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Original article

In vivo changes in carbonic anhydrase activity and histopathology of gill and liver tissues after acute exposure to chlorpyrifos in rainbow trout

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Chlorpyrifos is an organophosphate pesticide widely used in agriculture and aquaculture. This study investigated its effects on carbonic anhydrase (CA) enzyme activity and histopathology of rainbow trout gill and liver. The fish were exposed to 2.25 (25 % of 96 h LC_{50}), 4.5 (50 % of 96 h LC_{50}), and 6.75 μg L^{-1} (75 % of 96 h LC_{50}) of chlorpyrifos for 24, 48, 72, and 96 h. CA activity was measured in liver and gills and histopathological changes were examined by light microscopy. The most common liver changes at most of the chlorpyrifos concentrations were hyperaemia and degenerative changes. Gill tissues were characterised by lamellar hyperaemia, lamellar oedemas, clumping, cellular degeneration, hyperplasia, and lamellar atrophy. CA enzyme activity in the gills decreased at all concentrations at 48, 72, and 96 h after exposure to chlorpyrifos ($p<0.05$). Similarly, there was a time-dependent decrease in CA activity at all of the concentrations in liver tissues ($p<0.05$). The present study indicated that chlorpyrifos inhibits CA enzyme activity and causes histopathological damage in gill and liver tissues.

KEY WORDS: *acute toxicity; fish; histology; light microscopy; organophosphate pesticides*

Chlorpyrifos is an organophosphate pesticide widely used in agriculture, aquaculture, and fishery pest control (1, 2). Numerous environmental issues have arisen so far due to the excessive use of this chemical compound (3), as it, among other consequences, causes toxic effects in non-target aquatic organisms, especially fish (4). Fish are used to assess the health of aquatic environments and physiological changes occurring as a result of pollution and multiple studies have already established that chlorpyrifos has various detrimental effects on them (5-10), such as neurotoxicity via acetylcholinesterase inhibition (11), biochemical and histopathological alterations (12, 13), oxidative stress (12, 14), genotoxicity (15), and olfactory and neurobehavioral injuries (16). It therefore poses a serious threat to aquatic organisms as well as to human health (17). Rainbow trout has been selected for this study, because it is a sensitive indicator of aquatic pollution (18) and one of the most studied fish species due to its importance as food in terms of nutritional and economic value (19).

Carbonic anhydrase (CA) is a zinc metalloenzyme found in the tissues of most eukaryotes and has important physiological functions such as respiration, gas balance, lipogenesis, ureagenesis, bone resorption, or body fluid generation in various tissues (20, 21). CA plays an important role in the excretion of metabolic carbon dioxide in fish and catalyses the reversible hydration/dehydration of carbon dioxide to bicarbonate and protons (22, 23). Therefore, any inhibition of this enzyme leads to unfavourable effects for living organisms. Additionally, CA can also be used as a biomarker of toxicity (24).

Histological techniques are used to assess the toxic effect of pollutants such as pesticides and heavy metals in the aquatic environment (25). Little information is available on chlorpyrifos toxicity and effects on CA sensitivity in fish. This study was designed to determine CA activity levels and histopathological changes in gill and liver tissues of rainbow trout after acute exposure to chlorpyrifos.

MATERIALS AND METHODS

Our experiments were performed on rainbow trout, *Oncorhynchus mykiss* (body mass 171±5.73 g and average length 19.47±0.94 cm). They were obtained from the Ataturk University Faculty of Fisheries and Inland Water Fish Breeding and Research Center. Experiments were carried out in 4 fiberglass tanks (each 400 L) each containing 15 fish. The tanks were filled with dechlorinated tap water (temperature 10-12 °C, pH 7.1±0.3, dissolved oxygen 8.2±0.5 mg L^{-1}, water hardness 174.6±5.19 mg L^{-1} $CaCO_3$, SO_4^{-2}=0.36 mg L^{-1}, PO_4^{-3}=trace, NO_3^-=1.51 mg L^{-1} and NO_2^-=trace) and acclimated to laboratory conditions for 15 days. During acclimation, the fish were fed 2.5 % body weight with commercial trout pellets (Sibal Group, Sinop, Turkey).

Chlorpyrifos was at 99.2 % purity and the study used its commercial formulation [480 g L^{-1} chlorpyrifos, O,O-diethyl-O-(3,5,6-trichlor-2-pyridyl) phosphorothioate] purchased from a distributor company (Platin Chemistry, Turkey). The stock solution of chlorpyrifos was prepared by dissolving in distilled water.

Exposure to chlorpyrifos

The chlorpyrifos LC_{50} value for rainbow trout was set at 9 µg L^{-1} (26). The concentrations used for this study were 25 % (2.25 µg L^{-1}), 50 % (4.5 µg L^{-1}), and 75 % (6.75 µg L^{-1}) of the LC_{50} value. These concentrations were chosen because they were lower than the lethal concentrations for rainbow trout, and also may occur in a polluted environment. The fish were exposed to these concentrations for 24, 48, 72, and 96 h. At the end of each exposure period, fish were randomly selected from the control and exposed groups and sampled. The fish were sacrificed by cervical section and the liver and gill tissues were immediately removed. A portion of the tissues was washed with physiological saline (0.9 % NaCl) and stored at -20 °C until analysis for CA activity. The other portion of the tissues was fixed in 10 % formalin solution for histopathological examination.

Determination of CA activity

Liver and gill tissue samples were washed three times with 0.9 % NaCl. Each tissue was homogenised with buffer 25 mmol L^{-1} Tris–HCl+0.1 mol L^{-1} Na_2SO_4 (pH 8.7) by homogenizer and the supernatant was centrifuged at 4 °C, 15000 *g* for 60 min. Enzyme activity was assayed by following CO_2 hydration according to the protocol established by Wilbur and Anderson (27). CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (t_o-t_c/t_c) where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Histopathology procedures

After the routine histopathology process, paraffin sections were stained in 5 µ with hematoxylin and eosine (HE). Histopathological changes were semi-quantitatively assessed under a light microscope (Olympus BX51 with DP72 camera attachment system, Tokyo, Japan). The scores were derived as semi-quantitative according to severity and extent of changes and are reported as follows: none:−; mild:+; moderate:++; and severe:+++.

Statistical analyses

All data are expressed as mean±SEM. Statistical analysis of data was done using one-way analysis of variance (ANOVA) and LSD test and analysed using SPSS version 10.0. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Aquatic ecosystems are often faced with problems caused by contaminants released into the environment (28). Various non-target organisms, especially fish, are exposed to pesticides such as chlorpyrifos and this may cause many adverse effects, including biochemical alterations (29). In order to add to risk assessment studies conducted thus far, we strived to obtain information about the effects of chlorpyrifos on rainbow trout, one of the most studied species owing to its previously mentioned importance.

CA activity in liver and gill tissues

A time-dependent decrease in enzyme activity was evident, as CA activity decreased at all of the applied concentrations in all liver and gill tissues after 96 h ($p<0.05$). Exposure to 4.5 and 6.75 µg L^{-1} of chlorpyrifos for 72 h also caused a statistically significant decrease in liver and gill CA activity ($p<0.05$). Furthermore, the 6.75 µg L^{-1} concentration decreased CA activity after 48 h in both tissues and only in gill tissues after 24 h, while the 4.5 µg L^{-1} concentration also affected only gill tissue after 24 h when compared to the controls ($p<0.05$) (Figures 1 and 2). There was no statistically significant difference at either of the concentrations after 24 h in liver tissues when compared to the control (Figure 1).

Many chemical substances lead to changes in metabolism by changing enzyme activity, particularly via inhibition of a specific enzyme (30). There are many studies in the literature about the effects of pesticides on CA activity in different fish species and rainbow trout in particular (9, 23, 31, 32). For example, the pesticides deltamethrin, diazinon, propoxur, and cypermethrin were tested on rainbow trout gill CA activity by Ceyhun et al. (33) exhibiting inhibitory effects *in vivo* and *in vitro*. In another study, Dogan (23) reported that pesticides such as lambda-cyhalothrin, deltametrin, diozinon, dorzolamide, and brinzolamide caused inhibitory effects on CA activity in rainbow trout blood. CA is known to play an important role in the excretion of metabolic CO_2 as

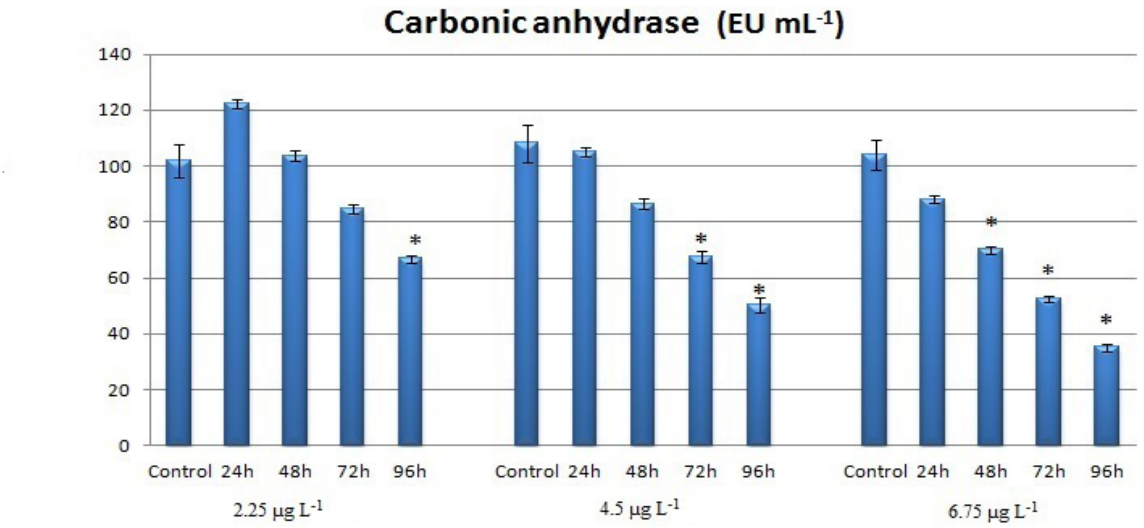

Figure 1 *The effects of chlorpyrifos on liver carbonic anhydrase enzyme activity of rainbow trout. Values are expressed as mean±S.E.M. Significant difference from control values* *p<*0.05. EU-enzyme units*

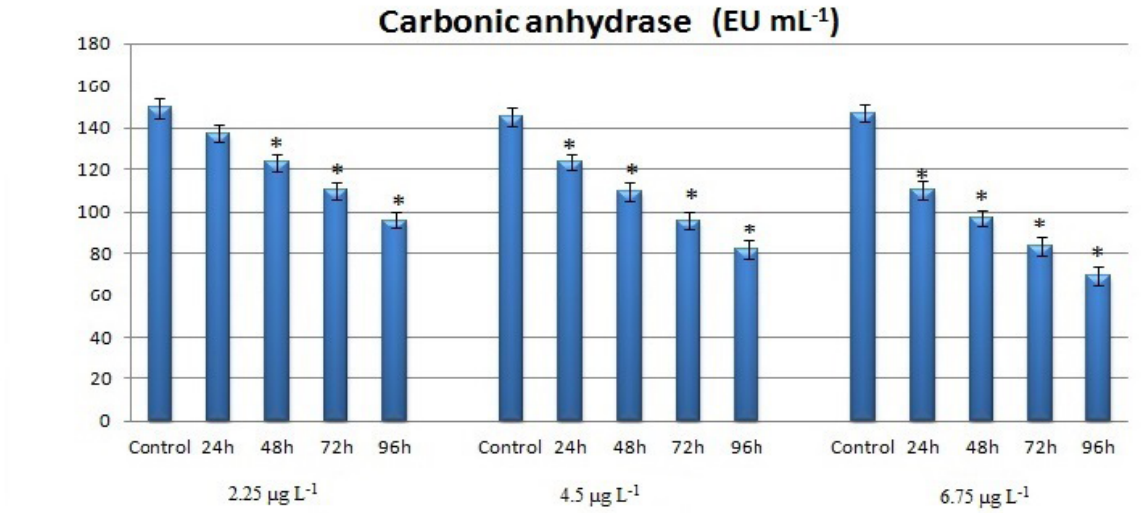

Figure 2 *The effects of chlorpyrifos on gill carbonic anhydrase enzyme activity of rainbow trout. Values are expressed as mean±S.E.M. Significant difference from control values* *p<*0.05. EU-enzyme units*

well as in CO_2 exchange between tissues and blood in fish and in catalysing the reversible hydration/dehydration of CO_2 to bicarbonate and protons (22, 23, 34).

Our results have shown that there was a time-dependent decrease in enzyme activity after exposure to chlorpyrifos at certain concentrations. This decrease can be explained by a decrease in CO_2 hydration. It has been reported that, in catalysis, CO_2 hydration is defined by the attack of a Zn^{2+} bound hydroxide on CO_2 to yield a Zn^{2+} bound HCO_3^- species. HCO_3^- is subsequently replaced by water to yield a Zn^{2+}-bound water molecule (23). In our case, the supply of HCO_3^- decreased with H^+ excretion (35). Accordingly, we can say that chlorpyrifos inhibited the enzyme at very low concentrations due to the electronegative atoms in the pesticide's chemical structures (9).

Gill and liver histopathology

In the present study, no histopathological changes were established in the control liver tissues and at 2.25 µg L^{-1} of chlorpyrifos (Figure 3). Hyperaemia and degenerative changes were observed at 48, 72, and 96 h of exposure to 4.5 and 6.75 µg L^{-1} of chlorpyrifos (Figure 4) (Table 1).

Fish liver histopathology is an indicator of chemical toxicity and a useful way to study the effects of the exposure of aquatic animals to toxins present in the aquatic environment (36). The effects of different pesticides on liver in various fish species have already been reported in other studies. Chlorpyrifos caused damages such as melanomacrophage aggregations, cellular atrophy, pyknotic nucleus, cytoplasmic vacuolation, cytoplasmic and nuclear degeneration, cellular rupture, necrosis, and nuclear and cellular hypertrophy in the liver tissues of the common carp, while phosalone induced histopathological changes such as nuclear degeneration, cytoplasmic vacuolation, hypertrophy, and congestion (37, 38).

No histopathological changes were observed in the gill tissues of the control group (Figure 3). Gill tissues showed lamellar hyperaemia at 48, 72, and 96 h of exposure to 2.25 µg L^{-1} and at 24, 48, 72, and 96 h at the two other concentrations (Table 1). Lamellar oedemas were intensive in all of the groups (Figures 5, 6, and 7). Apart from these, there were also other histopathological changes such as lamellar atrophy, hyperplasia, cellular degeneration, and clumping to a varying degree (Table 1).

Environmental pollutants cause pathological changes in fish physiology (39). Gills are especially suitable for histopathological examination to determine the effects of pollution because these tissues are frequently those that are adversely affected by contaminants in the aquatic environment; for instance through osmoregulatory function and reduced oxygen consumption (40-43). In support of our observation, Pal et al. (37) observed numerous lesions in the gill tissues of common carp exposed to chlorpyrifos. Ba-

Table 1 *Histopathological comparison of control and experimental groups none:−, mild:+, moderate:++ and severe:+++*

Lesion	Control				2.25 µg L^{-1}				4.5 µg L^{-1}				6.75 µg L^{-1}			
	Hours															
	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Liver tissue																
Hyperaemia	-	-	-	-	-	-	-	-	-	+	+	++	-	+	++	+++
Degenerative changes	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+
Gill tissue																
Lamellar hyperaemia	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Lamellar oedema	-	-	-	-	++	++	++	++	++	++	++	++	++	++	++	++
Clumping	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Cellular degeneration	-	-	-	-	-	-	+	+	-	+	+	+	-	+	+	+
Hyperplasia	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+
Lamellar atrophy	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+

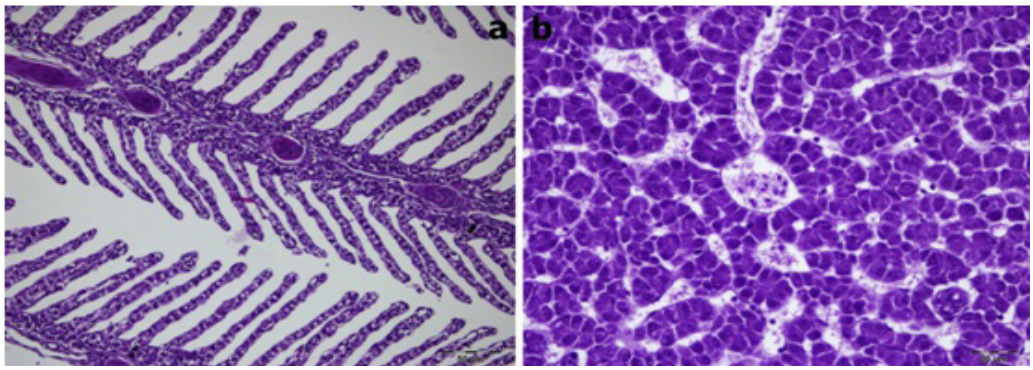

Figure 3 *Normal histological appearances of gill (left) and liver (right) sections control group*

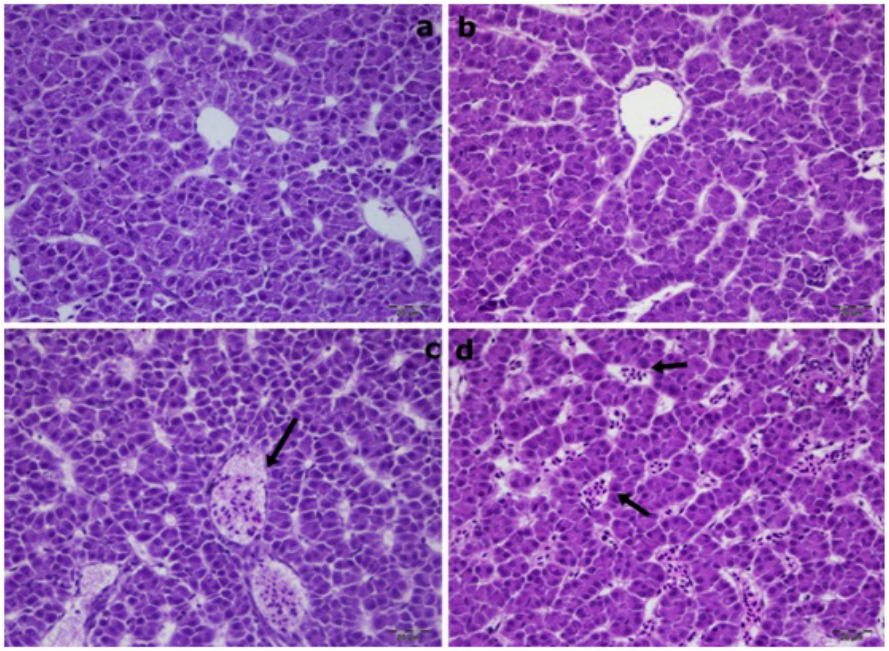

Figure 4 *There was no histopathological change in the liver tissues at 2.25 µg L^{-1} of chlorpyrifos (a and b). Dilated and hyperaemic central veins (arrow in c) and sinusoids (arrows in d) at 48, 72, and 96h of 4.5 and 6.75 µg L^{-1} of chlorpyrifos*

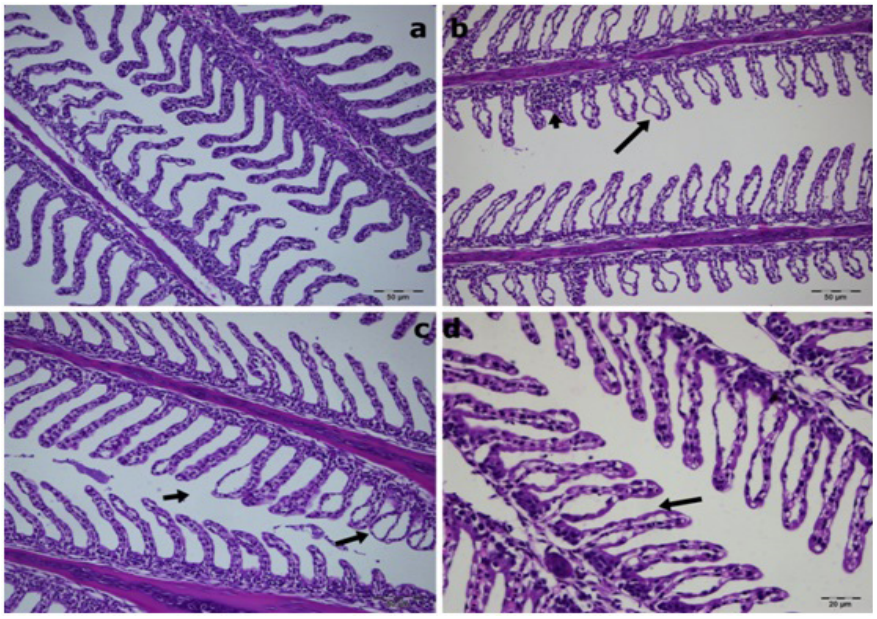

Figure 5 *Gill tissue sections from fish exposed to 2.25 µg L^{-1} of chlorpyrifos. Curled lamellas (a) at 24 h and severe oedematous changes (arrows in b-d) at 48, 72, and 96 h, respectively*

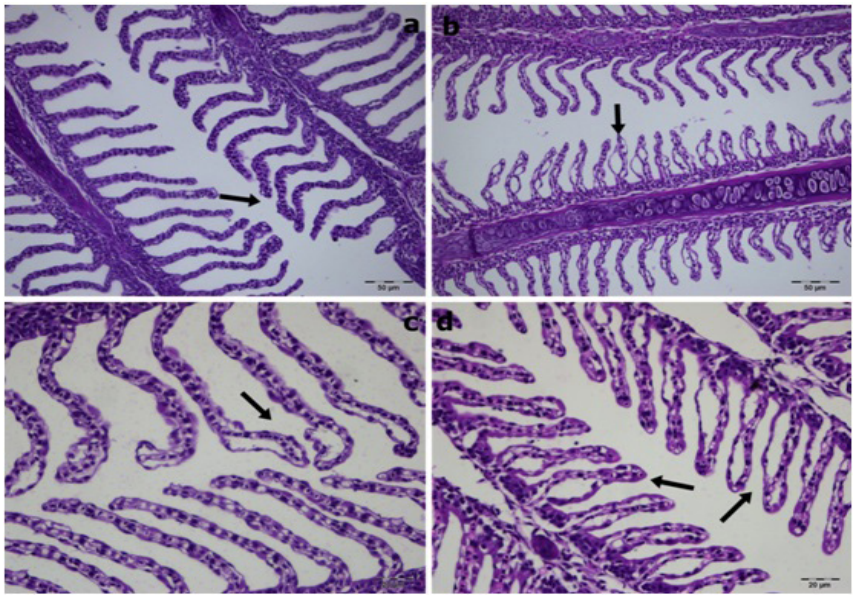

Figure 6 *Gill tissue sections from fish exposed to 4.5 µg L^{-1} of chlorpyrifos. Curled lamellas and clumpings (arrow in a) at 24 h severe oedematous changes (arrows in b-d) at 48, 72, and 96 h, respectively*

Omar et al. (44) observed hypertrophy, epithelial lifting, desquamation and lamellar fusion in the gill tissues of *Aphanius dispar* exposed to pesticide temephos which is known as a non-systemic organophosphorus pesticide. Our study has shown results very similar to these.

In conclusion, our results generally suggest that chlorpyrifos inhibits the CA enzyme and causes histopathological damages in gill and liver tissues under *in vivo* conditions, which proves that fish in both cultured and natural environments are sensitive to this pesticide and that chlorpyrifos contaminations would cause fish deaths. Therefore, stricter control must be applied to the use of this pesticide.

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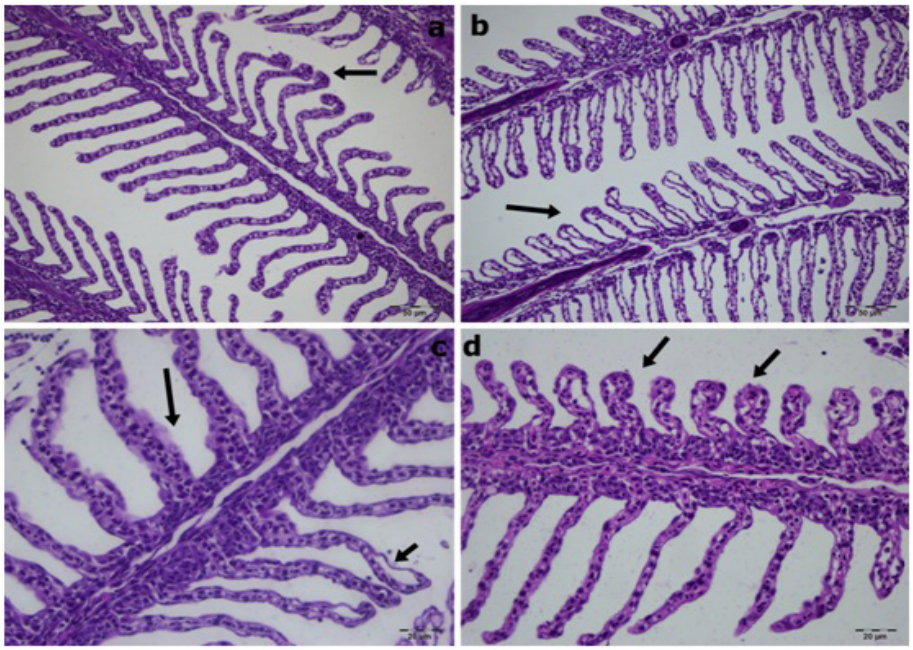

Figure 7 *Gill tissue sections from fish exposed to 6.75 μg L^{-1} of chlorpyrifos. Curled lamellas and clumpings (arrow in a) at 24 h. Oedematous lamellas (arrow in b) at 48 h, hyperplastic (long arrow in c) at 72 h, and capillary dilatation (arrows in d) in shortened lamellae at 96 h*

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Sažetak

Promjene u razini ugljikove anhidraze i histopatologiji škrga i jetre kalifornijske pastrve nakon izlaganja klorpirifosu

Klorpirifos je organofosforni pesticid široke primjene u poljoprivredi i ribarstvu. U ovome radu istražili smo njegov učinak na aktivnost enzima ugljikove anhidraze te histopatologiju škrga i jetre u kalifornijske pastrve. Ribe su bile izložene klorpirifosu u koncentracijama 2,25 μg L^{-1} (25 % 96-satnog LC_{50}), 4,5 μg L^{-1} (50 % 96-satnog LC_{50}) i 6,75 μg L^{-1} (75 % 96-satnog LC_{50}) tijekom 24, 48, 72 i 96 sati. Aktivnost ugljikove anhidraze mjerena je u jetri i škrgama, a histopatološke promjene promatrane su svjetlosnom mikroskopijom. Najčešće promjene u jetri pri većini koncentracija bile su hiperemija i degenerativne promjene. Na tkivu škrga primijećeni su hiperemija i edemi u škržnim listićima, sljepljivanje i degeneracija stanica, hiperplazija te atrofija škržnih listića. Aktivnost ugljikove anhidraze u škrgama smanjila se pri svim koncentracijama nakon 48, 72 i 96 sati izloženosti (p<0.05). Također je uočeno i smanjenje aktivnosti ugljikove anhidraze u jetri ovisno o duljini izloženosti pri svim koncentracijama (p<0.05). Dobiveni rezultati upućuju na to da klorpirifos inhibira aktivnost ugljikove anhidraze i izaziva značajna histopatološka oštećenja u škrgama i jetri.

KLJUČNE RIJEČI: *akutna toksičnost; histologija; pesticidi; ribe; svjetlosna mikroskopija*

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