pollution in the aquatic environment [Bilandžić et al., 2011].

Copper is an essential trace metal to all aquatic biota and human beings, but even a small excess amount of it is extremely toxic to aquatic organisms [Das and Khangarot, 2011]. This concern has been taken into account by different directives such as US Clean Water Plan for marine waters (EPA) and the EU Water Framework Directive (EU WFD). Thus, the environmental quality standard (EQS) has been defined as 4.8 μg L^{-1} dissolved Cu at acute conditions (CMC: Criterion Maximum Concentration) and 3.1 μg L^{-1} dissolved Cu at chronic conditions (CCC: Criterion Continuous Concentration) in salt water [US EPA, 2016]; and if DOC (dissolved organic content) is taken into account the standards applied at long term in transitional and coastal waters are 3.76 μg L^{-1} dissolved Cu, where DOC ≤ 1 mg L^{-1} and 3.76 + (2.677 × ((DOC/2) - 0.5)) μg L^{-1} dissolved Cu, where DOC > 1 mg L^{-1} [EU WFD, 2015]. Among the different physiological and biochemical disturbances induced by copper, it was shown that exposure to this metal negatively affects glycolysis [Carvalho and Fernandes, 2008], Krebs cycle [Couture and Kumar, 2003], ionic and osmotic regulation [Pinho and Blanchini, 2010], oxygen consumption and growth [Manyin

and Rowe, 2009], among others. Most of these effects can be directly or indirectly associated with an insufficient production of energy to maintain cell metabolism and homeostasis. At extreme conditions, this situation can even lead to death [Lauer et al., 2012].

Current awareness of the potential hazards of trace metals in the aquatic environment has attracted much interest and fish species are generally considered the most feasible organisms for pollution monitoring in aquatic systems. Fish bioaccumulation and other biomarkers may be applied to elucidate the aquatic behaviour of environmental contaminants as monitoring tools, allowing to identify certain substances at low levels in water and to assess exposure to or effect of them on biota [Van der Oost et al., 2003].

Earlier studies have revealed that Cu toxicity in fish is associated with the induction of oxidative stress [Gaetke, 2003; Regoli and Giuliani, 2014], leading to cellular injuries [Sanchez et al., 2005; Gravato et al., 2006; Craig et al., 2007; Eyckmans et al., 2011; Machado et al., 2013]. Furthermore, other studies have shown that the exposure of fish to copper results in histopathological alterations, mainly in gills and liver [Arellano et al., 1999; Ortiz et al., 1999; Paris-Palacios et al., 2000; Cerqueira and Fernandes, 2002; Oliva et al., 2009; Oliva et al., 2013].

The present study aimed to comprehensively assess the potential induction of oxidative and histopathological damage and the accumulation in fish tissues by one of the priority metals for public health as Cu. Different toxicologically relevant fish tissues were selected based on key physiological functions and differentially related to environmental exposition and accumulation of xenobiotics, i.e., gills (related to xenobiotic uptake), liver (related to xenobiotic metabolism), muscle (which accounts for the higher mass of fish body) and brain (related to possible neurotoxic and behavioral effects. The histopathological alterations were studied in liver and gills because this type of histological analysis shows

significant information. Thus, short-term experiments at different concentrations of metal were performed using juveniles of European seabass (*Dicentrarchus labrax*, Linnaeus 1758). This species is an autochthon euryhaline fish, which inhabits in estuaries, coastal water and rivers in and around Europe, principally in the Mediterranean Sea, the Black Sea and the Eastern Atlantic Ocean. It is one of the most important commercial fish cultured in the Mediterranean region, with a high global aquaculture production of 161059 T in 2013 [FAO, 2013], and consequently a high human consumption [Fernandes et al., 2009]. Additionally, it has been previously used as a sentinel species in monitoring studies and ecotoxicological assays [Giari et al., 2007], including the evaluation of oxidative stress-related responses after 17β-estradiol exposure [Ahmad et al., 2009], anthropogenic exposure [Maria et al., 2009] and temperature effect [Vinagre et al., 2012].

Though assays about waterborne Cu toxicity in fish have been reported elsewhere [Sanchez et al. (2005), Gravato et al. (2006), Craig et al. (2007) Eyckmans et al. (2011), and Machado et al. (2013)], they mainly involved fresh water, tap water, soft water, salt water (salinity 24 ppt) and river water. Neither covers experiments with seawater and the study of an integrative and comprehensive list of biological and chemical parameters.

Furthermore, although waterborne Cu toxicity in fish has been also reported through some studies performed in copper-contaminated aquatic ecosystems [Hansen et al., 2006a; Hansen et al., 2006b], it is often difficult to assign a specific response to a particular pollutant because usually complex mixtures of chemicals are present. Thus, unlike what occur in field studies, the interest of carrying out toxicity studies in the laboratory is that the effect of a unique metal can be isolated and accurately related to definite exposure concentrations.

2. MATERIAL AND METHODS

2.1. Fish and experimental design

Specimens of *Dicentrarchus labrax* (Linnaeus, 1758) (weight range: 41.80-65.82 g and length: 16.0-18.0 cm) were obtained from the aquaculture facilities of Central Service for Aquaculture Research (University of Cadiz, Spain). The fish were disease-free and did not have any history of previous chemical exposure. Before the experiments, fish were acclimated in large aquaria containing marine water supplied by seawater well under laboratory conditions with oxygen-saturated water at 20 ºC and 12/12 dark/light cycle for four weeks. Fish were fed three times per day with commercial dry pellets using automatic feeders (2% body weight; L2 Optibass 1P, Skretting España, Burgos, Spain). No mortality was observed during the acclimatization period.

They were randomly grouped into five duplicated groups (*n*=12) in 30-L tanks and exposed under laboratory conditions to 0 (control), 0.01, 0.1, 1 and 10 mg L^{-1} nominal concentrations of copper solution prepared by dissolving $CuSO_4 \cdot 5H_2O$ (purchased from Sigma-Aldrich, USA); six specimens from each tank were collected after each exposure time, at 24 and 96 h. Test concentrations were selected based on previous literature data reported in metal toxicity bioassays. Twelve animals were place in each tank with continuous slight aeration. Fish were not fed during the experimental period. Living and dead specimens were counted daily and the last ones were removed immediately to avoid fungal and bacterial infection of the other fish during the breakdown. At the end of the exposure period, fish were anaesthetized in 0.1% 2-phenoxyethanol, weighed and measured; then, they were sacrificed and bled. Liver, gills, muscle and brain were carefully excised, dissected on ice and flash frozen in liquid nitrogen followed by storage at -80ºC until analysis. In order to have enough amount of tissue for chemical analysis, the organ

samples were pooled in groups of 3 fish. For histological analysis, the samples were fixed in formalin (10% v/v formaldehyde (Panreac, Spain)) buffered at pH 7.2 with 0.1 mol L^{-1} phosphate (Analema, Spain) during 24 hours and washed in tap water for 1hours.

All experimental procedures were performed in compliance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Physical-chemical parameters of water

Temperature, pH, dissolved oxygen (DO), salinity and suspended solids (SS) of water from all tanks were measured in situ with an electrochemical portable device (HI 9828, Hanna Instruments, Spain). Water samples of 50 mL were taken from each tank to evaluate the dissolved organic carbon (DOC) using a TOC analyzer (Analytic Jena 3100, Germany). The mean values of these physical-chemical parameters in water are shown in Tables 1 and 2. As shown, the physical-chemical properties of the test water did not vary significantly among treatments throughout the experimental procedure. It is noteworthy that the experiments were accomplished at thermal optimum conditions for *D. Labrax*, as defined by different authors ranging from 20ºC to 25º C [Vinagre et al., 2012].

2.3. Trace metals analysis in water

Metal concentrations were analysed in water samples of 100 mL to check the actual metal concentration (Table 3) by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an Iris Intrepid spectrometer with argon gas humidifier, cyclonic spray chamber (Glass) and nebulizer type SeaSprays^{TM} (Thermo Elemental, Franklin MA) for tanks with 0.1, 1 and 10 mg L^{-1} Cu and differential pulse anodic stripping voltammetry (DPASV) using a Metrohm 757 VA Computrace Stand controlled by PC software (VA Computrace 2, Metrohm, Switzerland) for control and 0.01 mg L^{-1} Cu tanks. In case of

DPASV, the samples were previously digested during 2 hours using an UV digester (705 UV, Metrohm, Switzerland) and 50 µL of 30% H_2O_2 (Suprapur grade) per 15 mL of water sample. Analytical methods were checked using LGC 6016 (estuarine water) certified reference water sample by analysing three replicates for each sample, obtaining good recoveries (≥90%); blanks and limits of detection (LOD) were also evaluated (Table 4). The equilibrium software Visual MINTEQ 3.0 [Herndon and Branch, 1999; Gustafsson, 2010] was used to predict the metal speciation. Physical-chemical parameters of water and the values of Log $K_{1(\text{Cu-fulvic acid})}$=0.26 and Log $K_{2(\text{Cu-fulvic acid})}$=8.26 were used as complexation parameters. Because of high sensitivity required for trace metal concentrations, a strict protocol was followed to avoid contamination of solutions and samples when required. Low-density polyethylene bottles (LDPE, Nalgene) and polystyrene flasks were acid cleaned and used to store samples and reagent solutions. Sample handling and preparation of solutions were performed using polyethylene gloves under a class 100 laminar flow hood cabinet Crusair model 9005-FL (Cruma, Spain). All the clean material was kept in plastic bags to avoid lab air pollution. Water was purified by reverse osmosis with an Elix 3 (Milli-RO) system followed by ion exchange with an 18 MΩ cm deionised Milli-Q^{50} System (Millipore, USA).

2.4. Trace metals analysis in fish tissues

Fish tissue subsamples were taken from liver, gills, muscle (dorsal white muscle) and brain, making two pools of three specimens from each tank. Freeze-dried tissue samples (0.1-0.3 g) were acid-digested by microwave heating using 7 mL of 65% HNO_3 (Suprapur grade) for muscle and gills, and 4 mL of 65% HNO_3 (Suprapur grade) and 2 mL of 30% H_2O_2 (Suprapur grade) for liver and brain. After digestion, the samples were diluted up to 25 mL with Milli-Q deionised water and analysed by ICP-MS (X7 Series plasma scan

sequential inductively coupled mass spectrometer, Thermo Elemental, UK). Analytical parameters were evaluated and the accuracy of the applied methodology was satisfactorily evaluated using two certified reference biological materials of the National Research Council Canada (NRCC): DOLT-3 (dogfish liver) and DORM-2 (dogfish muscle) (Table 4).

2.5. Analysis of biomarkers

a) Tissue preparation

At time of assay subsamples of tissues were thawed and homogenized on ice in 1.5 mL of cold 0.1 mM phosphate buffer (pH 7) containing 0.5 mM EDTA using a tissue homogenizer (IKA homogenizer 10T Basic, Germany). Subsamples were centrifuged (Ortoarlesa Biocen 22R centrifuge, Spain) for 30 min at 12000 *g* (4 °C) and the supernatants were used for biochemical analyses.

b) Protein measurement

Protein quantifications were carried out according to the Bradford assay [Bradford, 1976] using bovine serum albumin as a standard.

c) SOD activity assay

Superoxide dismutase (SOD) activity was determined based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT) [Crouch et al., 1981], which was generated by xanthine oxidase. Briefly, the assay mixture consisted of 0.65 mL of phosphate buffer (50 mM, pH 7.8) with 5 mM EDTA, 0.1 mL of xanthine (50 mM), 0.1 mL of NBT (1 mM), 0.05 mL of xanthine oxidase (5.4 U mL^{-1}) and 0.05 mL aliquot of supernatant sample. The reaction was initiated by adding xanthine oxidase. The reduction of NBT by superoxide anion to blue formazan was measured at 560 nm. The rate of NBT reduction in the absence of tissue was used as the reference rate. One unit of SOD is

defined as the amount of enzyme required to decrease the reference rate to 50% of maximum inhibition. The SOD activity was expressed in units per mg protein.

d) CAT activity assay

Catalase (CAT) activity was determined according to the method of Claiborne [Claiborne, 1985] by measuring the decrease of H_2O_2 concentration in the presence of the supernatant sample at 240 nm for 2 min in phosphate buffer. Briefly, the assay mixture consisted of 1.95 mL of phosphate buffer (50 mM, pH 7), 1 mL H_2O_2 (15 mM) and 0.05 mL of tissue homogenate. CAT activity was calculated in terms of nmol H_2O_2 consumed per (min mg protein), using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

e) *GPx activity assay*

Glutathione peroxidase (GPx) activity was measured according to the procedure of Mohandas [Mohandas et al., 1984], as described by Athar and Iqbal [Athar and Iqbal, 1998]. The assay mixture consisted of 1.44 mL phosphate buffer (50 mM, pH 7.0), 0.1 mL EDTA (1 mM), 0.1 mL sodium azide (1 mM), 0.05 mL glutathione reductase (1 U mL^{-1}), 0.1 mL glutathione (GSH, 1 mM), 0.1 mL NADPH (0.2 mM), 0.01 mL H_2O_2 (0.25 mM) and 0.1 mL of supernatant sample in a final volume of 2 mL. Oxidation of NADPH was recorded spectrophotometrically at 340 nm for 3 min. The enzyme activity was calculated as nmol NADPH oxidized per (min mg protein), using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

f) LPO assay

Lipid peroxidation (LPO) was assessed by measuring the levels of lipid hydroperoxides (which results from the oxidative injury of saturated and unsaturated lipids) in the homogenate fraction of tissues. There were estimated according to the xylenol orange assay [Gay and Gebicki, 2000], using 0.1 mL of diluted supernatant sample (1:10 in water) and 0.9 mL of reaction mixture containing final concentrations of 25 mM H_2SO_4, 100 μM

Xylenol Orange (XO), 100 mM sorbitol and 250 μM ferrous ammonium sulfate. The pH of reaction mixture was adjusted to 1.8 with addition of Na_2HPO_4. Afterwards, the sample was centrifuged at 1000 *g* for 2 min and placed in the dark for 45 min. Absorbance was read at 560 nm. The molar extinction coefficient of 2.67×10^5 M^{-1} cm^{-1} was used.

2.6. Histopathological study

Six fish specimens for each Cu concentration and exposure time were studied and in the same way for each duplicate assay. The tissue samples for the histological exam were fixed in formalin (10%), washed in running tap water, dehydrated in alcohol and acetone, cleared in xylene and embedded in paraffin wax. Sections (6 μm thick) were cut and mounted on gelatinized slides using a rotary microtome (Leica RM 2125 model, Spain). Sections were rehydrated in distilled water and stained with haematoxylin/eosin (H/E) and haematoxylin/VOF (H/V) (light green, orange-G and acid fuchsine) [Gutiérrez, 1967]. A Leitz light microscope (170mm Leitz objectives: x4 (n.a. 0.12), x10 npl (n.a. 0.25), x25 npl (n.a. 0.50)) (Germany) was used for analysis. Pathology criteria were derived from several tissue alterations specified by the International Council for the Exploration of the Sea (ICES) [Davies and Vethaak, 2012] and additional pathology observed in the sampled specimens. Alterations were evaluated semi-quantitatively by ranking the severity of the alteration in the tissue. Ranking was as follows: grade 0 (no alterations), grade 1 (focal mild alteration), grade 2 (moderate alteration) and grade 3 (extended severe alteration). This ranking was used to establish an overall assessment value of the histopathological lesions for each organ of fish.

The histopathological condition was evaluated using the index of pathologies called IPAT. It is a modified index from histopathological condition index of Bernet et al. [Bernet et al., 1999]. Following these authors, an importance factor or condition weight (w) is assigned

for each alteration based on the biological significance of the lesion, that is, the grade in which a lesion may affect the normal functioning of a tissue or organ. The alterations are classified into three importance factors (condition weight):

1. Minimal pathological importance, the lesion is easily reversible as exposure to irritants ends.
2. Moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized.
3. Marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function.

In this work, the prevalence of lesions has been also included in the index calculation considering IPAT as a general index on pathological state of fish instead of an organ pathological index. The index was calculated for each metal concentration and exposure time taking into account the partial values obtained for each alteration and organ, by the following equation:

$$\sum_{i=1}^{n} \frac{alg_i \cdot p_i \cdot w_i}{100} \qquad (1)$$

where (alg_i) is the average lesion grade of the *i-th* alteration, (p_i) is the prevalence of this alteration, (w_i) is the condition weight and (n) is the number of pathologies observed (in this study, n =14).

Lesion observed in gills were: aneurysm (A), hypertrophy of lamellar epithelia (HPT), hyperplasia of lamellar epithelia (HPL), lamellar shortening (LS), lamellar fusion (LF), desquamation of lamellar epithelia (D) and epithelial lifting (EL). Lesions observed in liver were: steatosis (S), vacuolization (V), atrophy (AT), eosinophil granules (EG), necrosis (N), blood stagnation (BS) and hepatic parenchyma disorganization (HPD). According the lesion grade criteria (max=3) and the condition weight for each lesion (w_i=1 for all lesions

except w_i=2 for HPL and AT; and w_i=3 for N and V), the maximum value of the IPAT index was calculated. Thus, it was 60 for each metal concentration and exposure time. Thereby, assessment values ≤20 were assumed to be a low impact, values between 21 and 40 were assumed to be a moderate impact and values >40 were defined as high impact over health fish.

2.7. Statistical analysis

The experiment was performed with 12 fish per treatment tank and in duplicate (n=24 for each metal concentration), collecting 6 fish per exposure time and replicate. Measurements from fish tissues were done by triplicate from pools of 3 fish. The data are expressed as mean ± SD (standard deviation). Linear regression analysis was used to correlate two variables. Significant differences were determined by one-way ANOVA followed by the Duncan's *post-hoc* test. Significant differences were considered at $p<0.05$.

3. RESULTS

While no fish in the control group died during the experiment, 100% mortality was only observed in copper-exposed fish for the nominal dose of 1 mg L^{-1} after 96 h and for 10 mg L^{-1} after 24 h. A low mortality (16.7%) was observed for 1 mg L^{-1} Cu exposure after 24 h.

3.1. Trace metal concentrations in water and fish tissues

In order to check the evolution of the nominal metal concentrations in water of tanks throughout the experimental period, the actual metal concentrations were monitored by ICP-AES or DPASV at initial time, 24 h and 96 h (Table 3). At initial time, actual

concentrations of Cu in water matched those of nominal concentrations. However, along the experimental period the actual levels showed a trend toward a decrease in the upper Cu doses, as compared to the nominal levels (Table 3), probably due to changes in the speciation of copper in the water during the experiments. This evolution can produce poorly-soluble species easily adsorbed onto solid surfaces such as tanks [Kim et al., 2014] or even fish skin, disappearing from the water body. Thereby, the distribution of Cu species showed a high percentage of aqueous Cu-fulvic acid complexes for control, 0.01 and 0.1 mg L^{-1} Cu nominal concentrations (above 90% throughout the course of the experiments). For 1 and 10 mg L^{-1} Cu nominal concentrations, the percentage of organic copper species decreased because of the increase of the Cu carbonate ($CuCO_3$ aq) percentage, which ranged from 25 to 48% for 1 mg L^{-1} Cu nominal exposure and above 50% for 10 mg L^{-1} Cu nominal. Free Cu ions and $CuOH^+$ varied between 5% and 14% for the two higher exposures to metal, being lower than 5% for the others.

In addition, the levels of metal accumulation were measured in four different toxicologically relevant fish tissues: liver, gills, muscle and brain. Mean concentrations of Cu in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu for each test are shown in Figure 1. Mostly, the liver exhibited the highest metal concentration and they were arranged as follows: liver > gills > brain > muscle. As compared to controls, a significant 3.5-fold increase in Cu content was observed in gills after exposure to the maximal non-lethal doses of 1 mg L^{-1} Cu for 24 h and a 1.5-fold increase was found after exposure to 0.1 mg L^{-1} Cu for 96 h. No variation in metal content was recorded in liver, muscle or brain, in which control values were steadied. In regard to the time of exposure, no significant differences were observed between the 24 and 96 h periods in any organ for each non-lethal assay with copper.

3.2. Antioxidant enzymes

a) SOD activity

The variation in the SOD activity before and after exposure to Cu is depicted in the Figure 2. Gills showed clearly the highest basal activity. Exposure to increasingly higher Cu concentrations brought about increasingly higher SOD activities in gills, liver and muscle at 24 and 96 h. However, while this effect was significant in liver from 0.01 mg L^{-1} Cu dose, it only took place in gills and muscle from 0.1 mg L^{-1} Cu dose. Significant differences were observed in fish exposed to 0.1 mg L^{-1} Cu dose compared with the controls after 24 and 96 h, with 2-fold and 2.5-fold activity increases in gills and 1.9-fold and 3.1-fold in liver, respectively. In contrast to these results, in brain the SOD activity was significantly inhibited by Cu 0.1 and 1 mg L^{-1}.

b) CAT activity

As shown in Figure 3, remarkably higher levels of CAT activity were measured in the liver, although only the higher non-lethal doses of Cu at 24 h and 96 h were able to induce a significant increase in this organ as compared to controls (1.8-fold and 1.3-fold, respectively). All copper concentrations induced a significant increase of CAT activity in gills after 24 h exposure. However, it was significantly inhibited by 0.1 mg L^{-1} Cu after 96 h (0.7-fold). In addition, the CAT activities after 96 h were always significantly lower than those values obtained by 0.01 and 0.1 Cu mg L^{-1} after 24 h in this tissue. In muscle, an increase in CAT activity was only observed in fish exposed to 1 mg L^{-1} Cu for 24 h (1.4-fold), but it was inhibited after 96 h exposure at 0.01 and 0.1 mg L^{-1} of metal. Finally, the exposure to Cu induced an increase of CAT activity in brain from 0.1 mg L^{-1} dose after 24 h (1.5-fold for 0.1 mg L^{-1} Cu and 1.9-fold for 1 mg L^{-1} Cu), but it did not occur after 96 h; thus, these results were significantly different.

c) GPx activity

Exposure to copper resulted in a generalized stimulation of GPx activity. The higher basal levels were observed in gills (Figure 4), with a dramatic dose-dependent increase in GPx activity from the lowest Cu concentration, with 3.8-fold and 3.2-fold increases compared with control experiments for the higher non-lethal doses at 24 and 96 h, respectively. In liver, GPx activity was also increased 1.8-fold and 2.2-fold after exposure to Cu in these same experimental doses. In muscle and brain significant increases of GPx were only observed from the 0.1 mg L^{-1} dose with maximal increases for the higher non-lethal doses (in muscle: 1.9-fold and 1.7-fold after 24 h; in brain: 1.4-fold and 1.6-fold after 96 h).

d) Lipid peroxidation

The levels of LPO in the four studied organs of fish after the bioassays are shown in Figure 5. As a whole, the higher levels were observed in gills followed by liver, while similar lower contents were measured in muscle and brain. As compared with controls, the LPO levels were significantly higher after copper exposure in all organs; these increases were evidenced in gills from the lowest Cu concentration of 0.01 mg L^{-1} after both 24 and 96 h exposure to Cu; thus, they increased up to 3.1-fold for 1 mg L^{-1} after 24 h and 2.3-fold for 0.1 mg L^{-1} after 96 h. In the other organs this effect was observed only from the nominal dose of 0.1 mg L^{-1} at both 24 and 96 h. In addition, significant higher LPO was observed after 96 h in comparison with after 24 h for the same doses from the lowest Cu concentration in gills and for 0.1 mg L^{-1} Cu in muscle and brain. It must be noted that data are not available for higher copper concentrations because fish died.

3.3. Histopathological alterations

The occurrence of histopathological lesions was studied in gills and liver of *D. labrax* juveniles exposed to copper after 24 and 96 h. Six types of alterations were observed in gills: aneurysms (A), hypertrophy of lamellar epithelia (HPT), hyperplasia of lamellar

epithelia (HPL), lamellar shortening (LS), lamellar fusion (LF) and desquamation of lamellar epithelia (D) (see prevalence percentage of pathologies in Table 5 and representative microphotographs of some lesions in Figure 6). The prevalence percentage of lesions in gill was ≥75% for HPL after both exposure times for all metal concentrations, for LF at 0.01 mg L^{-1} Cu after 96 h, and for D at 0.1 mg L^{-1} Cu after 24 h. Percentage values of ≥62.5% were also found for A and LF at 0.1 and 1 mg L^{-1} copper doses, as well as for D at 0.01 mg L^{-1} Cu after 96 h. On the other hand, severe histopathological changes were observed in liver, being evidenced from the lowest experimental dose of 0.01 mg L^{-1} copper after 96 h exposition as steatosis (S), atrophy (AT), necrosis (N), blood stagnation (BS) and hepatic parenchyma disorganization (HPD) (Table 5 and Figure 6). A prevalence percentage of 100% was found for S damage at 0.1 and 1 mg L^{-1} Cu, BS at 1 mg L^{-1} Cu after 24 h and HPD at 0.1 mg L^{-1} Cu after 96 h. N had lower prevalence percentages. Notably, these alterations were highly related to the increase of copper dose and exposure time.

Significant correlations ($p<0.05$) were established between Cu concentrations in water/tissues and histological alterations in gills/liver. After 24 h exposure, positive correlations were established between Cu concentrations in water and the following hepatic lesions: S ($r = 0.499$), V ($r = 0.730$), AT ($r = 0.742$), HPD ($r = 0.695$) and EG ($r = 0.643$). After 96 h exposure, positive correlations were only established with S ($r = 0.640$), AT ($r = 0.633$) and N ($r = 0.701$). Positive correlations between Cu concentration in gills and A were established at both exposure times ($r = 0.639$ after 24 h and $r = 0.670$ after 96 h).

The assessment of the lesions found in gills and liver of *D. labrax* juveniles exposed to Cu allowed calculating the histopathological condition indices. The general IPAT index corresponding to each Cu dose and exposure time was calculated from the partial values

obtained for each alteration and organ as aforementioned in section 2.6. As shown in Table 6, IPAT indexes for non-lethal doses were highly related to the increase of copper dose and exposure time. A slightly moderate impact on health fish (20.1) at 0.1 mg L^{-1} Cu after 96 h was found but the other indexes showed a low impact on health fish (≤20).

4. DISCUSSION

To assess the effect of waterborne Cu-exposure in European seabass and to avoid any alteration in the experimental conditions that would result in distress of fish, a static model was selected and a wide range of metal concentrations (from 0.01 to 10 mg L^{-1}) was evaluated. It is not easy to establish canonic environmentally relevant concentrations of Cu, since it may greatly differ for the different bodies of water as dependent on a number of key chemical parameters such as pH, hardness, organic ligands and salinity, which may influence the copper speciation and hence its bioavailability and toxicity. Overall, seawater is high in carbonates, calcium and magnesium, and has higher pH than most freshwaters, reducing the potential for copper toxicity [Chen et al., 2006]. In a comprehensive review by Georgopoulos et al. [Georgopoulos et al., 2001], it is stated that Cu levels in surface water range from 0.5 to 1000 μg L^{-1}, with a median of 10 μg L^{-1}; shorter range of 0.069 to 20.0 μg L^{-1} is described for surface estuary, bay and coastal waters and 0.0063 to 2.8 μg L^{-1} for open surface seawater [Crompton, 2007]. It is a well-known fact that estuaries serve to trap much of the copper that flows out of rivers into oceans and thus much higher Cu concentrations may be found. A high concentration of copper in water principally originates from agricultural and industrial activities, being of concern among others the loading of copper in the marine environment from the mining activities and the use of

copper-based antifouling paints on both recreational and commercial watercraft [Hall and Anderson, 1999]. In this regard, total copper concentrations were found ranging 21-72 μg L^{-1} in a mining Atlantic region (Huelva, SW, Spain) [Vicente-Martorell et al., 2009] and values of 61, 14.1 and 86 μg L^{-1} of maximum dissolved copper were reported in marinas/harbors, estuaries and open sea/coastal areas of Europe, respectively [Hall and Anderson, 1999]. Therefore, the 0.01 and 0.1 mg L^{-1} nominal test concentrations may be reasonably considered as representative of those encountered in moderately and highly contaminated marine environments, respectively; while that of 1 mg L^{-1} is yet inside the range achieved in some surface waters near industrial areas affected by high copper containing spills.

The actual metal concentrations in water of the bioassay tanks were checked. While the measured levels of Cu in water matched the nominal ones at time 0, they showed a trend toward a decrease at the higher doses and the longer exposure times (though very far from an order of magnitude). This copper decrease was reported previously in a similar toxicity model [Dautremepuits et al., 2002; Sanchez et al., 2005] and may be due to adsorption to organic ligands or the walls of the tanks [Kim et al., 2014], or to diminished solubility and, therefore, precipitation. Indeed, the percentage of Cu-carbonate and Cu-hydroxyl species increased at the higher doses of metal having a lower solubility than free Cu ions. However, since it is coincident with those Cu treatments where lethality occurred, its contribution to the study results is rather limited.

The mortality found by exposure to Cu (100% for 10 mg L^{-1} Cu after 24 h and 1 mg L^{-1} Cu after 96 h; 16.7% for 1 mg L^{-1} Cu after 24 h) likely demonstrates the high potential of Cu to cause acute toxicity at the short term in *D. labrax,* as previously observed in other fish species. In a similar study with juvenile Senegalese sole (*Solea senegalensis)* only 0.01 and 0.1 mg L^{-1} copper concentrations resulted sublethal while 1 mg L^{-1} of metal showed a

mortality of 26.6% at 24 h and 100% at 96 h exposure [Oliva et al., 2009]. However, no mortality was observed in dogfish (*Scyliorhinus canicula)* after 48 h exposure to 2 mg L^{-1} copper but 4 mg L^{-1} copper produced 50% mortality [Torres et al., 1987].

Unlike other studies on Cu exposure, an outstanding aspect of this study was the simultaneous evaluation of the response of four representative organs with key physiological functions and differentially related to environmental exposition and accumulation of xenobiotics, i.e., gills (related to xenobiotic uptake), liver (related to xenobiotic metabolism), muscle (which accounts for the higher mass of fish body) and brain (related to possible neurotoxic and behavioral effects). During chronic Cu exposure, accumulation is observed in these organs as a consequence of re-distribution of Cu uptake across gills (a primary target for Cu) and the regulation in the liver by excretion can occur [Grosell et al., 1996; Handy, 2003; Hansen et al., 2006a].

The basal Cu content in the fish tissues, in order from highest to lowest, ranged as follows: liver > gills > brain > muscle. However, only gills showed an increase in Cu content for the maximal non-lethal doses after both 24 h and 96 h exposure. These results suggest that Cu concentrations were well maintained inside the homeostatic range in most of the body organs in fish exposed to the lower range of Cu concentrations; far from those leading to lethality. The values of bioconcentration factor (BCF= $([Cu]_{exposed}–[Cu]_{control})/[Cu]_{water}$) [Paris-Palacios et al., 2000] in gills were ranging from 8.2 to 40.6, very low as compared to typical values described for several species and metals to be in the range of 100-1000 [McGeer et al., 2003], also reflecting the homeostatic regulation of Cu [Sanchez et al., 2005]. Thus, prevention of Cu uptake might be a mechanism to avoid toxicity, at least at the lower levels of metal exposure. Similarly, no significant Cu accumulation was observed in gills and liver of saltwater guppies acutely exposed (96 h) to 0.005, 0.009 and 0.02 mg L^{-1} Cu [Machado et al., 2013]. By contrast, an increase of Cu in gills already one

day after transfer from a reference to a high Cu-content river (0.016 mg L^{-1}) was described in brown trout (*Salmo trutta*) [Hansen et al., 2006b]. Although no metal accumulation in liver, muscle or brain was found in this paper, it cannot be excluded that it might occur after longer exposure times. Sanchez et al. observed Cu accumulation in the liver of stickleback fish exposed to 0.2 mg L^{-1} at eighth day, but not at fourth day [Sanchez et al., 2005].

A wide range of environmental pollutants, as trace metals, are known to induce oxidative stress in aquatic biota including fish. The generation of reactive oxygen species (ROS) induced by trace metals is commonly associated with cellular injures due to alterations in DNA, proteins and membranes [Leonard et al., 2004]. To counteract the adverse effect of ROS, living organisms have a complex and effective antioxidant defense system comprising both enzymatic and non-enzymatic processes involving superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), ascorbic acid, reduced glutathione (GSH) and Vitamin E [Kelly et al., 1998; Lushchak, 2011]. In fish, antioxidant enzymes have been shown to be, either induced or inhibited by copper, dependent on the dose, the species and/or the route of exposure [Sanchez et al., 2005]. Variations in these antioxidant defenses can be very sensitive to reveal pro-oxidant conditions and have been used as biomarkers of oxidative stress in fish [Ahmad et al., 2006a; Ahmad et al., 2006b; Oliveira et al., 2008].

A few previous studies have shown that acute exposure of fish to Cu induce an increase in ROS production and subsequently changes in antioxidant enzymatic activities as SOD, CAT and GPx, which eventually may results in oxidative stress and cellular damage [Pedrajas et al., 1995; Roméo et al., 2000; Sanchez et al., 2005; Gravato et al., 2006; Craig et al., 2007; Eyckmans et al., 2011; Machado et al., 2013]. The ability of Cu to induce oxidative stress is directly related to its chemical nature as transition metal with changeable

valence that can catalyze ROS generation through the Fenton-like/Haber–Weiss reactions [Stohs and Bagchi, 1995; Gaetke, 2003; Regoli and Giuliani, 2014]. Furthermore, Cu can also contribute to ROS formation by acting as a cofactor of several enzymes with oxidase activity, as well as through the interference of metal-related processes or by direct inhibition of the components of the antioxidant system [Regoli and Giuliani, 2014; Sanchez et al., 2005].

SOD is considered as the first line of defense against superoxide radical by catalyzing its dismutation to H_2O_2. In this study, SOD activity was highly stimulated by Cu in muscle, gills and liver; however, it was inhibited in brain. These effects were observed at both the 24 and 96 hour exposures, being the increase of enzymatic activity slightly higher at the longer exposure. An adaptive increase in SOD activity has been often described in response to metal-induced oxidative stress [Regoli et al., 2003; Velma and Tchounwou, 2010], and specifically in response to acute Cu treatments. In gilthead seabream (*Sparus aurata)* the peritoneal injection with $CuCl_2$ increased the SOD activity and resulted in appearance of two new Cu,Zn-SOD isoforms [Pedrajas et al., 1995]. Sanchez et al. reported an increase on hepatic SOD activity after 4 days of Cu-exposure (0.025 mg L^{-1}) in stickleback [Sanchez et al., 2005] as well as Craig et al. in zebrafish (*Danio rerio*) after 48 h of Cu exposure (0.008 and 0.015 mg L^{-1}) [Craig et al., 2007]. In studies with brown trout, SOD activity increased after 24 h in liver and after 48 h in gills (0.016 mg L^{-1}) although returned to control levels thereafter [Hansen et al., 2006b]. Finally, the inhibition of SOD activity in the brain is coincident with a previous study reporting an elevation of SOD activity in liver, kidney and gills but a decrease in brain of *Carassius auratus* exposed to Mn for 96 h [Vieira et al., 2012].

CAT activity plays an important role in ROS detoxification and has been previously reported to be stimulated in metal-treated fish [Di Giulio et al., 1993]. In our model of Cu

exposure, the pattern of modulation of the CAT activity was likely dependent on the exposure time. After 24 h, CAT increased in all the organs. However after 96 h, CAT activity increased only in liver while a decrease was significantly detected in gills and muscle. The simultaneous increase of SOD and CAT may occur when productions of both superoxide and peroxide anions are increased up to some level [Machado et al., 2013]. However, if the first effect of Cu exposure is an increase in superoxide radicals, uncoupled reactions of ROS might happen, and CAT activity might be inhibited but not SOD one [Craig et al., 2007; Kono and Fridovich, 1982]. Both behaviors have been found in the literature. Thus, CAT activity was stimulated in the liver of the estuarine guppy *Poecilia vivipara* [Machado et al., 2013] as well as for three-spined stickleback after 4 days of Cu exposure as low as 0.025 mg L^{-1} [Sanchez et al., 2005]. A CAT inhibition has been described in liver of *D. Labrax* exposed to mercury and urban pollution [Maria et al., 2009] and in gills of zebrafish after 48 h of 0.015 mg L^{-1} Cu exposure [Craig et al., 2007], being considered the inhibitory response as a signal of contamination. And finally, the response of CAT to Cu in tilapia (*Oreochoromis niloticus)*, was inhibited and stimulated after 96 h depending on the tissue and the metal exposure, being significantly increased in liver at 0.1 and 1 mg L^{-1}, but in gills and brain it was increased at higher concentrations (0.5, 1, 1.5 mg L^{-1}; and 1, 1.5 mg L^{-1}, respectively) and decreased at lower concentrations (0.1 mg L^{-1}; and 0.1, 0.5 mg L^{-1}, respectively) [Atli et al., 2006]. Otherwise, no significant stimulation of CAT activity could be observed in liver, gills or muscle of pale chub (*Zacco platypus)* after 96 h Cu exposure up to 0.02 mg L^{-1} [Kim et al., 2014].

GPx is a selenium-containing enzyme that catalyzes the reduction of both hydrogen peroxide and organic peroxides. Both this function and its widespread cellular distribution point to GPx as an important factor of the antioxidant defense system against oxidative damage in animal cells [Li et al., 1999] and particularly in fish [Van der Oost et al., 2003;

Oliva et al., 2012]. However, as deduced from the literature revised, it has been often omitted when addressing the oxidative effects of Cu in fish. The increase of the GPx activity can be related to the effect of Cu exposure to oxidize membrane lipids in fish, and its function over the detoxification of lipoperoxidation products counteracting its stimulation [Sanchez et al., 2005]; thus, GPx plays a key role in protecting membranes against LPO. In this paper, GPx was the only activity being stimulated in all the organs after both 24 and 96 h treatment, which emphasizes its key role in the defense against Cu-induced oxidative stress in *D. labrax*. GPx activity resulted drastically increased in gills and liver while the muscle and brain showed lower but significant rates of induction. The coupled induction of CAT and GPx frequently occur under low stress, but at severe oxidation conditions an increase of GPx and depletion of CAT may be produced [Regoli and Giuliani, 2014], as showed in our studies after 96 h in gills and muscle. In the same way, a slight GPx induction versus CAT decrease in liver of *D. Labrax* affected by some contaminants as mercury has been reported [Maria et al., 2009]. By contrast, Sanchez et al. did not found enhanced hepatic GPx activity until day 8 of exposure to 0.2 mg L^{-1} in stickleback [Sanchez et al., 2005].

LPO is a valuable indicator of oxidative damage of cellular components [Monteiro et al., 2010]. In our study, the hydroperoxides levels were used as a biomarker of ROS-induced cellular injury. They were significantly increased in all the organs by Cu effect at both 24 and 96 h exposure time, outstanding the gills response even at lower metal concentration. Similarly, the acute waterborne copper exposure of guppy *Poecilia vivipara* resulted in the stimulation of lipid peroxidation in liver, gills and, in a lesser extent, in muscle after 96 h of 0.005-0.02 mg L^{-1} Cu exposure in salt water [Machado et al., 2013]. LPO also occurred at significant levels in liver but not in gills of pale chub by 0.02 mg L^{-1} Cu after the same exposure time [Kim et al., 2014]. Taking together our results related to oxidative stress it

can be assumed that in the short term a generalized state of oxidative stress was induced by Cu since the antioxidative response displayed was not sufficiently efficient to avoid oxidative injury to cellular biomolecules, as deduced from the enhanced levels of LPO. Furthermore, a differential response was observed among the studied organs. Gills appeared to be the most sensitive, showing the highest levels of stimulation of lipid peroxidation and antioxidative enzymes; and mainly from low environmentally relevant Cu concentrations as 0.01 mg L^{-1}. This may be explained because, as deduced from their anatomical and physiological characteristics, this organ shows the higher contact with the waterborne metal, which is especially important in short-term exposures. It is noteworthy that this is the only organ showing a significant increase of Cu content throughout the experimental period. It is also important to state that even though no significant Cu accumulation was detected in the liver, muscle or brain, they showed significant effects related to oxidative stress in the manner as in gills for 0.01 mg L^{-1} Cu dose. Importantly, they showed a response clearly dependent on copper concentration in the water. Therefore, the evaluation of these parameters may provide early reliable biomarkers of waterborne Cu exposure, even before the detection of Cu accumulation. The assessment of peroxidative damage may be considered of particular interest because regardless of the up or down regulation of enzymatic activities or other elements involved in antioxidant defense, it denotes the occurrence of a cellular damage that may be responsible of toxic deleterious effects in fish. Interestingly, the levels of hydroperoxides and GPx activity, the most related antioxidative activity, were dramatically enhanced in all the organs from the 0.1 mg L^{-1} Cu exposure.

Since cellular oxidative damage, as assessed by the levels of hydroperoxides, showed an increasing trend with the water metal concentration, it could be assumed that a higher levels of oxidative stress might have contributed to the high mortality rate observed at the

higher Cu concentration; though other injuries not directly associated with the oxidative stress cannot be excluded. In this regard, the failure of gills appears to be of particular importance since it may lead to alterations in key functions as osmoregulation and gas exchange and, eventually, to death. A causal relationship between the amount of metal accumulated in gills and lethal tolerance for copper was described by Anderson and Spear [Anderson and Spear, 1980]. It has been suggested that the mechanisms for Cu toxicity in fish occurs mainly at gills through the competition of Cu for Na^+ at apical Na^+ channels and the eventual inhibition of normal Na^+ uptake in freshwater ecosystems [Craig et al., 2007]; in seawater the interference of Cu affects the active transport mechanisms of Na^+ and Cl^- extrusion by branchial epithelium [Evans, 1987]. Thus, alterations in blood ionic levels, as well as gills Na,K-activated ATPase activity and ionic fluxes in this tissue can take place in fish.

In this study, Cu exposure of *D. labrax* juveniles after 24 and 96 h resulted in histopathological alterations in gills and liver. Previous studies have shown that the exposure of fish to copper treatments ranging from 0.01 to 1 mg L^{-1} is associated with structural damages in gills [Arellano et al., 1999; Ortiz et al., 1999; Cerqueira and Fernandes, 2002; Oliva et al., 2009; Oliva et al., 2013]. In our study, aneurysms (A), hyperplasia of the lamellar epithelia (HPL) and lamellar fusion (LF) were the histopathological lesions with the higher prevalence percentage observed in gills, outstanding HPL in all studied conditions even from the lowest 0.01 mg L^{-1} Cu sublethal concentration. Pathologies such as hypertrophy of the lamellar epithelia (HPT), lamellar shortening (LS), desquamation of lamellar epithelia (D) and epithelial lifting (EL) were also observed, although with lower prevalence percentage (Table 5). Among them, positive correlations were established between Cu concentrations in water and gill aneurysms at 24 h exposure, as well as between Cu concentration in gills and the observation of gill

aneurysms at 24 and 96 h exposure. All these epithelial changes appear to be related to an inflammatory response of the tissue as defense mechanism and may lead to irreparable damage of gill morphology and function and even to fish mortality [Cerqueira and Fernandes, 2002]. In a similar model of acute Cu exposure, lifting and peeling of the lamellar epithelia as well as lamellar fusion, hyperplasia and aneurysms [Arellano et al., 1999; Oliva et al., 2009] were observed in Senegalese sole (*Solea senegalensis)* at 0.1 mg L^{-1} Cu; whereas in curimba (*Prochilodus scrofa)* gills damage after 96 h to 0.03 mg L^{-1} was characterized by epithelial lifting, hypertrophy of pavement, cell swelling, chloride and mucous cell proliferation [Cerqueira and Fernandes, 2002]. A subchronic exposure of mummichog (*Fundulus heteroclitus)* to 0.8 mg L^{-1} of copper produced desquamation of gill epithelia, lamellar telangiectasis, as well as necrosis after 30 days [Ortiz et al.,1999]. In a field study over a polluted estuary, copper concentrations in seawater correlated with lifting and desquamation of lamellar epithelia in Senegalese sole gills, with a higher lesion grade for aneurysms and hyperplasia [Oliva et al., 2013].

Furthermore, in this study *D. labrax* showed severe histopathological changes in the liver related to Cu concentrations and exposure time. These included multivesicular steatosis (S), vacuolization (V), atrophy (AT), eosinophil granules (EG), necrosis (N), blood stagnation (BS) and hepatic parenchyma disorganization (HPD) (Table 5). Interestingly, some of them (S, AT, N, BS and HPD) were already evidenced from the experimental dose of 0.01 mg L^{-1} after 96 h with a prevalence percentage of ≥62.5%; however vacuolization (V) was only significant for the higher non-lethal doses. Steatosis (S) was outstanding in all conditions and could indicate a failure of lipid metabolism; atrophy (AT), blood stagnation (BS) and hepatic parenchyma disorganization (HPD) had a similar behavior except at the lowest non-lethal dose after 24 h, and with several moderate alterations (grade 2 or grade 2.5) specially for atrophy. Positive correlations were established between

Cu concentrations in water at 24 h exposure and S, V, AT, HPD and EF; at 96 h exposure, positive correlations were established only between Cu concentrations and S, AT and N. Similarly, certain disorganization of hepatic parenchyma and signs of vascular degeneration was observed in Senegalese sole exposed to at 0.01 mg L^{-1} Cu after 96 h, while more serious injuries including necrotic zones, a disorganization of total hepatic parenchyma, blood stagnation, and expansion of the capillaries were observed at 0.1 and 1 mg L^{-1} Cu [Oliva et al., 2009]. Arellano et al. observed ultrastructural alterations such as partial disruption of the microvilli and endothelial lining of the sinusoids, steatosis and blood stagnation of Senegalese sole hepatocytes exposed to 0.1 mg L^{-1} Cu after 7 days [Arellano et al., 1999]; in contrast to our results, these effects were associated with increasing hepatic Cu concentrations. Massive hepatic necrosis was observed in liver of tench (*Tinca tinca*) exposed for 12 days to 75 mg L^{-1} Cu, probably after the vacuolar degeneration caused by the alteration of membrane structural lipid [Roncero et al., 1992]. Mummichog fish subchronically exposed to 0.8 mg L^{-1} Cu for 30 days also showed hepatic necrosis and vacuolization of hepatic parenchyma. Similarly to our study, Paris-Palacios et al. [Paris-Palacios et al., 2000] evaluated both biochemical and structural perturbations in zebrafish (*Brachydanio rerio)* exposed for 14 days to sublethal concentrations of 0.04 and 0.14 mg L^{-1} Cu and found that the liver developed large lysed areas and hepatocytic alterations in reference to slightly Cu accumulation. Interestingly, this was associated with increase in hepatic anti-oxidative defenses, including glutathione content, catalase and glutathione-*S*-transferase (GST) activities; which is in accordance with the enhanced antioxidative SOD, CAT and GPx activities reported in our studies in liver.

Several studies have shown enhanced lipid peroxidation in aquatic organisms exposed to high concentrations of metals, with higher levels in liver, gills and muscle [Monteiro et al., 2010; Carvalho et al., 2012] and, notably, a causal association between lipid peroxidation

and histopathological alterations was also noted [Abdel-Moneim et al., 2013; Kaptaner et al., 2014]. In fish from contaminated sites of Nile delta, liver necrosis associated with lipid peroxidation, which in turn correlated with the high mortality observed at the higher Cu concentrations [Abdel-Moneim et al., 2013]. Furthermore, the significant gills response described in our study for LPO values could also be associated with the histopathological alterations found such as aneurysms (A), hyperplasia of lamellar epithelia (HPL) and lamellar fusion (LF). Interestingly, a similar association between lipid peroxidation and histopathological alterations was also described by Abdel-Moneim et al. [Abdel-Moneim et al., 2013] for hyperplasia and hypertrophy of the respiratory epithelium, lamellar fusion and aneurysms in gills.

The IPAT index **[**Bernet et al., 1999] corresponding to each non-lethal copper dose and exposure time was evaluated to summarize the impact of the histopathological alterations on health fish (Table 6). The prevalence percentages of lesions were highly Cu dose-dependent, although the maximum value of IPAT index only showed a slightly moderate impact on health fish (20.1) at 0.1 mg L^{-1} Cu after 96 h. However, data obtained predict that copper concentrations ranging between 1 (after 96 h) and 10 mg L^{-1} (after 24 h) would yield IPAT indexes with a moderate/high impact, causing the mortality observed in these experiments and for which the indexes could not be evaluated. Thus, the importance of the histopathological alterations described in our study at the very short time with the sublethal doses must not be discarded, because they might derive in severe physiological and life-threatening dysfunctions at a longer term.

5. CONCLUSIONS

The finding of this study showed that sublethal nominal concentrations of Cu as low as 0.01 mg L^{-1} (average measured Cu concentration of 0.009±0.001 mg L^{-1}) were able to induce oxidative damage principally in gills as well as histopathological alterations in liver and gills, even without a significant accumulation of metal. Although these parameters could return afterward to normal values through an acclimation process, they might lead to permanent deleterious effects on fish physiology, and subsequently on their health. To appreciate the significance of these results in the context of the environmental setting, it must be considered that this Cu concentration is excessively close to EQS defined by US EPA in salt water (4.8 μg L^{-1} of dissolved Cu) at acute conditions (CMC) and EU WFD in transitional and coastal water with DOC > 1 mg L^{-1} (3.76 + (2.677 × ((DOC/2) - 0.5)) μg L^{-1} dissolved Cu) [US EPA, 2016; EU WFD, 2015]. Hence, this might call for environmental protection agencies to consider more conservative standards for seawater Cu. Furthermore, particular attention must be paid to the use of Cu levels as high as 2.0 mg L^{-1} used as algaecide to control aquatic vegetation in fish culture systems [Paris-Palacios et al., 2000; Jančula and Marsalek, 2011]. On the other hand, our results also highlight *D. labrax* as particularly sensitive to Cu toxicity and therefore as a good species for biomonitoring purposes. But it also advocates for a high control of Cu levels during aquaculture practices of this species and for the protection of fish populations living in sites with very high Cu concentrations, i.e. in a natural habitat of *D. Labrax* between the Atlantic Ocean and the Mediterranean Sea, where Cu reached up to 0.046 mg L^{-1} of dissolved Cu with 3.7 mg L^{-1} of DOC average [Vicente-Martorell et al., 2009].

In summary, the results of this comprehensive study suit with the initial hypothesis that the exposure to waterborne Cu induces oxidative stress and a differential, enzymatic-dependent antioxidant response in different organs of *D. labrax*. In addition, histopathological perturbations related to Cu exposure were also found to occur in gills and

liver. These effects were rapidly observed from very low concentrations of 0.01 mg L^{-1} of copper and even before metal accumulation were evidenced. Therefore, the combination of oxidative stress parameters, particularly LPO and GPx activity, and histological alterations in gills and liver of this species might provide a good model fish and reliable early biomarkers for monitoring Cu pollution in seawater.

ACKNOWLEDGEMENTS

This work was supported by the "Ministerio de Ciencia e Innovación" (Project CTM2010-17474) and the "Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía" (Project RNM-6641). The authors would like to extend their gratitude to the director Rosa Vázquez Gómez and the workers of the Laboratory of Marine Cultures, Faculty of Marine and Environmental Sciences (University of Cadiz, Spain) for technical assistance.

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Table 1. Average values of physical-chemical parameters in water during the exposure tests [a]

$[Cu]_{Nominal}$ ($mg\ L^{-1}$)	T (ºC)	pH	$O_{2\ dissolved}$ ($mg\ L^{-1}$)	Salinity ($g\ L^{-1}$)	Suspended Solids ($mg\ L^{-1}$)
Control	21.2±1.1	8.1±0.2	7.13±1.21	38.40±0.89	28.74±0.61
0.01	20.6±0.4	8.1±0.3	7.38±0.31	39.11±0.28	29.22±0.19
0.1	20.6±0.4	7.9±0.2	7.33±0.24	39.13±0.29	29.24±0.18
1	20.5±0.6	8.0±0.2	7.24±0.40	38.88±0.03	29.07±0.03
10	20.6±0.5	8.1±0.4	7.29±0.37	38.61±0.14	28.90±0.08

[a] Measurements at 0, 24 and 96 h from two replicate tanks

Table 2. Dissolved organic carbon (DOC) in water from the exposure tests

$[Cu]_{Nominal}$ (mg L^{-1})	DOC (mg L^{-1})		
	0 h	24 h	96 h
Control	4.71±0.19 [a]	6.13±0.92	8.04±2.04
0.01	"	6.12±0.10	7.63±0.22
0.1	"	6.07±0.19	7.35±0.94
1	"	7.47±0.51	[b]
10	"	9.71±1.50	[b]

[a] DOC was the same for all water samples at 0 h

[b] No DOC measures because fish died

Table 3. Actual copper concentrations in water from the exposure tests

$[Cu]_{Nominal}$ (mg L^{-1})	$[Cu]_{Measured}$ [a] (mg L^{-1})		
	0 h	24 h	96 h
Control	0.0018±0.0002	0.0072±0.0001	0.0061±0.0003
0.01	0.009±0.001	0.0103±0.0004	0.0088±0.0003
0.1	0.098±0.006	0.089±0.005	0.090±0.002
1	0.957±0.015	0.999±0.007	0.557±0.008
10	10.46±0.18	3.552±0.023	3.972±0.067

[a] Mean concentrations of metal in water from two replicate tanks and three measures for each one; control, 0.01 and 0.1 mg L^{-1} Cu samples were measured by DPASV; 1 and 10 mg L^{-1} Cu samples were measured by ICP-AES

Table 4. Analytical quality control data for copper analysis in water and fish tissues (n=10 for LOD; n=3 for analysis of reference material)

Sample	Reference material	Method	LOD of method ($\mu g\ L^{-1}$)	Blank concentration ($\mu g\ L^{-1}$)	Found concentration ($\mu g\ L^{-1}$)	Certified concentration ($\mu g\ L^{-1}$)
Water	LGC 6016	DPASV	0.069	1.944	189.3 ± 3.2	190.0 ± 2.0
		ICP-AES	6.3	<LD	189.3 ± 3.8	190.0 ± 2.0
Sample	Reference material	Method	LOD of method ($\mu g\ L^{-1}$)	Blank concentration ($\mu g\ L^{-1}$)	Found concentration ($mg\ kg^{-1}$)	Certified concentration ($mg\ kg^{-1}$)
Muscle [a]	DORM-2 (dogfish muscle)	ICP-MS	0.049	0.142	2.23 ± 0.10	2.34 ± 0.16
Liver [b]	DOLT-3 (dogfish liver)	ICP-MS	0.004	0.023	31.7 ± 0.6	31.2 ± 1.0

[a] 0.2 g of sample digested with 7 mL 65% HNO_3

[b] 0.2 g of sample digested with 4 mL 65% HNO_3 and 2 mL 30% H_2O_2

Table 5. Prevalence percentage of pathologies and average lesion grade (Alg_i in parenthesis) in gills and liver of *D. labrax* exposed to copper [a]

[Cu] $_{Nominal}$ (mg L^{-1})	Time (h)	Prevalence percentage/(Alg_i) in Gills							Prevalence percentage/(Alg_i) in Liver						
		A	HPT	HPL	LS	LF	D	EL	S	V	AT	EG	N	BS	HPD
0.01	24	12.5 (0.5)	12.5 (1)	87.5 (1.5)	12.5 (1)	50 (1)	50 (2)	25 (3)	62.5 (1)	12.5 (1.5)	25 (1)	25 (1)	0	50 (2)	25 (1)
	96	50 (0.5)	37.5 (1)	75 (1)	37.5 (1)	75 (1)	62.5 (1)	50 (1)	62.5 (2)	37.5 (1.5)	75 (2)	50 (1)	62.5 (1)	62.5 (1)	75 (2)
0.1	24	62.5 (1)	37.5 (1)	87.5 (1.5)	50 (1)	62.5 (1)	75 (1)	25 (1.5)	100 (2)	37.5 (1)	62.5 (1)	12.5 (1)	0	62.5 (1.5)	50 (1.5)
	96	62.5 (1)	50 (1)	87.5 (1.5)	50 (1.5)	62.5 (1)	37.5 (1)	12.5 (0.5)	100 (2)	62.5 (1.5)	87.5 (2.5)	12.5 (1)	62.5 (1)	87.5 (1.5)	100 (2)
1	24	62.5 (1)	25 (1)	87.5 (1.5)	62.5 (1)	62.5 (1.5)	12.5 (0.5)	12.5 (1)	100 (1.5)	87.5 (1.5)	62.5 (2)	87.5 (1)	12.5 (0.5)	100 (1.5)	87.5 (1.5)

[a] *Lesion grade criteria*: grade 0 (no alteration), grade 1 (focal mild alteration), grade 2 (moderate alteration) and grade 3 (extended severe alteration). *Lesions in gills*: A (aneurysm), HPT (hypertrophy of lamellar epithelia), HPL (hyperplasia of lamellar epithelia), LS (lamellar shortening), LF (lamellar fusion), D (desquamation of lamellar epithelia), EL (epithelial lifting). *Lesions in liver*: S (steatosis), V (vacuolization), AT (atrophy), EG (eosinophil granules), N (necrosis), BS (blood stagnation), HPD (hepatic parenchyma disorganization).

Table 6. IPAT index in *D. labrax* for different copper concentrations and time exposure [a]

[Cu] $_{Nominal}$ (mg L^{-1})	Time (h)	Partial values for Gills							Partial values for Liver							IPAT
		A (1)	HPT (1)	HPL (2)	LS (1)	LF (1)	D (1)	EL (1)	S (1)	V (3)	AT (2)	EG (1)	N (3)	BS (1)	HPD (1)	
0.01	24	6.25	12.5	262.5	12.5	50	100	75	62.5	56.25	50	25	0	100	25	8.4
	96	25	37.5	150	37.5	75	62.5	50	125	168.75	300	50	187.5	62.5	150	14.8
0.1	24	62.5	37.5	262.5	50	62.5	75	37.5	200	112.5	125	12.5	0	93.75	75	12.1
	96	62.5	50	262.5	75	62.5	37.5	6.25	200	281.25	437.5	12.5	187.5	131.25	200	20.1
1	24	62.5	25	262.5	62.5	93.75	6.25	12.5	150	393.75	250	87.5	18.75	150	131.25	17.1

[a] Partial values were obtained as each average lesion grade multiplied by its prevalence and condition weight (w_i) (values in parenthesis). *Lesions in gills*: A (aneurysm), HPT (hypertrophy of lamellar epithelia), HPL (hyperplasia of lamellar epithelia), LS (lamellar shortening), LF (lamellar fusion), D (desquamation of lamellar epithelia), EL (epithelial lifting). *Lesions in liver*: S (steatosis), V (vacuolization), AT (atrophy), EG (eosinophil granules), N (necrosis), BS (blood stagnation), HPD (hepatic parenchyma disorganization).

Figure(s)

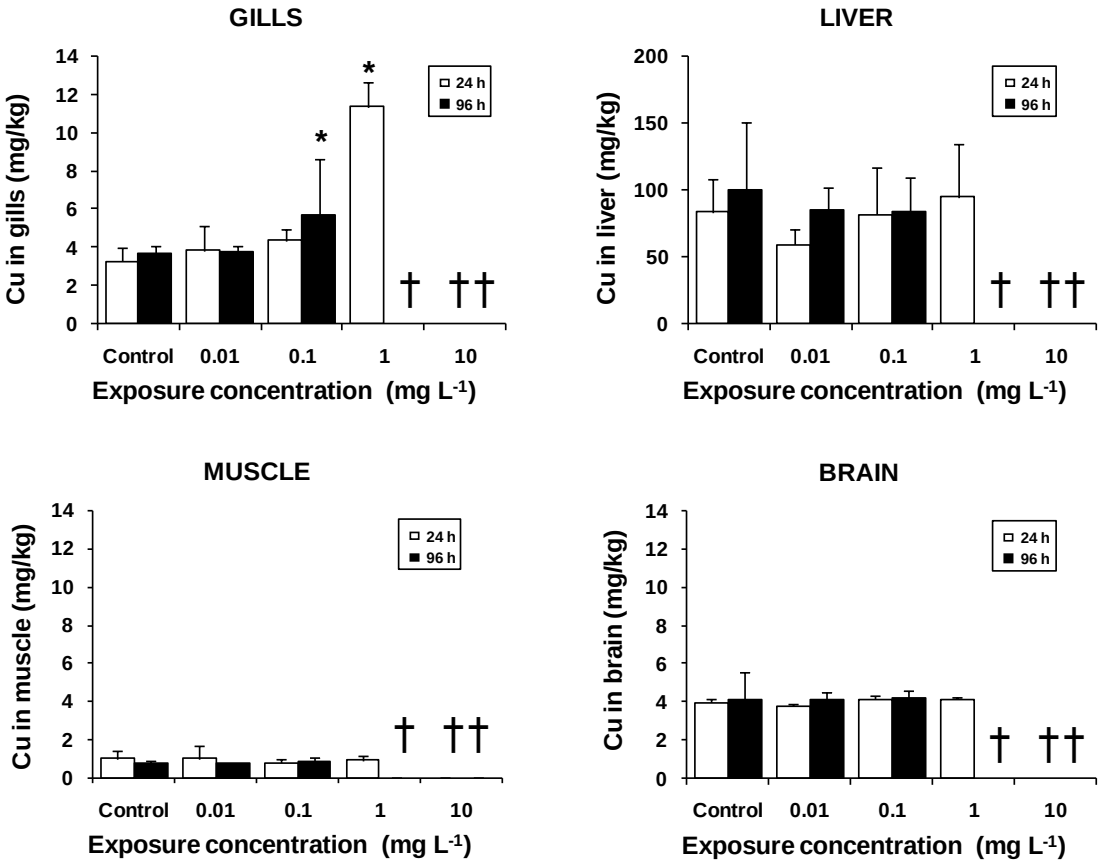

Figure 1. Mean concentrations of Cu in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (full mortality was recorded for 10 mg L^{-1} Cu after 24 and 96 h and for 1 mg L^{-1} Cu after 96 h, denoted as dagger; data are shown as mean ± SD)

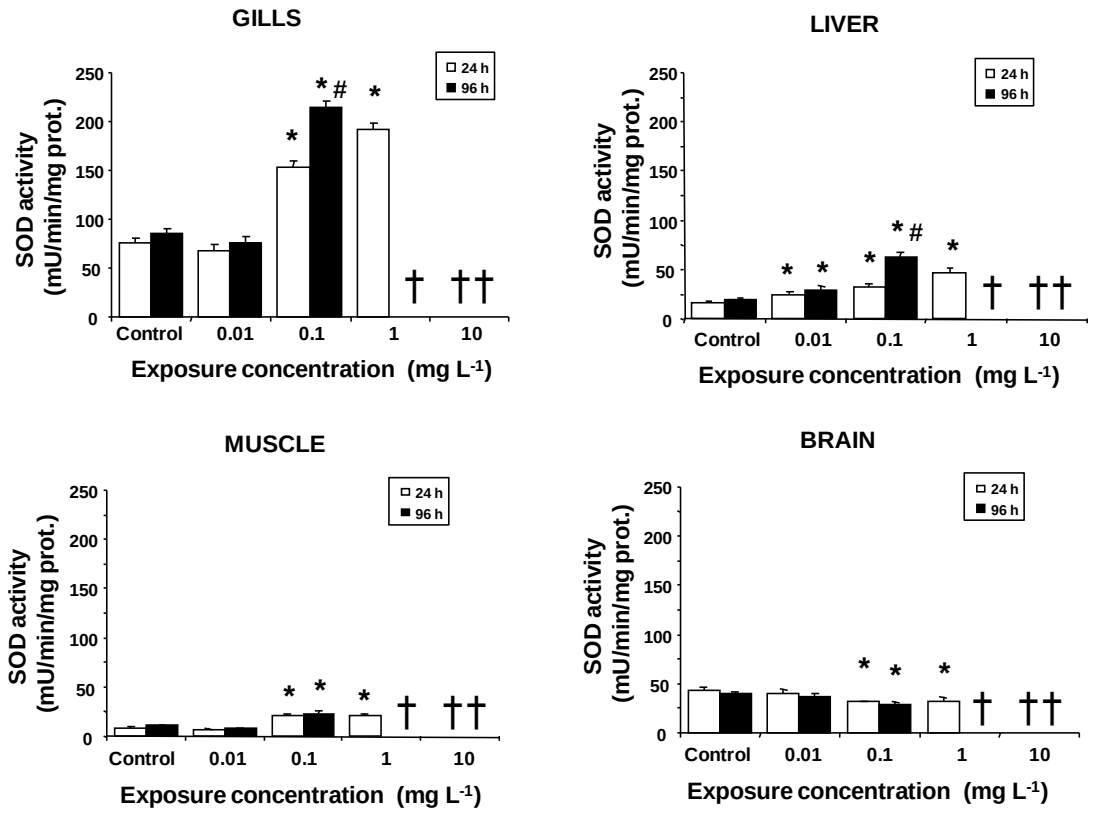

Figure 2. Mean values of SOD activity in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

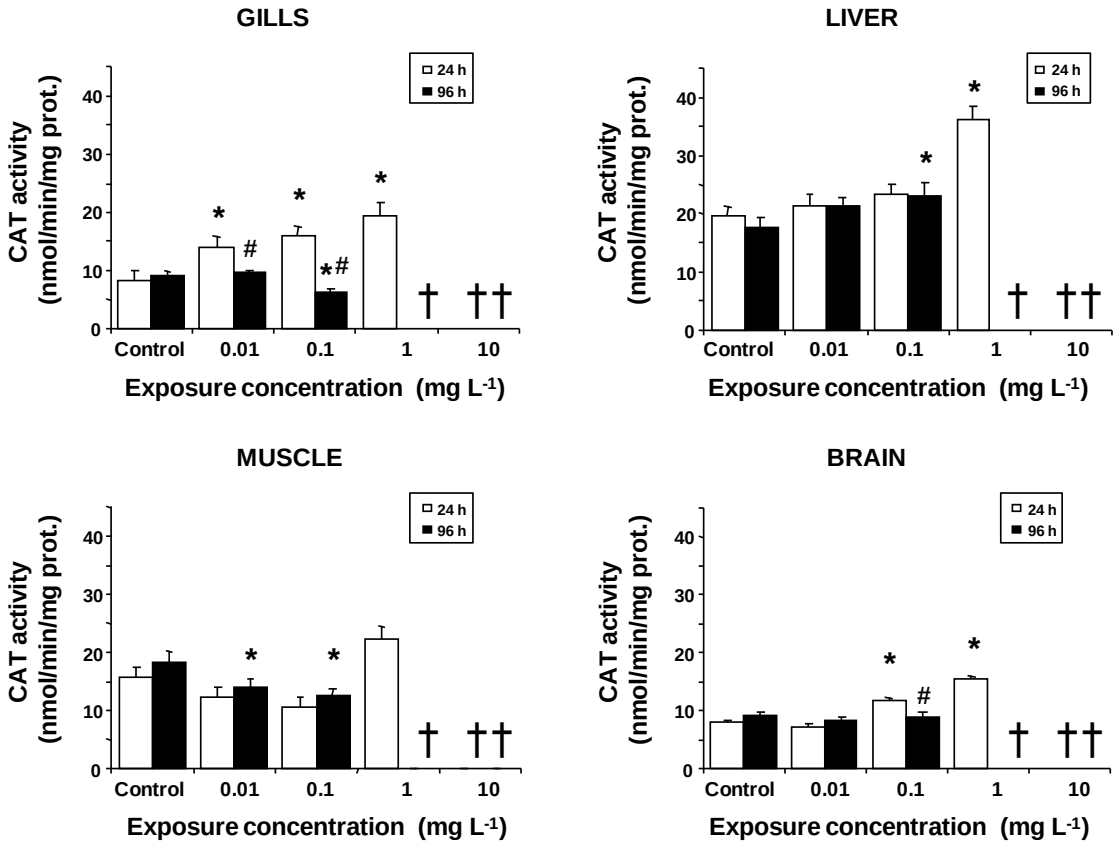

Figure 3. Mean values of CAT activity in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

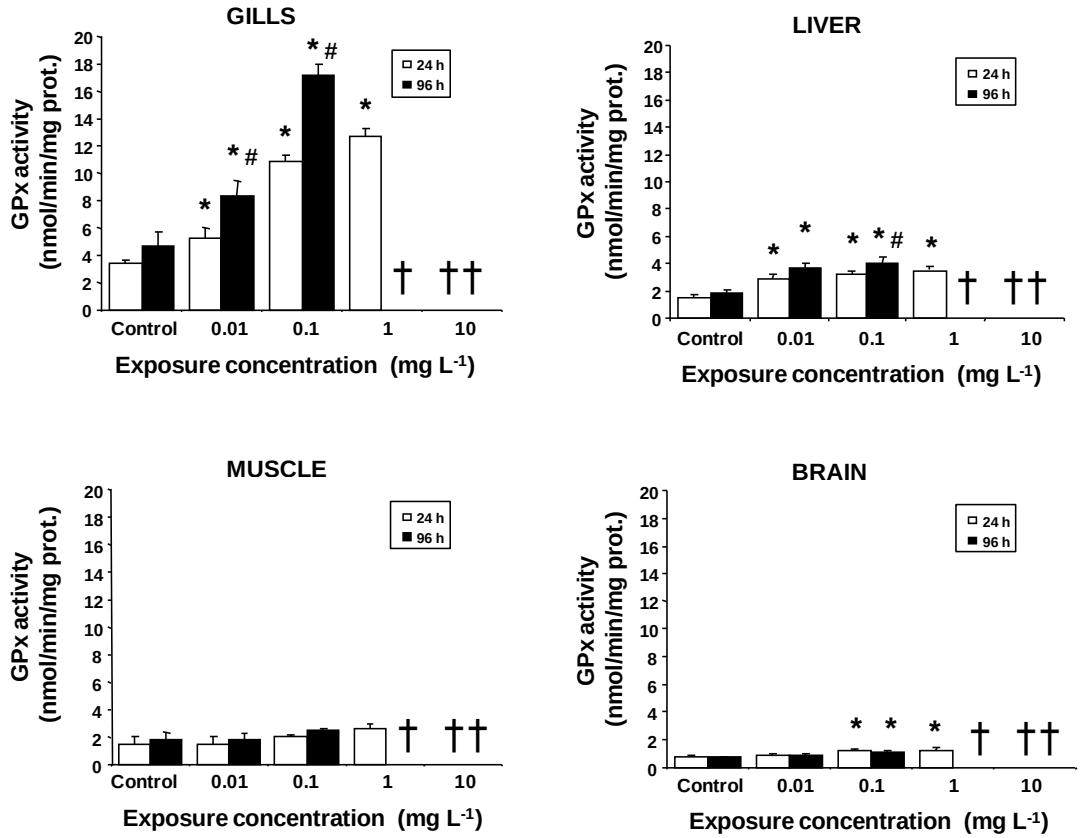

Figure 4. Mean values of GPx activity in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

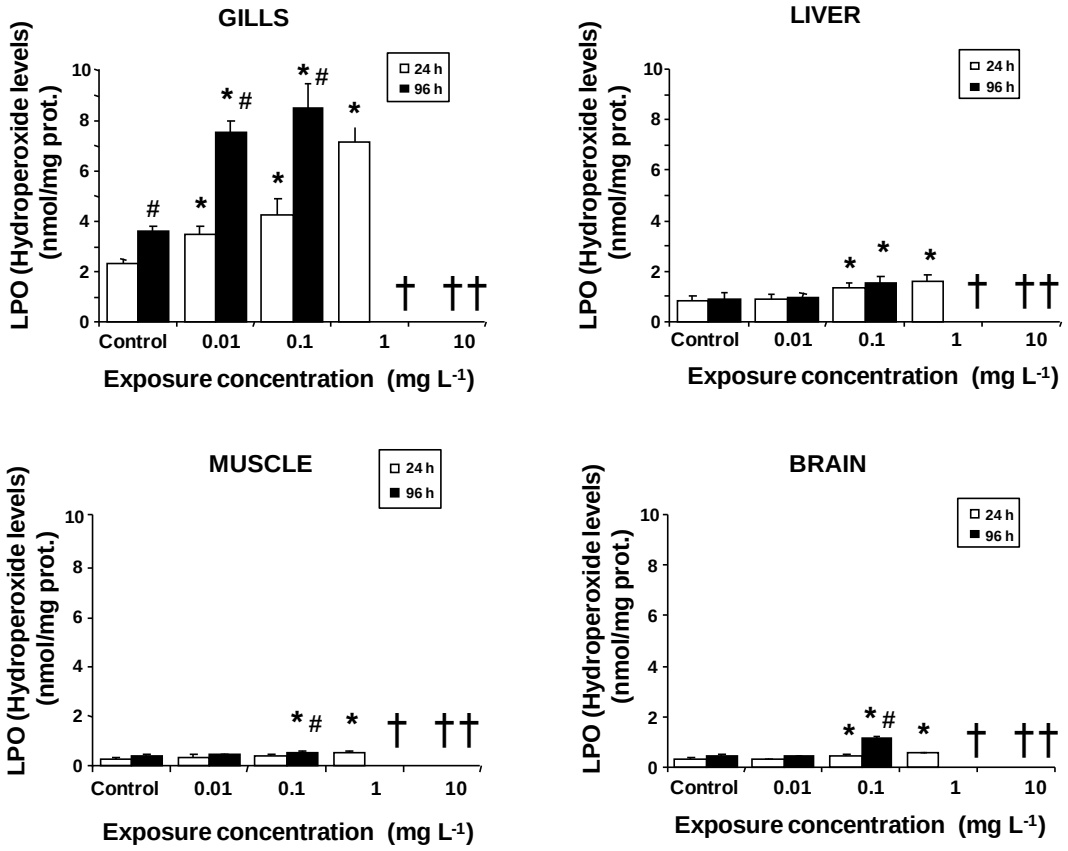

Figure 5. Mean values of LPO in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

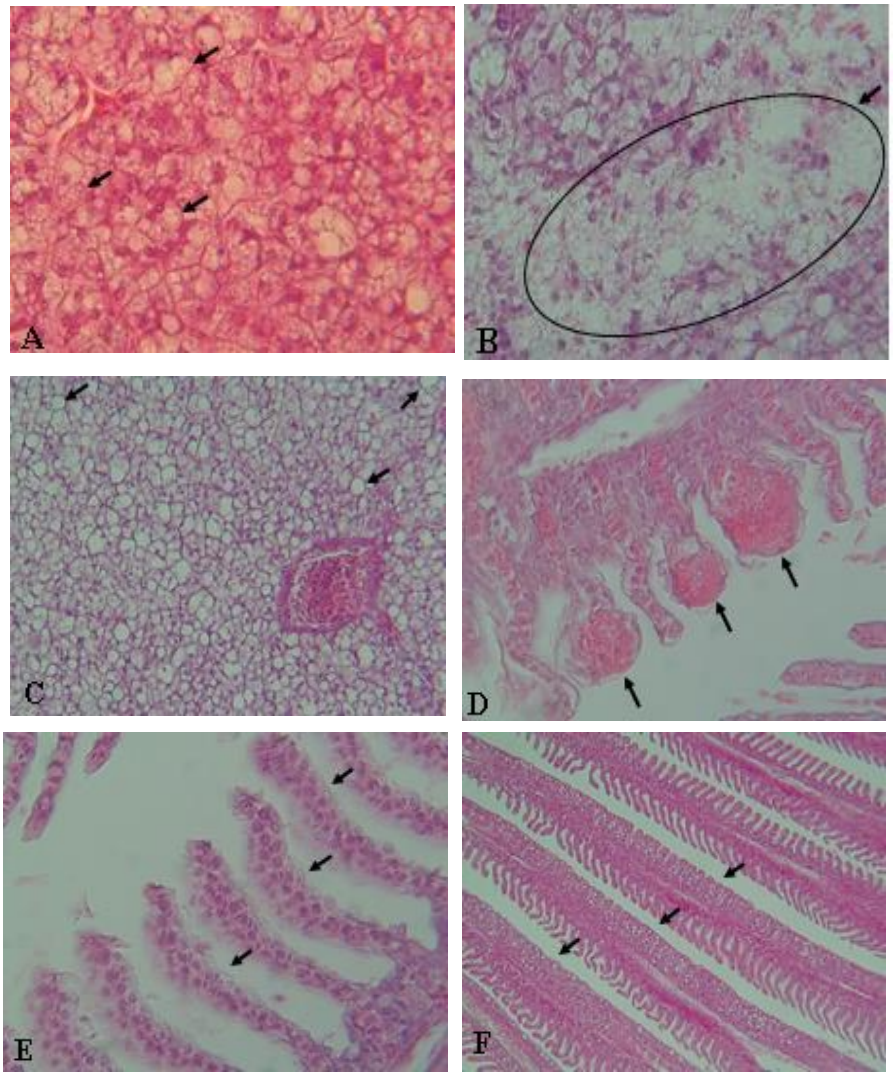

Figure 6. Histopathological alterations in tissues of *D. labrax* after exposure to Cu. In liver: A) steatosis H/E (x25); B) necrosis H/E (x25); C) vacuolization H/E (x10). In gills: D) aneurysm H/E (x25); E) hyperplasia of lamellar epithelia H/E (x25); F) lamellar fusion (LF) and shortening H/E (x4)

FIGURE CAPTIONS

Figure 1. Mean concentrations of Cu in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (full mortality was recorded for 10 mg L^{-1} Cu after 24 and 96 h and for 1 mg L^{-1} Cu after 96 h, denoted as dagger; data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

Figure 2. Mean values of SOD activity in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

Figure 3. Mean values of CAT activity in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

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Figure 5. Mean values of LPO in fish tissues of *D. labrax* after 24 and 96 h exposure to Cu (data are shown as mean ± SD). Significant differences are as follows: (*) $p<0.05$ vs. control; (#) $p<0.05$ vs. 24 h

Figure 6. Histopathological alterations in tissues of *D. labrax* after 96 h exposure to 0.1 mg L^{-1} Cu (H/E staining). In liver: A) steatosis (x25); B) necrosis (x25); C) vacuolization (x10). In gills: D) aneurysm (x25); E) hyperplasia of lamellar epithelia (x25); F) lamellar fusion and shortening (x4)