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Biochemical and histological changes in the liver and gill of Nile tilapia *Oreochromis niloticus* exposed to Red 195 Dye

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Abstract

The present study investigates the biochemical and morphological responses induced in the liver and gills of Nile tilapia *Oreochromis niloticus* by exposure to various Red 195 dye concentrations (0.05, 0.1 and 0.2 mg/L) for various durations (7, 14 and 21 days). The histology and antioxidant activities of catalase (CAT), glutathione reductase (GR) and glutathione S-transferase (GST) were monitored and evaluated during exposure and after recovery in clean water. The results revealed that CAT activity decreased after 7 and 21 days of exposure to 0.2 mg/L concentrations and increased only after 7 days of exposure to high concentrations compared to the liver and gill control samples, respectively. Both organs were, however, noted to undergo a decrease in GST activity after 7 days of exposure to low Red 195 dye concentrations. Compared to the control, the gills of the tilapia exposed to 0.05 and 0.1 mg/L concentrations for longer periods underwent an increase in GST activity. Similarly, GR activity was higher in the liver of tilapia exposed to this dye at the sampling days, except for the highest concentration (0.2 mg/L) after 21 days of exposure. The GR activity in the gills decreased significantly after 7 and 21 days of exposure to 0.05 and 0.1 mg/L concentrations, respectively. The results of the recovery group revealed that the liver and gills displayed insignificant differences in antioxidant enzyme activities. The liver and gill tissues of the fish exposed to Red 195 showed several histopathological changes. Liver damages included an increase in cytoplasmic vacuolization, disruption of endothelial lining, metabolic zonation, necrotic cells, rupture of hepatocytes membrane, and decline of higher eosinophilia. The gills also exhibited some necrotic cells, edema, lifting of filament and lamellar epithelium, and vascular disorders, such as extreme vasodilatation and proliferation of filament epithelium. A correlation between the biochemical and histological changes of the

liver and gill tissues was established, attributing the tissue and cell damages to the accumulation of hydrogen peroxide or production of other radicals via a fenton reaction.

Keywords: Oxidative stress; Tilapia; Red 195 Dye; Liver; Gill; Histopathology.

1. Introduction

Increasing concerns have been expressesed over the serious environmental and health effects associated with the growing amounts of toxic contaminants from domestic, industrial and agricultural activities released into the aquatic environment worldwide. Synthetic dyes represent one of the major groups of toxic compounds used in various industries, including the textile and leather processing industries.[1,2] The indiscriminate discharge of synthetic dyes into aquatic systems can cause adverse effects on marine ecosystems and human life. Those toxic contaminants can accumulate in various aquatic organisms, including fish, which constiute an important component of human nutrition, thus presenting serious risks to human health.. Reactive Red 195 azo dye is characterized by having one or more azo bonds (-N=N), bound to substitutable aromatic rings. The latter rings of these dyes are responsible for intense colour, water solubility and degradation resistance under conventional wastewater treatment[3], since some of these azo dyes and their metabolites can be mutagens or carcinogens.[4]

Due to its low-cost and ease of use compared to natural dyes, Red 195, , has become one of the most widely used dyes in various industries worldwide, including the Tunisian textile industry. The wastewaters carrying this dye present strong color and high COD values since about 10-15% of dyes are dispersed in the effluent during the dyeing process, thus posing devastating effects on the ecological balance of the recipient environment and human health.[5] In fact, several studies carried out in Tunisia have demonstrated the mutagenic and

genotoxic effects of various dyes, including acid violet 7, acid yellow 17, and acid orange 52.[6,7,8,9] Several works have also studied the effects of different dyes on fish samples.[10,11,12] Nevertheless, little work has been performed to investigate the effects of Red 195 dye on the biochemical and morphological responses of fish.

Fish have been widely used as models to evaluate the health of aquatic ecosystems, biomonitor environmental stress, and identify possible ways to mitigate and prevent the effects of pollution.[12] Liver plays an important role in vital functions of basic metabolism[13] and represents the major organ for the accumulation, biotransformation and excretion of contaminants in fish.[14] The gills are the main targets of direct contamination, as they play a significant role in metal uptake, storage and eventually, transfer to the internal compartments via blood transport.[15] The study of their biochemical and histological changes has provided valuable insights for the monitoring of fish exposure to contaminants in laboratory and field studies.[16] The exposure to contaminants in aquatic ecosystems can enhance the intracellular formation of reactive oxygen species (ROS), which can bring oxidative stress and damage to various biological systems.[17,18] Oxidative stress occurs as an outcome of imbalance between ROS generation and elimination. ROS can be detoxified by enzyme defense systems, including superoxide dismutase (SOD), catalase (CAT) and selenium-dependent glutathione peroxidase (GPx), or by non-enzymatic systems through the scavenging action of reduced glutathione (GSH) and several other molecules, such as vitamins C, A and E. Organic peroxides, on the other hand, can be detoxified by the activity of glutathione *S*-transferase (GST).[19] Several studies showed that changes in the levels of antioxidant enzyme activities could be used as possible biomarkers in different aquatic organisms.[20,21,22]

. Nile tilapia, *Oreochromis niloticus*, has commonly used as a target biological model for the study of xenobiotic biotransformation in toxicology studies. This poular freshwater

finfish species belonging to the cichlid family has several distinctive features that support its suitability for use as a model species for the assessment of aquatic pollution. It is kown by its high growth rates, distinct ability to adapt to diverse diets, great resistance to diseases and handling practices, easy reproduction in captivity and prolific rate of breeding and multiplication, and tolerance to various environmental conditions.[23] In fact, tilapia has previously been used as a sentinel organism for contaminants in various toxicological studies.[24] Alterations in the biotransformation enzymes of this indicator species have also been used in various biomonitoring studies to reflect and assess environmental contaminants.[25]

The liver and gill are the main and main sensitive target organs of xenobiotic toxicity and damage for fish. [26] They also play a major role in the biotransformation of xenobiotics. The sensitivity of these tissues to pesticide-induced stress is a function of the disturbed balance between the degree of oxidative stress and the antioxidant capability. [26,27] Previous studies have shown that pesticides alter enzymatic and non-enzymatic antioxidant systems and induce oxidative stress in animals.[27,28,29] The exposure to low doses of fipronil for prolonged periods has previously been reported to induce oxidative stress in the blood serum of pregnant rats and their offsprings.[30] The oxidative stress-induction potential of exposure to different sublethal concentrations of chlorpyrifos has previously been investigated in the brain, liver and gill tissues of guppy fish (*Poecila reticulate*). [31] In fact, morphological alterations in the liver and gill tissues of fish are useful indicators for responses to environmental stressors or toxicants. The liver is the main organ of detoxification. Due to their lipophilicity, chlorpyrifos have a high rate of gill absorption. This could be a contributing factor to the sensitivity of fish to this organophosphorous pesticide.[31,32] The oxidative damages and toxicity effects induced by deltamethrin (DLM) intoxication were also

investigated in the liver, kidney and gills in freshwater Nile tilapia (*Oreochromis niloticus*). [33,34]

Considering the scarcity of data on the contamination status and ecological impacts of fish exposure to Red 195 dye, the present study was undertaken to investigate and evaluate the potential biochemical changes in the activity of antioxidant enzymes and morphological responses in the liver and gill of tilapia *Oreochromis niloticus* exposed to different concentrations of Red 195 dye for various durations under laboratory conditions.

2. Materials and methods

2.1. Fish and experimental design

The fish samples (n = 96) used in this study were healthy adult tilapia *O. niloticus* of both sexes (mean total length 17.14 ± 2.36 cm; mean total weight 121.54 ± 10.17 g) obtained from the aquaculture station of the University of Trás-os-Montes and Alto Douro, Portugal. The samples were randomly distributed in 12 tanks of 100L, with water recirculation rate of 5L/min, temperature range 25 ± 1°C, and controlled photoperiod of 12D:12L for 21 days. Three groups of tilapia were exposed to different concentrations of Red 195 dye (0.05, 0.1 and 0.2 mg/L), and the other group, serving as a control, was placed under similar conditions without the xenobiotic. Each exposure condition was performed in triplicate. Two fish samples were removed from each tank each week. The tanks were provided with Supplemental Aeration to maintain dissolved oxygen concentration near saturation. The fish were fed daily to visual satiation with a diet that had been tested in a previous study.[35] The following water quality parameters were provided: 25±1 ◦C of temperature; pH 7.7±0.2; 6±1 mg/L of dissolved oxygen; 376µS/cm of conductivity; 0.08±0.06mg/L of ammonium, and 0.01±0.01mg/L of nitrite. The Red 195 dye concentration in the water-tanks was controlled

spectrophotometrically (at λ = 520 nm) and absorbance was measured using a standard curve (0-0.2 mg/L).

After 21 days of exposure period, the remaining fish samples were transferred to Red-195-dye-free water for 30 days to study recovery responses. They were then anesthetized with 1 mL of 2-phenoxiethanol L^{-1} water (Sigma, Barcelona, Spain), measured, weighed, and euthanized by decapitation. Their livers and gills were removed, and one randomly chosen part of each organ was fixed for microscopy (see below). The remaining part was deeply frozen for further enzymatic assays. The tissues were crushed and homogenized with phosphate buffer KH_2PO_4 at pH 7.4 and centrifuged at 16000 g for 20 min at 4 ° C. The supernatant was used for the subsequent enzymatic assays. All experimental procedures were performed in accordance with the Portuguese law of animal welfare (Portaria No. 1005/92) and EU Directives (2010/63/EU) on the care and use of laboratory animals.

2.2. Analytical techniques

Catalase activity (CAT) was measured by a Clark-type oxygen electrode (Hansatech®).[36] The reaction medium consisted of potassium phosphate buffer (50 mM KH_2PO_4 pH7.0) and hydrogen peroxide (1M) at a final volume of 1 mL. The medium buffer was previously submitted to a nitrogen stream to decrease dissolved oxygen. A sample volume of 50 µL (diluted 100 times) was added to the reaction medium, and the mixturewas incubated at 25°C for 2 min. Reaction was initiated by the addition of hydrogen peroxide (12 mM) at a final volume of 1 mL. After 2 min of thermostatic incubation at 25°C and stabilization, H_2O_2 was added to the reaction medium, and the new slope was measured. After 30 s, the enzyme extract (diluted 100 times) was added, and the new slope measured. A

control test was performed without the addition of sample to determine the autodismutation of H_2O_2. CAT activity was expressed as mmol H_2O_2/min/mg of protein.

Glutathione reductase (GR) activity was determined by a Varian - Cary ® 50 spectrophotometer according to the method described by Carlberg and Mannerviek (1975).[37]The reaction medium consisted of potassium phosphate buffer (100 mM KH_2PO_4 and 0.5 mM EDTA, pH 7.4), 100 mM oxidized glutathione (GSSG) and 10 mM NADPH. GSSG was added after 2 min of sample incubation to initiate the reaction. Enzyme activity was measured at 340 nm and 25 °C by NADPH oxidation. The result was expressed as mM NADPH oxidized/min/mg of protein using the molar coefficient extinction (ε) of $6.22x10^3$ $mM^{-1}cm^{-1}$.

Glutathione S-transferase (GST) activity was determined by a Varian - Cary® 50 spectrophotometer.[38] The reaction medium contained potassium phosphate buffer (100 mM KH_2PO_4, pH 7.0), 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), 10% Triton X-100 (v/v), and 100 mM GSH at a final volume of 2 mL. Recording started after 2 minutes of incubation at 25 ° C. The reaction was initiated 1 minute after the onset of recording with the addition of GSH (100 mM). The variation in absorbance was determined at 340 nm, and enzyme activity was expressed in mM CDNB conjugated /min/mg of protein ($\varepsilon = 9.6\ x10^3\ M^{-1}cm^{-1}$).

Protein content was determined according to the method of Bradford (1976)[39] using bovine serum albumin as standard. All chemicals used in the enzymatic activity assays were of analytical purity and purchased from Sigma Chemical Co.

2.3. Histology

Two gills of each fish were randomly collected and fixed in buffered formaldehyde (Panreac, Barcelona, Spain) for 24 h, dehydrated in graded ethanol series, and embedded in

paraffin wax. Sagittal sections (5μm of thickness) were cut and mounted on glass slides. Sections were deparaffinized in xylene, hydratated in ethanol and stained with hematoxylin-eosin (HE). The liver was quickly dissected, sliced into 3-mm-thick slabs, and immersed in buffered formaldehyde for 24 h, dehydrated, and embedded in paraffin; a minimum of 5 pieces were obtained. Histological sections (5 μm of thickness) were cut and stained with H&E. The histopathological changes induced by the treatment in the gill and liver organs were photographed and analyzed by light microscopy (Nikon® Labophot).

2.4. Statistical analysis

The data were analyzed by the STATISTICA 6.0 software (StatSoft, 2001). The mean ± standard error was calculated for each experimental group. Differences between groups were tested using one-way ANOVA, followed by a Student– Newman–Keuls multiple comparison test at a 5% level of significance.

3. Results

3.1. Biochemical analysis

The changes in the activities of antioxidant enzymes in the liver and gill are shown in Fig. 1 and Fig. 2, respectively. The results revealed that Red 195 exposure at all concentrations (0.05, 0.1 and 0.2 mg/L) and for all periods altered the normal functioning of hepatic activity tilapia by consistently decreasing CAT activities (Fig. 1A) as compared to the control. Statistically significant differences were, however, observed only for the highest concentration and for two different periods (7 and 21). Furthermore, GST activities (Fig. 1B) were noted to undergo a decrease after 7 days of exposure with all concentrations, although statistically significant values were observed only for the group exposed to the lowest concentration as compared to the control group. After 14 days of exposure, no significant

differences were observed between the experimental groups, except for the one exposed to Red 195 at a concentration at a concentration of 0.1 mg/L, which showed a slight decrease. After 21 days of exposure to a concentration of 0.2 mg/L, however, a significant increase in GST activities was observed as compared to the control. The results also revealed that glutathione reductase activity (Fig. 1C) underwent a significant increase after 7 and 14 days of exposure. After 21 days of exposure, however, this increase was statistically significant only for the lowest concentration (0.05 mg/L).

Furthermore, the results revealed that, compared to the control, the CAT activity in the gills (Fig. 2A) underwent a significant increase after 7 days of exposure to 0.1 and 0.2 mg/L of Red 195. No statistically significant differences in CAT activity were, however, observed for longer periods of exposure (14 and 21 days) with all concentrations (0.05, 0.1 and 0.2 mg/L) when compared to the control. Further results showed an unexpectedly significant decrease in GST activity after 7 days of exposure to the lowest concentration of Red 195 (Fig. 2B). While GST activity displayed no statistically significant differences after 14 days, they underwent a significant increase after 21 days exposure to 0.05 and 0.1 mg/L of Red 195. Interestingly, the profile displayed by GR activity after 7 and 14 days of exposure was similar to the one observed for GST activity (Fig. 2C). After 21 days of exposure, however, the 0.1 mg/L concentration was noted induce a significant decrease in GR activity.

After 30 days of depuration, the antioxidant enzyme activities in the liver and gills did not show significant differences when compared to the ones observed after 21 days of exposure (data not shown).

3.2. Histology

The histological observations of control *O. niloticus* liver tissues (Fig. 3A, 3B) showed normal architecture, with the hepatocytes exhibiting a quite homogeneous cytoplasm

and a large central or subcentral spherical nucleus. The liver of the fish exposed to Red 195 (Fig. 3C–3F) showed several histopathological changes whose severity was noted to increase with the the increase of Red 195 concentration and time of exposure. The hepatic parenchyma of fish exposed to 0.1 mg/L of Red 195 showed lower eosinophilia and some degree of cytoplasm vacuolization (Fig.3C), which clearly increased with the time of exposure. The disruption of the endothelial lining was also evident after 7 days of exposure (Fig.3C), becoming more prevalent after 21 days of exposure. An apparent presence of metabolic zonation was observed, with the vacuolization of hepatocyte being farthest from the afferent vascular area (Fig.3D). Necrotic cells appeared in the periphery of vascular regions, and a rupture was observed in membrane of hepatocytes . These histopathological changes were more clearly evident after long periods of exposure (21 days), being accompanied by a higher prevalence of macrophage aggregates. However, the hepatic parenchyma of the fish exposed to 0.2 mg/L of Red 195 showed reduced eosinophilia and expansion of cytoplasmic vacuolization after both 7 and 21 days of exposure (Fig. 3E,3 F). The endothelial lining displayed a higher degree of rupture, with high levels of necrotic cells in the vascular periphery. A rupture in the hepatocytes membrane was also observed. Figure 3G and 3H show the liver tissues from the recovery group (RG) of fish previously exposed to 0.1 and 0.2 mg/L of Red 195, respectively. Although some degree of hepatocyte cytoplasm vacuolization was observed, there were signs of regeneration. In general, an improvement in the hepatic parenchyma structure was detected for the RG. In some fish, the liver showed normal architecture, and the hepatocytes exhibited a quite homogeneous basophilic cytoplasm.

Further histological observations revealed that the gill tissues of the *O. niloticus* control group (Fig. 4 A,4B) showed normal architecture. The gill filaments consisted of a

central axis with a connective tissue and venous sinus. The filaments were covered by a stratified epithelium, intersected by lamellae (Fig. 4A, 4B). The gills of the fish exposed to Red 195 (Fig. 4C–4F) for 7 and 21 days showed histopathological changes, with the highest rates of severity being observed after long periods of exposure. The gills of fish exposed to 0.1 mg/L of Red 195 for 7 days (Fig. 4C) showed lamellar epithelium lifting associated with a high level of filament epithelium proliferation (FEP). After 21 days of exposure to the same Red 195 concentration (Fig. 4D), the filament epithelium proliferation induced, in some instances, lamellar fusion. Moreover, the results revealed the vasodilatation of the lamellar vascular axis and some necrotic cells in the deep region of the filament epithelium.

The histopathological changes observed for the gills of fish exposed to 0.2 mg/L of Red 195 were similar to those detected for the fish exposed to 0.1 mg/L. The severity rate was, however, clearly higher after 21 days of exposure to the highest concentration of Red 195. Exposure to 0.2 mg/L for 7 days induced edema (Fig.4E), which presumaly induced filament lifting and lamellar epithelium (Fig.4F). Vascular disorders, such as extreme vasodilatation and filament epithelium proliferation, were also observed. Furthermore, after 21 days of exposure, the presence of necrotic cells became more evident, mainly in the deep region of the filament epithelium (Fig.4F).

After the depuration period, the gills of the fish previously exposed to 0.1 and 0.2 mg/L showed a clear recovery of the filament microarchitecture (Fig. 4G, 4H). In some fish, the gill epithelium recovered its normal structure. Although epithelial repair was not complete, clear signs of compensatory responses were still evident in most of the fish samples.

4. Discussion

Biochemical and histopathological responses have commonly been used as effective biomarkers for evaluating the effects of pollutants on the health and ecological status of aquatic biota. Accordingly, the present work aimed to evaluate the toxicity effects of Red 195 dye on *O. Niloticus* gill and liver through the study of oxidative stress responses and histopathological changes. The results revealed that the oxidative stress-response enzymes evaluated in this study displayed different responses to this dye. The findings indicated that, compared to the control, the tilapia fish exposed to Red 195 dye for all the periods assayed in this work underwent a decrease of CAT activity (Fig. 1A) in the liver. This is in agreement with the results previously reported for other xenobiotics.[35,40]The results showed, however, that CAT activity in the gills (Fig. 2A) increased after 7 days exposure, which is in agreement with the results previously observed for bivalves [41,42,43,44] and fish exposed to several pollutants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) .[45,46]

Catalase activity is responsible for the conversion of hydrogen peroxide in water. However, the microsomal production of oxyradicales seems to be stimulated by a wide variety of xenobiotics including B(a)P, aroclor 1254,[47] and HAPs,[48] as well as pharmaceutical products, such as Ubiprofen.[49] The results of the present study revealed that longer periods of exposure to Red 195 dye induced a decrease in CAT activity. This could be attributed to the accumulation of hydrogen peroxide, which causes other damages to the tissue and cell. In fact, it is possible to have the production of other types of radicals via a fenton reaction.

Furthermore, GST activity plays a central role both in the maintenance antioxidative defences [50] and removal of pollutants by increasing their solubility in water through conjugation with GSH.[51,52,53] The tilapia fish exposed to Red 195 displayed an increase in GST activity exclusively in the liver and only after 21 days of exposure to a high

concentration 0.2 mg/L. Conversely, the hepatic GST activity of tilapia species exposed to oxyfluorfen was previously reported to undergo an increase in the first week, but to exhibit a subsequent decrease with longer periods of exposure.[24] However, an increase in hepatic GST activity was reported for fish exposed to PCBs, PAHs, and PCDDs (Polychlorinated dibenzodioxins).[54,55] Other studies showed that exposure to PCDD, PAHs, and organochlorine pesticides (OCPs) caused a decrease in enzymatic activity.[56,57] In the gills, GST activity increased after 14 days of exposure to 0.2 mg/L of Red 195, although a significant increase was observed with all concentrations after 21 days of exposure. These observations suggested that the GST was able to eliminate the xenobiotic at this tissue. This result is in agreement with the experimental variations observed for the GST activity in the gills of Fulvia fragilis.[44] However, a decrease in GST activity was previously reported for the gills of *Donax trunculus* originating from a Tunisian polluted site.[58] Decreased GST activities have also been observed in other species of bivalves,[59] gastropods[60] and fish.[61]Several xenobiotics can disrupt phase II enzymes of the cellular metabolic processes, inducing signal transduction events that can lead to various cellular and physiological responses. For pharmaceuticals, this takes place primarily through the various biotransformation reactions to be eliminated.[49,62,63] Several studies have shown that the effects of GST may be caused by other pollutants, such as B(a)P and aroclor[47] In fact, different assumptions have been made about the metabolic pathways and mode of action of this dye on GST activity . The change observed in GST activity can reflect the ability of red 195 to affect the enzymes involved in the maintenance of tissue redox balance.[64] The changes of GST activity may correspond to the induction of a detoxifying enzyme in the gills of the fish exposed to the dye to respond to the invasion of the organism by the dye via its conjugation with a thiol group.[65]

The results showed an increase in hepatic GR activity for all periods of exposure and at all concentrations, except for the 0.2 mg/L concentration for 21days of exposure. Peixoto et *al*. (2006)[24] showed that GR activity increased in the liver of tilapia treated with oxyfluorfen at all sampling days. However, Matos et *al*., (2007)[25] reported that carbamate treatment induced an increase in GR activity only after 14 days of treatment. While several studies showed that the exposure to different contaminants induced an increase in GR activity,[66,67] other studies reported on a significant decrease in this activity, including the study on *O. niloticus* originating from a site contaminated with PCBs and HCHs (hexachlorocyclohexane).[68] Unlike the case of GR activity in the liver, the results showed that GR activity in the gills decreased after 14 days treatment, with statistically significant values observed for the groups treated with Red 195 at concentrations of 0.1 and 0.2 mg/L.. This result suggested the activation of gill detoxification processes, presumably due to the presence of organic contaminants.[69]

The literature indicates that the adverse effects of pollutants on marine biota can be evaluated by histopathological alterations in fish organs, such as the liver and gills[61] since they reflect the overall health of the fish population in the ecosystem.[70] Morphological changes are generally indicative of irreversible damage. The results of the present study revealed that the the most prominent histological alterations observed in the liver of Nile tilapia fish exposed to Red 195 were necrosis, hepatocytes cytoplasm vacuolization and macrophage aggregates. These histopathological changes were more clearly evident after long-term exposures, with a high level of necrotic cells. This result is in accordance with several reports in the literature.[71,72] The higher rates of necrosis in fish exposed to different pollutants can be an internal response of the organism, as a consequence of the metabolic stress induced by the increase of xenobiotic availability during this period.[72] This is

consistent with the results observed for enzymatic activity. In fact, the results showed that CAT activity was inhibited, this reflecting the accumulation of H_2O_2 which, in turn, could lead to higher possibilities for the formation of other radicals and lipidic peroxidation. However, the fact that GR activity increased for all concentrations and with all periods of exposures suggested the presence of more GSSG. GST activity was, on the other hand, noted to undergo an increase or a decrease depending on the dose and duration of exposure. These variations highlighted the state of conjugation reactions of GSH. Exposure to red 195 was noted to alter the GSH/GSSG ratio in tilapia. In fact, GST constitutes a complex family of proteins that play significant roles both in the normal cellular metabolism and the detoxification of a wide variety of xenobiotic compounds.

The vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver.[73] The vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchyma cells and the rate of their release into the circulation.[74] Vacuole formation was considered as a cellular defense mechanism against substances injurious to hepatocytes, and this mechanism could be responsible for collecting the harmful elements and preventing them from interfering with the biological activities of these cells.[75] An increase in the density of the macrophage aggregates is generally related to important hepatic lesions,[76] such as degenerative and necrotic processes. Several studies have reported on other changes in the liver of *O. niloticus* and other fish species, resulting from exposure to different toxic chemicals.[77,78,79,80,81] After the depuration period, the liver tissues of some fish samples previously exposed to Red 195 dye showed signs of regeneration. These results suggested that the toxic effects induced by Red 195 dye could be reversed after a depuration period of 30 days. In fact, the toxic effects of other toxicants, such as carbaryl, in *O. niloticus* species

were previously shown to be relatively reversible within 15 days of exposure after stimulus withdrawal.[25]

Furthermore, the results revealed that the gill from the control *O. niloticus* fish displayed a typical structural organization. The histopathological changes of the gills from the fish exposed to Red 195 dye showed higher levels of severity after a long period of exposure, including lamellar epithelium lifting, epithelium proliferation, necrotic cells, lamellar axis vasodilation, fusion of lamellar, and edema in the filament. As an histological change, the lifting of lamellar epithelium could presumably be induced by the incidence of severe edema.[82,83] Edema, together with the lifting of lamellar epithelium, could serve as a defense mechanism since epithelial separation from the lamellae increases the distance across which waterborne pollutants need to diffuse to reach the bloodstream.[84]

The cell proliferation observed with the thickening of gill filament epithelium in *O. niloticus* exposed to Red 195 dye could lead to the lamellar fusion. Similar results were previously reported in fish species exposed to other pollutants.[85,86] Azevedo et *al*. (2013)[72] showed that, in *Cathorops spixii,* if the pollutant pressure decreases, the lamellar fusions could be considered a reversible lesion with low severity. However, in cases of stress continuity, lamelar fusion could compromise the breathing of the fish and ultimately contribute to asphyxia or neoplasic events.[72] Miranda et *al*. (2008)[71] reported on similar results for fish species from aquatic systems under exposure to organochlorine compounds. The epithelial lifting, edema and lamellar fusion are defense mechanisms that reduce the branchial superficial area in contact with the external milieu. These mechanisms also increase the diffusion barrier to the pollutant.[87]

Lamellar axis vasodilatation was also reported for tilapia exposed to Red 195. Garcia-Santos et *al*. (2006)[88] reported that this lesion could induce changes in the normal structure

of pillar cells, with a consequent loss of their support function, which could presumably be responsible for the emergence of lamellar aneurysms in fish.

The cellular damages observed in the gills can adversely affect the gas exchange and ionic regulation processes, as a consequence of the increased distance between water and blood due to epithelial lifting.[89]

Similar histological alterations in the gills were previously observed in some fish species due to the exposure to different types of pollutants, including drugs.[83] Accordingly, this could suggest that alterations are non-specific and can be induced by different types of contaminants.

The gill epithelium of the recovery group of fish previously exposed to Red 195 showed a relatively normal structure. Although the epithelial repair was not complete, clear signs of compensatory responses were evident. These results confirmed the findings observed for the liver of the *O. niloticus* fish.

Taken together, the results of the present study showed for the first time that low concentrations (0.05, 0.1, 0.2 mg/L) of Red 195 dye could induce oxidative stress in *O. niloticus*. The oxidative stress was demonstrated by biochemical and histological changes in the liver and gill of tilapia. Biological responses were proved by increasing or inhibiting the activities of some antioxidant enzymes (CAT, GST and GR) and by histopathological changes observed in tilapia exposed to Red 195 dye. The results obtained for the recovery group suggested that the toxicity produced by Red 195 dye was, to some extent, reversible within 30 days after the removal of the stimulus. The biochemical and histological changes in the liver and gill were noted to depend on the concentration and period of exposure to Red 195. Overall, the results call for extreme caution to the adverse negative impacts of this dye on wild fish. Further studies are needed to explore the practical application of using bacterial

species in the bioremediation of effluents containing this dye and some cases that involve the persistence of this dye in the water.

Competing interests

The authors declare that they have no competing interests.

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Legend of figures

Fig. 1: Liver antioxidant activities (means ± SD) in Nile tilapia *O. niloticus* exposed to different concentrations of Red 195 (0.05; 0.1 and 0.2 mg/ L). The activity levels of treated fish were compared with control group in each sampling day. Data are means±SD of six independent experiments. *Values statistically different from control $p < 0.05$; **Values statistically different from control $p < 0.01$; ***Values statistically different from control $p < 0.005$.

Fig. 2: Gill antioxidant activities (means ± SD) in Nile tilapia *O. niloticus* exposed to different concentrations of Red 195 (0.05; 0.1 and 0.2 mg/ L). The activity levels of treated fish were compared with control group in each sampling day. Data are means±SD of six independent experiments. *Values statistically different from control $p < 0.05$; **Values statistically different from control $p < 0.01$; ***Values statistically different from control $p < 0.005$.

Fig. 3: Histological images of Nile tilapia (*O. niloticus*) liver. **(A, B):** Control fish, showing a normal architecture, with the hepatocytes (H) exhibiting a quite homogeneous cytoplasm and a large central or subcentral spherical nucleus (arrow). S: sinusoid; VE: vein. **(C-F):** Liver from fish exposed to Red 195 dye; **(C):** (7th day, 0.1 mg/L) showing cytoplasmic vacuolization (VA), disruption of the endothelial lining (arrow); **(D):** (21st day, 0.1 mg/L) showing increased vacuolization (VA), severely disruption of the endothelial lining (arrow), a metabolic zonation, Necrotic cells (N) and rupture of hepatocytes membrane (arrowhead), macrophage aggregates (MA); **(E):** (7th day, 0.2 mg/L) and **(F):** (21st day, 0.2 mg/L) showing higher eosinophilia reduction, increased cytoplasmic vacuolization, the endothelial lining showed a higher degree of rupture (arrow), with high level of necrotic cells and rupture of the hepatocytes membrane (arrowhead); **(G-H):** Recovering Group (RG) from fish liver previously exposed to 0.1 and 0.2 mg/L of Red 195 showing the signs of regeneration with

the maintenance of hepatocyte cytoplasmic vacuolization and an improvement in the hepatic parenchyma structure.

Fig. 4: Histological images of Nile tilapia (*O. niloticus*) gills. **(A, B):** Control fish, showing a normal appearance of gill filament epithelium (FE) and lamellae (LA). (CT): connective tissue and (CVS): venous sinus. **(C- F):** Gills from exposed fish to Red 195 dye; **(C):** (7th day, 0.1 mg/L) showing lifting (LI) of lamellar epitheliumand filament epithelium proliferation (FEP); **(D):** (21st day, 0.1 mg/L) present some necrotic cells (N) in the deep region of the filament epithelium, vasodilatation (V) and filament epithelium proliferation that in some instances conduced to lamellar fusion (LAF); **(E):** (7th day, 0.2 mg/L) showing edema (ED), Vascular disorders such as extreme vasodilatation (V) and Proliferation of filament epithelium (FEP); **(F):** (21st day, 0.2 mg/L) presence of necrotic cells (N) and lifting (LI) of filament and lamellar epithelium became evident, mainly in the deep region of the filament epithelium.**(G-H):** Recovering Group (RG) from fish gill previously exposed to 0.1 and 0.2 mg/L showing a clearly recover of the filament microarchitecture. In the majority of the fish, although the epithelial repair was not complete, clear signs of compensatory responses were evident. Bar = 50 µm.

Fig. 1

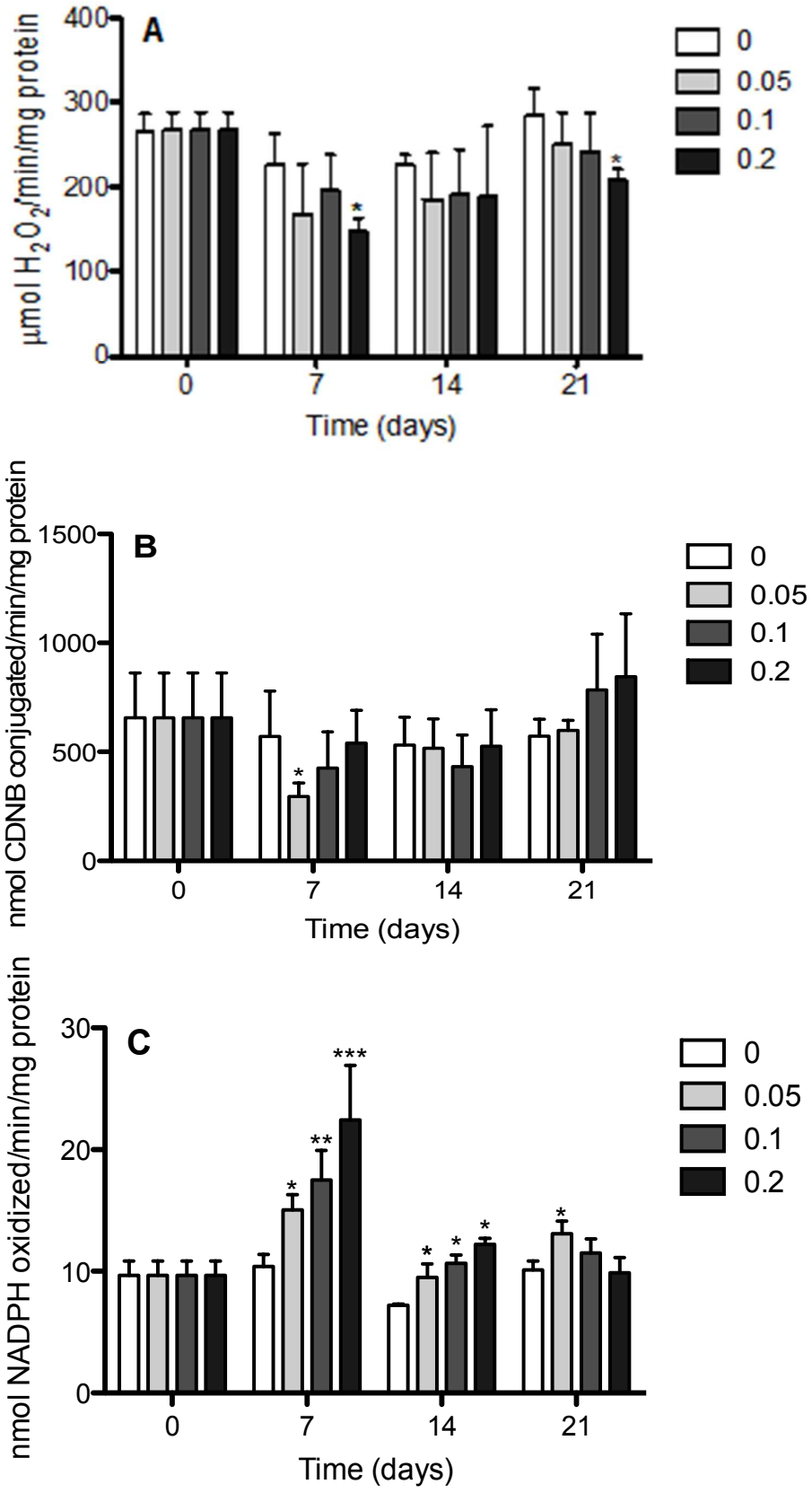

Fig. 2

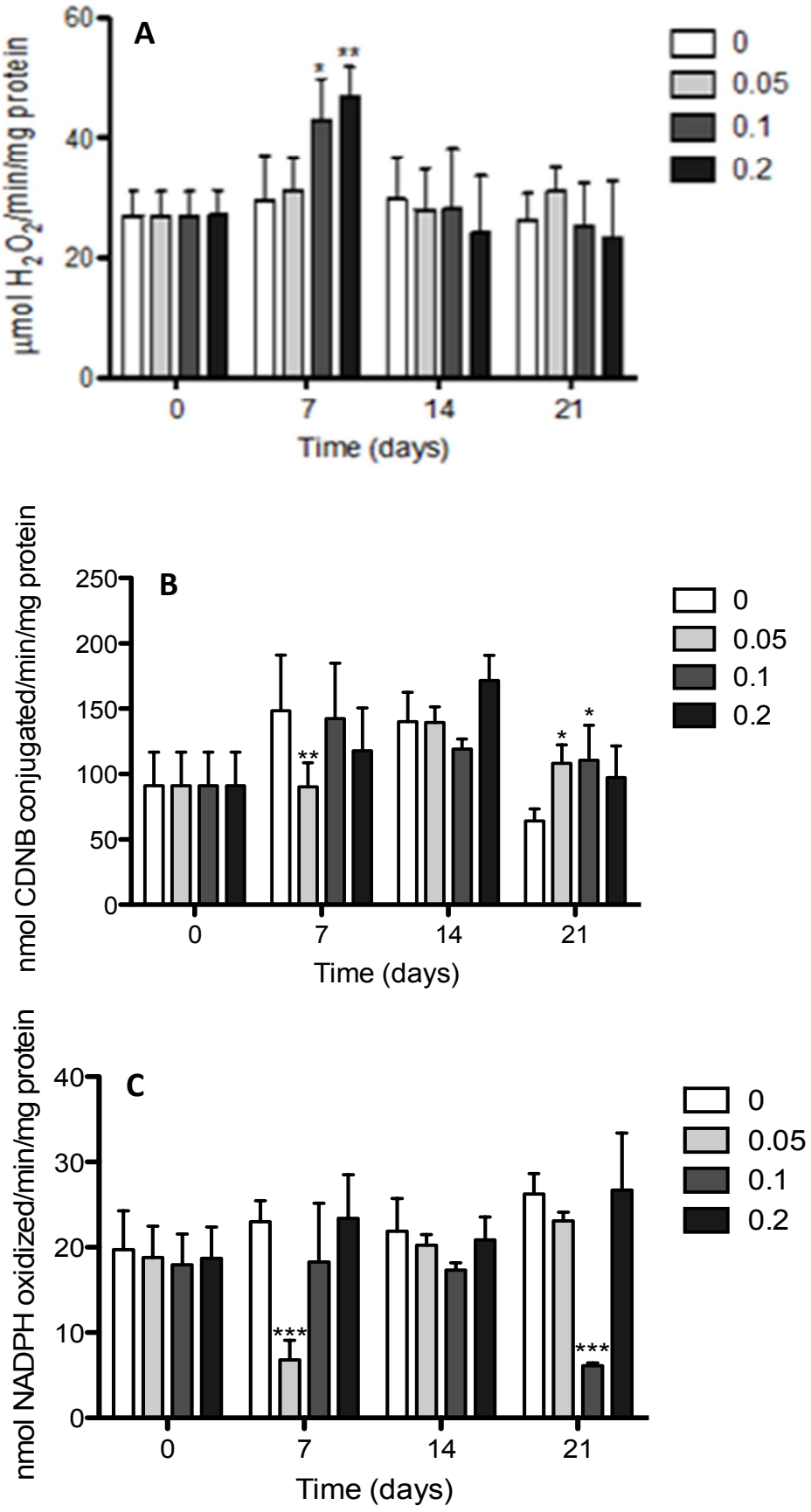

Fig. 3

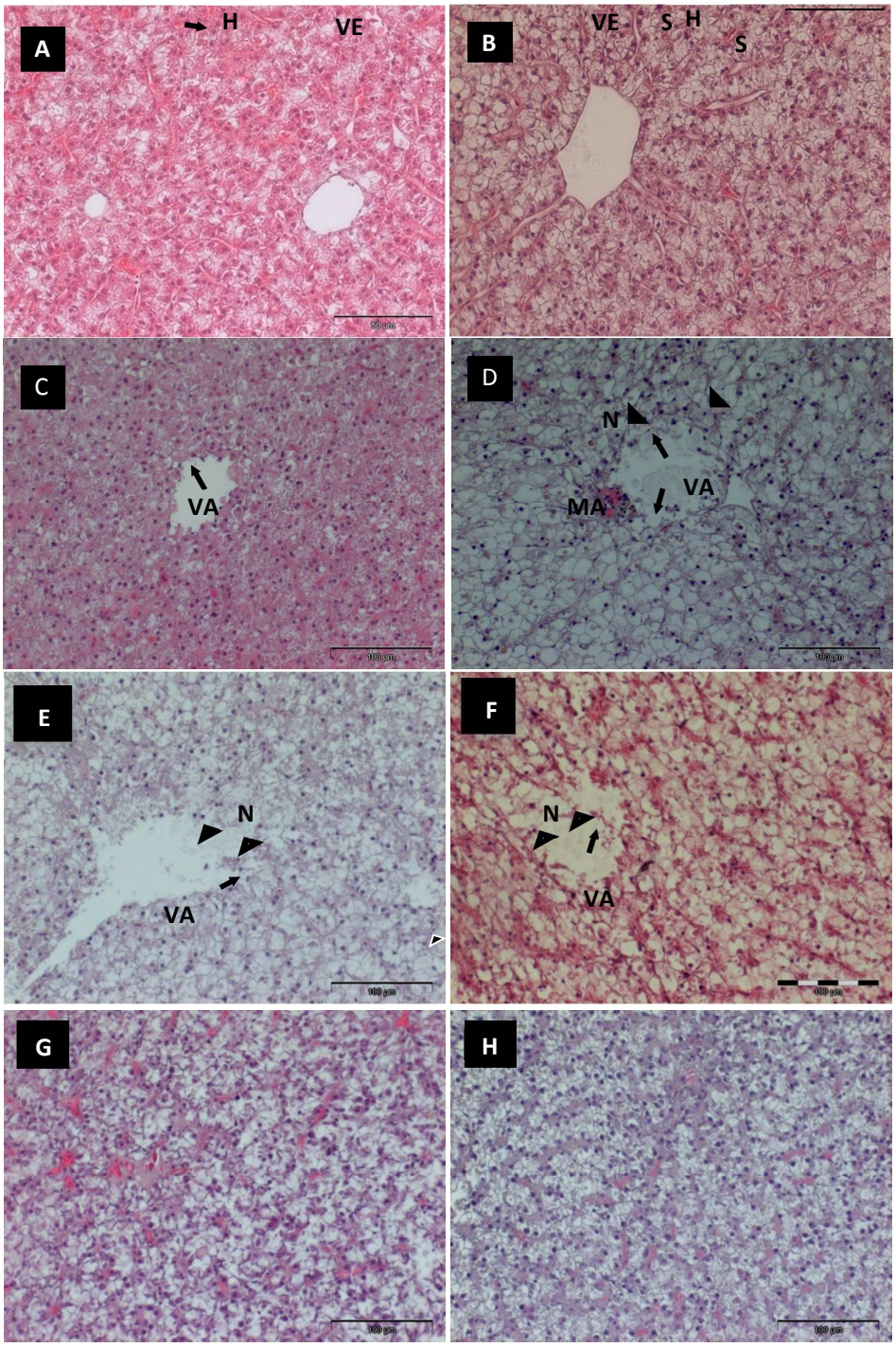

Fig. 4