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Biochemical and histological hepatic changes of Nile tilapia *Oreochromis niloticus* exposed to carbaryl

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Abstract

The purpose of this study was to evaluate biochemical and morphological responses induced by carbaryl in the liver of Nile tilapia (*Oreochromis niloticus*) exposed during 21 days to sublethal concentrations (0.25 and 0.5 mg L^{-1}), testing also recover for 14 days in clean water, after 14 days exposure. The activities of the following enzymes were measured: superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST), glutathione reductase (GR), and reduced (GSH) and oxidized glutathione (GSSG). Globally, our data showed that exposure to carbaryl decreased the SOD, CAT, GR, and GST activities, except for the SOD and GST activities after 14 days exposure to 0.25 mg L^{-1}. In contrast, after 14 days exposure the GR activity of the hepatic tissue from carbaryl-treated fish showed significant elevation in relation to the control. When fish were left to recover, a positive response was seen in the GSH and GSSG contents. The results of the recovery group suggest that the toxicity produced by carbaryl is reversible to some extent within 15 days. The liver histological analysis showed differences between fish concerning the cellular vacuolization degree (VD) of the hepatocytes. In fish exposed to carbaryl it was observed an increasing hepatocellular basophilia. No other histological alterations were observed when fish was exposed to carbaryl, except a few necrotic foci at day 7. The sections stained with PAS reaction showed that the vacuolization was always not due to glycogen deposits, thus suggesting lipid accumulation. The combined increased basophilia and glycogen depletion is a common, although non-specific, liver response to many toxicants. In short, this work shows a relation between histological and biochemical changes in liver and carbaryl exposure. The effects of carbaryl were observed at different concentrations.

Keywords: Oxidative stress; Tilapia; *Oreochromis niloticus*; Carbaryl; Liver; Histology

1. Introduction

The aquatic ecosystem is continuously being contaminated with toxic chemicals from domestic, industrial and agricultural activities. Pesticides are one of the major classes of toxic compounds used in agricultural activities, namely organophosphorous and carbamates [1]. Carbamates are systemic and contact pesticides used as substitutes for organochlorine insecticides because of their high efficiency and low persistence in the environment [2]. Carbaryl (1-naphthyl-*N*-methyl carbamate) is a broad spectrum carbamate insecticide that is reported to have a low persistence in soil and water [3,4]. Carbaryl has been used for the control of pests in forestry and agriculture, as well as to control the crustacean predators of shellfish and aquatic weeds in bays and estuaries [5].

The biochemical parameters in fish are sensitive for detecting potential adverse effects and relatively early events of pollutant damage [6]. Thus, it is important that pollutant effects can be determined and interpreted to

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delineate mechanisms of pollutant action and possibly ways to mitigate adverse effects. A review of literature indicates that biochemical changes in fish under pesticide exposure were extensively reported [7–9]. However, it is relevant that data on the effects of the carbaryl using aquatic organisms as experimental models is not well-established [10–12].

This liver plays an important role in several vital functions of basic metabolism and it is also the major organ of accumulation, biotransformation and excretion of contaminants in fish, including degradation and bioactivation of pesticides [13,14]. The evaluation of biochemical and histological changes in fish liver has become an important tool for monitoring environmental exposure of fish to contaminants in experimental studies [15,16]. The exposure to contaminants in aquatic ecosystems can enhance the intracellular formation of reactive species of oxygen, which induce oxidative damage to biological systems [17,18]. Bagchi et al. [19] found that different classes of pesticides may induce in vitro and in vivo generation of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and the hydroxyl radical. Oxidative stress happens when an imbalance occurs between production and elimination of ROS. The ROS can be detoxified by an enzyme defense system, comprising superoxide dismutase (SOD), catalase (CAT), and selenium-dependent glutathione peroxidase (GPx), or non-enzymically systems by the scavenging action of reduced glutathione, while organic peroxides can be detoxified by the activity of glutathione *S*-transferase [20]. Several studies demonstrated that changes in the levels of antioxidant enzyme activities can be used as possible biomarkers in different aquatic organisms [21,22].

The lack of information on the effects of carbaryl on fish has prompted us to undertake this study. As to the target species, tilapia was chosen because it is a good biological model for toxicological studies due to diverse characteristics, namely their high growth rates, efficiency in adapting to diverse diets, great resistance to diseases and to handling practices, easy reproduction in captivity and prolific rate, and, finally, good tolerance to a wide variety of environmental conditions [23].

Hence, this study was undertaken to examine the effect of carbaryl on biochemical aspects of Nile tilapia *Oreochromis niloticus*, during exposure and after recovery in clean water. The liver histology and hepatic activities of SOD, CAT, glutathione *S*-transferase (GST), glutathione reductase (GR), and the amount of reduced (GSH) and of oxidized glutathione (GSSG) were measured in liver after experimental exposure to two sublethal concentrations of commercial grade carbaryl.

2. Materials and methods

2.1. Fish and experimental design

Nile tilapia *O. niloticus* (Bouaké strain) were originally obtained from the Institute Nationale de Recherche Agronomique (Rennes, France) and raised in the Aquaculture Station of the University of Trás-os-Montes and Alto Douro (UTAD, Portugal) for three generations. Fish were kept in 100 L recirculating tanks (water flow rate of 5 L min^{-1}) filled with dechlorinated tap water. Water composition was in agreement with European Community instructions (84/449/EEC Directives, Annex 5). The water parameters were within the normal ranges: pH 7.1; alkalinity 60 mg HCO^{3-} L^{-1}; conductivity 63 μS cm^{-1}; 14 mg Na^{+} L^{-1}; 2.3 mg K^{+} L^{-1}; 4.1 mg Ca^{2+} L^{-1}; 6.5 mg Mg^{2+} L^{-1}; 19.5 mg Cl^{-} L^{-1}; 27 mg $NO_3{}^{-}$ L^{-1} (nitrate); 0.5 mg $NO_2{}^{-}$ L^{-1} (nitrite); hardness 74.5 mg $CaCO_3$ L^{-1}; 6.2 mg dissolved O_2 L^{-1}; 21 mg CO_2 L^{-1}; 0.1 mg H_2S L^{-1} (hydrogen sulfide); 0.7 mg $NH_4{}^{+}$ L^{-1} (ammonia); and 12 mg suspended solids L^{-1}. Supplemental aeration was provided to maintain dissolved oxygen near saturation, the temperature was kept at 25 ± 1 °C and the photoperiod controlled (12D:12L).

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the UTAD for the use of laboratory animals. The water quality parameters mentioned above were assessed at collection days during the experimental period, with no significant changes being observed. Adult male and female *O. niloticus* (39.1 ± 1.0 g of body weight and 12.8 ± 1.3 cm of total length) were randomly distributed through 9 tanks of 100 L, at a density of 12 animals per unit. Food was withheld from 24 h prior to the experiment. During the experiment fish were fed daily to visual satiation with a diet tested in a previous study [24].

Two groups were exposed (in triplicate) to 0.25 and 0.5 mg L^{-1} commercial carbaryl for 21 days; these served as the exposed groups. The higher level of exposure was one-third the 96 h LC50 concentration for commercial carbaryl. After 14 days of exposure period 12 fish were transferred to carbaryl-free water for 14 days to study the recovery response. A third group of 12 control fish was kept in toxicant free water. Both control and experimental tanks were water renewed (90%) every two days. Sampling of exposed and control fish ($n = 6$/group) was done after 45 min (day 0), 7, 14, and 21 days after starting the experience. Tilapia were anaesthetized by immersion in a 0.2 ml L^{-1} aqueous solution of 2-phenoxyethanol (Sigma, Spain), and thereafter measured and weighed. Then, fish were rapidly killed by decapitation and the liver immediately removed and processed, one part being fixed for microscopy (see below), and other frozen for later assay of enzymes.

2.2. Analytical techniques

The activities of SOD, GR, and GST were measured in the hepatic cytosolic fraction from *O. niloticus* of all groups considered in this study. Livers were homogenised in ice-cold sodium phosphate buffer 50 mM, Na_2EDTA 0.1 mM, pH 7.8. The fractions were obtained after centrifugation at 10,000*g* for 20 min. SOD (EC.1.15.1.1) activity was assayed according to Paya [25], with minor

modifications. The nitrotetrazolium blue chloride (NBT, Sigma N-6876) was used as detection molecule instead of cytochrome *c*. Assays were conducted in the presence of 100 nM potassium phosphate buffer (pH 7.8), EDTA 10 mM, NBT 10 mM, hypoxanthine 10 mM (Sigma H-9377), and xanthine oxidase 0.023 U mol^{-1} (Sigma X-4500). The reduction of NBT was measured at 560 nm and constant temperature (25 °C). The rate of NBT reduction in the absence of tissue was used as reference rate. One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50% of maximum inhibition. GR (EC 1.5.4.2) activity was measured by the method described by Carlberg and Mannervick [26] and Smith et al. [27]. This method was based on the absorbance decrease of NADPH at 340 nm. Reaction solution contained buffer phosphate 200 mM (pH 7.5), Triton X-100 10%, DTNB 20 mM (Sigma D-8130), NADPH 100 mM (Sigma N-7505), and GSSG 200 mM (Sigma G-4376). The temperature of incubation was constant (25 °C). GST (EC 2.5.1.18) activity was measured according to Habig et al. [28]. Reaction mixture contained 2 mL of potassium phosphate buffer 100 mM, Triton X-100 10%, CDNB 100 mM (Sigma C-6396), and GSH 100 mM (Sigma G-4251). Reaction was started by adding sample, and absorbance at 340 nm at room temperature was monitored. The GST activity was expressed according to Uguz et al. [29]. Concentrations of GSH and GSSG were also measured in the hepatic cytosolic fraction by a fluorometric assay according the methodology described by Hissin and Hilf [30]. One molar Tris–HCl, 5 mM EDTA (pH 8.0), 10 mM H_2O_2 were mixed and the rate of H_2O_2 consumption at 240 nm and 37 °C was used for quantitative determination of CAT (EC 1.11.1.6) activity [31]. The protein content was determined according to Bradford [32], with bovine serum albumin as standard. All chemicals used in the enzymatic activity were of analytical purity and were obtained from Sigma Chemical Co.

2.3. Histology

When the liver was quickly dissected, 3 mm thick slabs were made. Some pieces were then fixed in Bouin's fixative for 24 h (at room temperature), dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Histological sections (5 μm thick) were cut and stained either with haematoxylin-eosin (H&E) or with the periodic acid Schiff (PAS) reaction. In addition to the qualitative analysis, evaluation of the hepatocellular cytoplasm vacuolization degree was made using a semi-quantitative approach, according to the following five grades and general criteria [33]: Grade 0 (none)—absence of hepatocellular vacuolization; Grade 1 (low)—on average, <25% of the hepatocyte cytoplasm shows vacuolization; Grade 2 (moderate)—on average, $25\% < x < 50\%$ of the hepatocyte cytoplasm shows vacuolization; Grade 3 (high)—on average, $50\% < x < 75\%$ of the hepatocyte cytoplasm shows vacuolization; Grade 4 (extreme)—>75% of the cytoplasm (virtually total) is vacuolated.

2.4. Statistical analysis

Regarding biochemical data, differences among groups were tested by ANOVA followed by a Tukey's multiple comparison test at a 5% significant level. Differences between vacuolisation degrees were tested by the non-parametric Kruskall–Wallis ANOVA and Mann–Whitney U tests. The Spearman's correlation non-parametric test was used for evaluating the strength of the relationship between pairs of variables. All tests were performed using the software STATISTICA 6.0 (StatSoft, Inc., 2001).

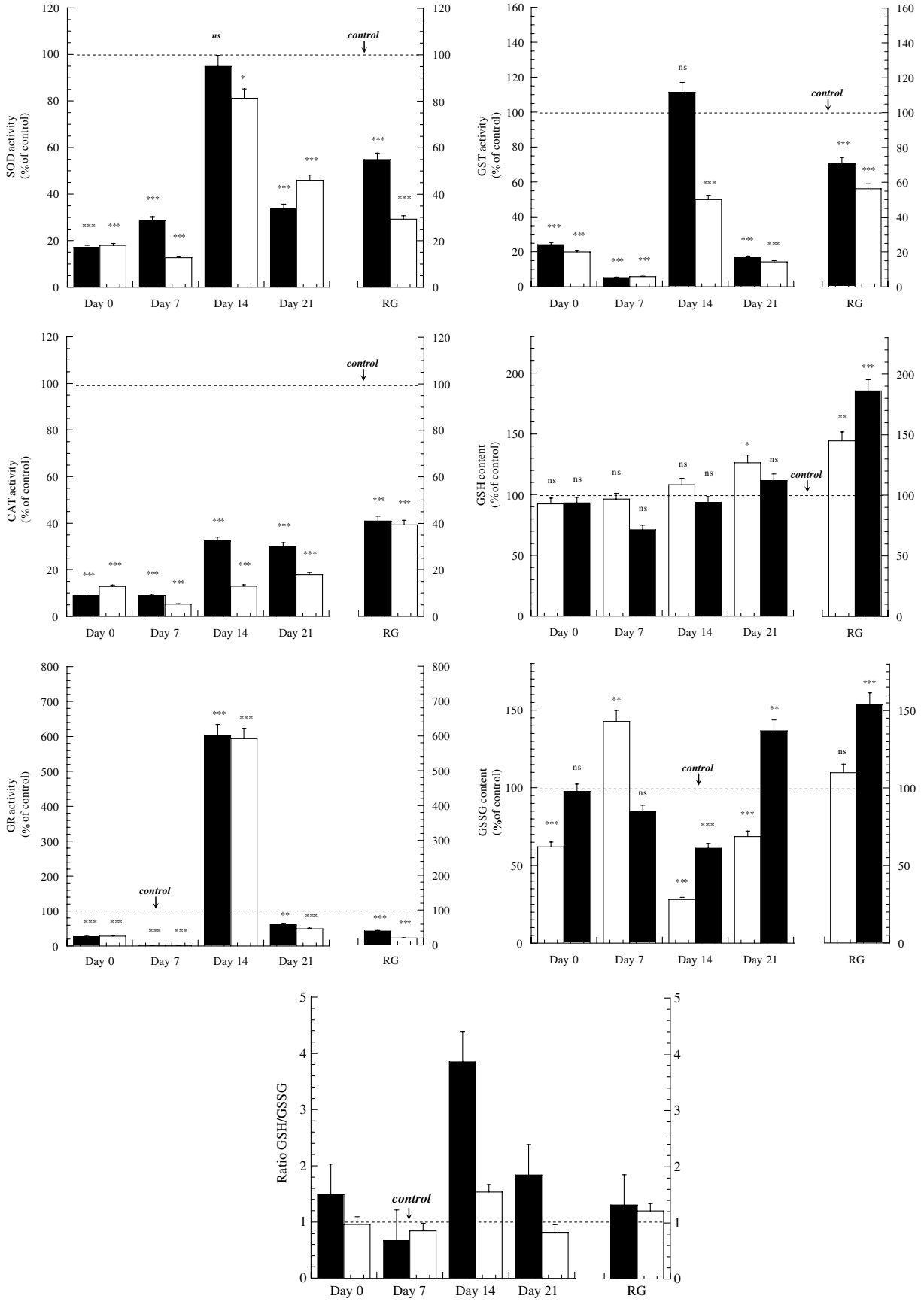
3. Results

3.1. Biochemical analysis

Neither mortality nor visible disease signals were observed in the fish exposed to sublethal concentrations of carbaryl. Changes in the activities of antioxidant enzymes in experimental fish are shown in Fig. 1. Exposure of tilapia *O. niloticus* to carbaryl alters the normal functioning of hepatic activity, by consistently decreasing the SOD, CAT, GR, and GST activities (except for SOD and GST activities after 14 days of exposure to the lower concentration, *i.e.*, 0.25 mg L^{-1}, as well as for GR activity also at 14 days of exposure to both concentrations). In fact, after 14 days of exposure the GR activity in carbaryl-treated fish showed significant elevation in relation to the control. Also, the GST activity in fish exposed to 0.25 mg L^{-1} at 14 days was significantly higher than in the other exposure time but not significantly higher than control value. It is notable that at day 0 fish exposed to carbaryl showed significant lower values in relation to control. The GSH/GSSG ratio in hepatic tissue showed a decrease after carbaryl exposure (at 7 days) followed by an increase at the 14th (both concentrations) and 21st (0.25 mg L^{-1}) days. When fish were transferred into clean water, there was a recovery response, as revealed by the GSH and GSSG content. The SOD and CAT activities of the recovery group (RG) (0.5 mg L^{-1}) were significantly higher when compared with the other exposure times, except for SOD when compared with the 14th day of exposure. The GSH content at 21 days and in the RG was significantly higher when compared with the other exposure days in both concentrations. Finally, concerning the GSSG content the lowest values were observed at 14 days, whereas the RG showed higher values.

3.2. Histology

The homogeneous parenchyma of *O. niloticus* liver from control fish (Fig. 2a) was composed of hepatocytes arranged in a typically complex three-dimensional architecture. Fish exposed to carbaryl (Fig. 2b–f) showed no histo-


SOD activity (% of control)
GST activity (% of control)
CAT activity (% of control)
GSH content (% of control)
GR activity (% of control)
GSSG content (% of control)
Ratio GSH/GSSG
control
ns
Day 0
Day 7
Day 14
Day 21
RG

logical modifications, like inflammation or perceivable changes in the pool of pigmented macrophages. However, it was observed necrosis in fish exposed to carbaryl (0.25 mg L^{-1}) at the 7th day (Fig. 2c). The sections (overall weakly) stained with PAS reaction showed, indirectly, that the vacuolization was mainly due to lipid accumulation. Fig. 2g represents the recovering group from fish exposed to 0.5 mg L^{-1}, showing that the hepatocyte nuclei in the RG were more peripheric when compared to control group. The semi-quantitative analysis of the histological results suggests there are differences in the VD, as function of the day of exposure (Table 1). The VD was low at 7th and 14th days, whereas at 21st and RG the values were similar to those of day 0. No significant correlations between VD and GSH or VD and GSSG were observed.

4. Discussion

Pesticide-induced oxidative stress is the final manifestation of a multi-step pathway, resulting in an imbalance between pro-oxidant and antioxidant defence mechanisms [34]. Oxidative stress has been shown to be associated with exposure to several pesticides [35]. Herein, we studied the influence of carbaryl on toxicity related to oxidative stress. Despite that carbamate insecticide is reported to have a low persistence in water, very little is known about the toxic mechanism of carbaryl on aquatic organisms.

The results showed an effect of carbaryl on the enzymatic activities and in GSH and GSSG content in relation to control. It was also observed a decrease of enzymatic activity in relation to control group at day 0 in fish exposed to both concentrations of carbaryl. This decrease may derive from the sudden change in the fish environment, since they were moved from maintenance tanks with clean water to the aquaria with dissolved carbaryl. Although tilapia remained in these tanks for only 45 min before being sampled, the fact is that there was a quite rapid adaptative response to carbaryl exposure, compared to controls that did not show any change. There are indeed reports of very rapid gene transcription in fish after toxicant exposure, such as the case of CYPIA1 under the dioxin TCDD activation [36,37].

The inhibition of CAT activity may be the result of H_2O_2 increase in the cell which can be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of O_2 followed by dismutation of $O_2^{-\cdot}$ [38]. Some pesticides caused an increase in CAT activity [39,40], while a decline in this activity was observed by Babo and Vasseur [41].

Several studies suggest that carbaryl might affect either the biotransformation or oxidative stress defence system in different classes of animals [2,42,43]. Our results shown that at 14 days of exposure a metabolic change occurred, which may be due to the generation of reactive species in other metabolic pathway to that one of carbaryl. One of the multiple metabolites resulting from carbaryl degradation could be the responsible for that response. The 1-naphtol is the principal metabolite resulting from carbaryl degradation in some biological models, and can induce oxidative stress in the liver and erythrocytes of rats [44,45]. The accumulation of this metabolite in significant concentrations between 7 and 14 days of exposure and its degradation products may be the reason for the increase of enzymatic activities observed after 14 days of exposition to carbaryl.

In an oxidative stress situation, it would be normal to observe an increase in GR activity in order to re-establish the levels of GSH that is oxidised [46,47]. The present study shows an increase in the GR activity only in 14th day. On the other hand, the GSH content was rather stable during the exposure period. It may be an evidence that GSH is being synthesized by GSSG reduction and not because its need due to its involvement in other metabolic processes, like participating in ascorbic acid metabolism, maintaining intercellular communication and generally preventing protein–SH groups from oxidizing and cross-linking [20]. The GSH/GSSG ratio is a good indicator of oxidative stress. For example, the GSSG/GSH ratio in contaminated bivalve molluscs from Spanish coast under oxidative stress conditions showed a significant increase [48]. In our case, after 14 days of exposure the GSH/GSSG ratio was higher than the control group, whereas in the remaining periods it was alike the control. In summary, considering a determined biological model, increased enzymatic activities levels are evidence that the antioxidant system is attempting to protect the organism against any damage caused by oxidative stressors [49]. However, in the present study this increase was not observed.

In relation to the histological results, the homogeneous parenchyma of *O. niloticus* liver from control fish was quite similar to that described by Figueiredo-Fernandes et al. [50]. The occasional necrotic foci were not significant in number and frequency. The data concerning the cellular VD did not show significant changes when the fish were exposed to carbaryl. Our results are similar to those obtained by Tos-Luty et al. [51] in rats exposed to carbaryl, in the sense that only slight histological changes were seen.

Fig. 1. Liver antioxidant activities (means ± SD) in Nile tilapia *O. niloticus* ($n = 6$) exposed to sublethal concentrations of carbaryl: (■ 0.25 mg L^{-1} and □ 0.5 mg L^{-1}). The activity levels of treated fish were compared with control group in each sampling day, including recovery group (RG). The control groups values were the following: SOD 4.11 ± 0.05 U/mg protein; CAT 4.95 ± 0.14 U/mg protein; GST 3.61 ± 0.03 U/mg protein; GR 6.49 ± 0.20 U/mg protein; GSH—2.74 ± 0.03 μmol g^{-1} tissue; GSSG—0.15 ± 0.01 μmol g^{-1} tissue; GSH/GSSG was 18.77 ± 1.52 μmol g^{-1} tissue. [$^{***}p \leqslant 0.001$; $^{**}p \leqslant 0.01$; $^{*}p \leqslant 0.05$; ns, not significant].

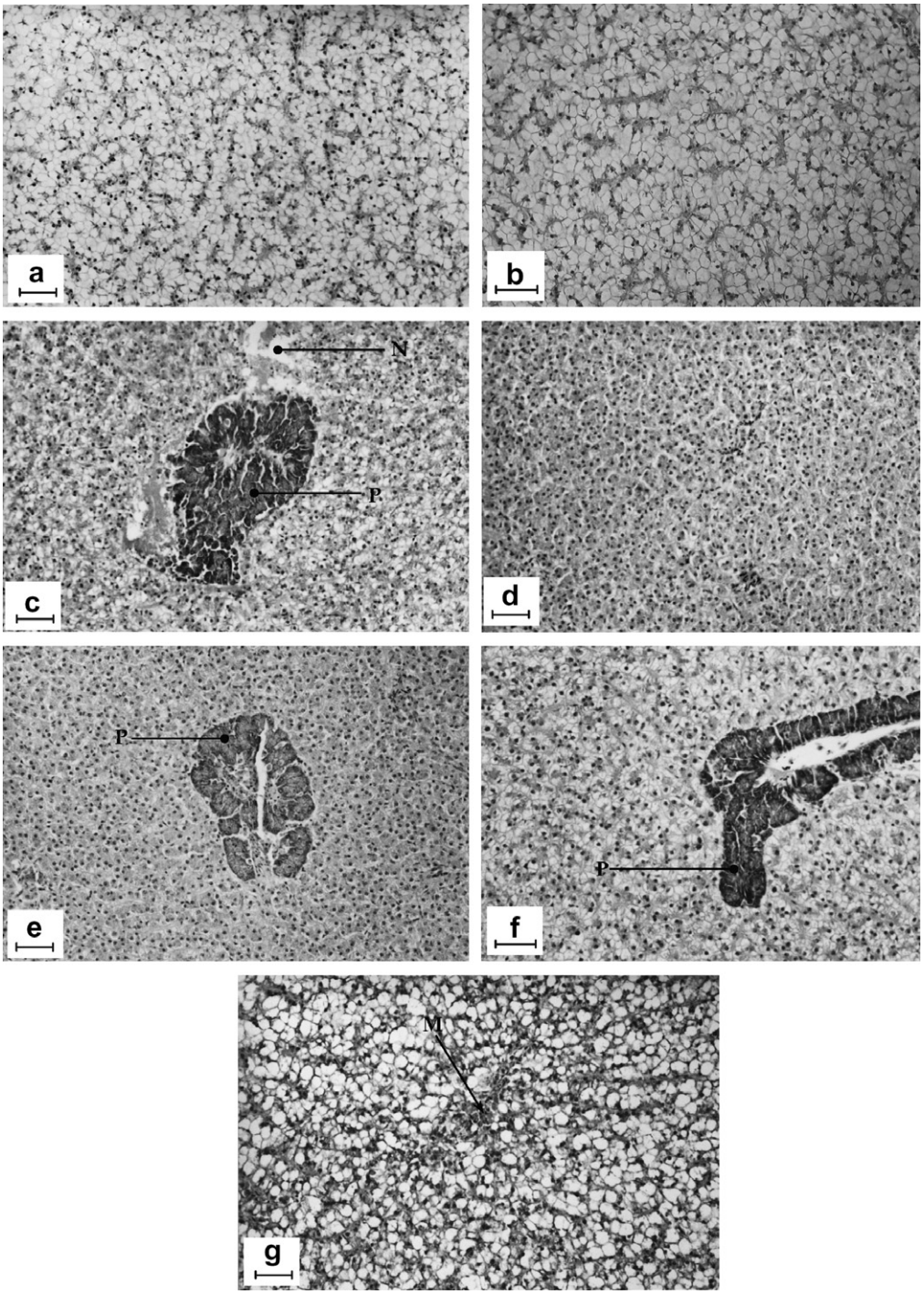


Fig. 2. Histological images of Nile tilapia (*Oreochromis niloticus*) liver. Control group (a), day 0 group (b) and fish exposed to carbaryl (c—7th day, 0.25 mg L^{-1}; d—7th day, 0.5 mg L^{-1}; e—14th day, 0.25 mg L^{-1}; f—21st day, 0.25 mg L^{-1}; g—recovering group from fish exposed to 0.5 mg L^{-1}). The series of images illustrate the gradual increase in the basophilia of the hepatic parenchyma, as seen by the increasingly darker hepatocytes. The darkest areas in the images correspond to exocrine cells of the pancreas, typically surrounding afferent veins (b, d, and e); exogenous pancreas (P) and necrosis (N); H&E, bars = 50 μm.

In fish exposed to carbaryl it was observed a relation between the parenchyma heterogeneous vacuolization appearance and the duration of exposure, but also a concomitant increase in the hepatocellular basophilia. The combined increased basophilia and loss of hepatic glycogen is a common, although non-specific, liver response to many toxicants [52,53]. Most commonly, the greater cell basophilia invariably results from an increased relative amount of rough endoplasmic reticulum in the cytoplasm, as previous correlative studies have demonstrated in fish hepatocytes [52,54,55].

This study proved that exposure to sublethal concentrations of carbaryl evoked in Nile tilapia hepatocellular changes suggestive of an adaptive response, for example

Table 1
The vacuolization degree of the hepatocytes as a function of exposure day and concentration of carbaryl ($n = 6$)

Day	Concentration (mg L^{-1})	Minimum	Maximum	Median	Mode
Control	0.25	1	3	3.00	3
	0.5	1	3	3.00	3
0	0.25	1	3	3.00	3
	0.5	1	3	3.00	3
7	0.25	1	3	2.50	3
	0.5	0	3	1.50	0
14	0.25	0	3	2.00	3
	0.5	1	3	2.00	2
21	0.25	2	3	3.00	3
	0.5	2	3	3.00	3
RG	0.25	2	3	2.50	2
	0.5	2	3	3.00	3

as revealed by the glycogen depletion and augmented basophilia. The biochemical data showed there was a very rapid response after the fish contact with the compound. The kinetics of the response was not linear in time, as, for certain biochemical targets we noticed a different behaviour at the day 14 and/or at the day 21. The results of the recovery data suggest that the toxicity produced by carbaryl is reversible to some extent within 15 days after stimulus withdraw. In conclusion, in this work histological and biochemical changes of liver have been related to carbaryl exposure and concentration, warning for the potentially negative impact of this insecticide for wild fish, especially in case of persistence of contact with carbaryl contaminated water.

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