

# Histopathological effects of atrazine on gills of Caspian kutum *Rutilus frisii kutum* fingerlings

Zahra Khoshnood<sup>1,\*</sup>, Shahla Jamili<sup>2</sup>, Saber Khodabandeh<sup>3</sup>

<sup>1</sup>Department of Experimental Sciences, College of Science, Dezful Branch, Islamic Azad University, PO Box 313, Dezful, Khuzestan, Iran

<sup>2</sup>Iranian Fisheries Researches Organization, Tehran, Iran

<sup>3</sup>Department of Marine Biology, Faculty of Marine Sciences, Tarbiat Modares University, Tehran, Iran

**ABSTRACT:** The use of chemical pesticides has increased environmental pollution and affects fishes as non-target organisms. To investigate the toxic effects of the widely used herbicide atrazine on Caspian kutum *Rutilus frisii kutum* fingerlings, fish were exposed to a sublethal concentration of half LC<sub>50</sub> for 96 h. The main alterations visible in the gill tissue were detachment of the epithelium of the lamellae, necrosis, lamellar fusion, hyperplasia, club shaped lamellae, collapse of the lamellae, shrinkage and curling of the lamellae, and ultrastructural alterations such as necrosis of the apical microridges of the pavement cells. Results also showed that the gill ionocytes were fewer in number and larger in size in the atrazine-exposed fish. Atrazine appears to be highly toxic to Caspian kutum fingerlings even at a sublethal concentration (12.47 mg l<sup>-1</sup>) and acute exposure. This toxicity could affect gill respiration and ion regulation function of fingerlings by damaging tissue, pavement cells, and ionocytes.

**KEY WORDS:** Ionocyte · Osmoregulation · Toxicology · Immunohistochemistry · Ultrastructure

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## INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a white crystalline solid synthetic herbicide which is used to kill broad-leaved weeds in agricultural and roadway applications (USEPA 2002). The herbicide reaches aquatic systems via atmospheric deposition and runoff from agricultural fields; it can be detected in nearly every surface water system in the world and has a half-life ranging from 2 to 800 d, depending on pH and other environmental factors. Concentrations of atrazine in the aquatic environment can exceed 20 mg l<sup>-1</sup> (Solomon et al. 1996).

In freshwater invertebrates, atrazine has been found to affect hydromineral balance and gill function in crabs (Waring & Moore 2004). Fish are very susceptible to bioaccumulation of environmental pollutants in their fatty tissues, as they take up lindane residues from the water through the gills and skin (Ortiz et al. 2002). The exposure to chemical contam-

inants can induce a number of lesions and injuries to different fish organs that are suitable for histopathological examination of damage to tissues and cells (Rabitto et al. 2005).

In fish, gills are vital organs that perform respiratory and osmoregulatory functions (Evans et al. 2005), and respiratory distress is one of the early symptoms of pesticide intoxication (Ortiz et al. 2002). Considerable interest has been shown in histopathological examinations following sub-lethal tests in fish. In test organisms exposed to sub-lethal concentrations of toxicants, tissue changes are a functional response which provides information on the nature of the toxicants (Davoodi et al. 2009).

The Caspian kutum *Rutilus frisii kutum* (Kamensky 1901), in the family Cyprinidae, is a native, economically important fish species of the Caspian Sea. This migratory anadromous fish spawns from March to April on aquatic weeds and gravel and sandy substrates in freshwater rivers and lagoons (Razavi 1995,

\*Corresponding author: zkhoshnood@gmail.com

Abdoli 1999). The kutum is of great importance in commercial markets of the region. As a result of over-fishing, urban and agricultural pollution, destruction of natural spawning rivers, and other factors (Abdoli 1999), this species is now considered endangered and is therefore used annually in stock recruitment programs by the Iranian government in an attempt to improve the Caspian Sea ecosystem.

The environmental concentration of atrazine in the study area varies depending on agricultural activities, rain regime, and season, among other factors. Kutum fingerlings produced annually in the stock recruitment facilities are not released at fixed sites; rather, they are released at different sites in different years, depending on site availability. For these reasons, estimating the effects of environmental concentrations of atrazine would be difficult in just one study. Due to intense agricultural and urban activities near the Caspian Sea, we felt it necessary to investigate the gill structural and ultrastructural histopathology which may occur in *R. frisii kutum* following exposure to a sublethal concentration of atrazine. The information obtained from this study may be useful for monitoring atrazine contamination in the environment by using fish histopathological biomarkers. Moreover, the results from the test species could be used as basic information for further research in the field of atrazine toxicity.

## MATERIALS AND METHODS

### Fish and experimental design

Caspian kutum fingerlings were obtained from Shahid Ansari Fish Proliferation and Culture Center, Rasht, Iran, in July 2011. Mean total length and mean body weight of fingerlings were 3.5 cm and 2.6 g. Following the determination of 96 h LC<sub>50</sub> of atrazine for the fingerlings (Khoshnood et al. in press), a sublethal concentration was determined as ½ LC<sub>50</sub> (12.47 mg l<sup>-1</sup>). Atrazine was dissolved in distilled water, filtered, and added to the aquarium following the method of Pluta (1989).

Fish were exposed to this sublethal concentration for 96 h in triplicate groups of 30 fish each (biomass ~0.43 g l<sup>-1</sup>) in glass aquaria, under laboratory conditions (see below). One triplicate group of fingerlings was held in clean water as the control group. No mortality was observed during the experiments in all groups, and no feeding was performed for all groups during the experiment. Water parameters were monitored daily throughout the experiment for all groups

(Eutech instruments pcd650), and the values were as follows: temperature: 14.5 ± 0.5°C, pH: 7.6 ± 0.1, dissolved oxygen: 8.5 ± 0.5 mg l<sup>-1</sup>, and a photoperiod of 12:12 h L:D. Water quality conditions (pH, temperature, O<sub>2</sub>) did not differ among treatments, and water was not changed during the experiment.

Atrazine was analyzed in the following exposure solutions: atrazine nominal concentration of 0 mg l<sup>-1</sup> on Day 0 for the control group and 12.47 mg l<sup>-1</sup> at the beginning (t<sub>0</sub>) and end (t<sub>24</sub>) of each 24 h exposure period in the atrazine-exposed group. These measurements were used to assess the potential contamination of the controls, as well as variability associated with the preparation of the solution and/or potential degradation of atrazine during the 24 h period of exposure. Chemical analyses at the end (t<sub>24</sub>) of each 24 h exposure period of the 96 h assay were performed on pooled water samples of all 3 replicates of each experimental group. Various volumes of exposure solutions were collected, depending on the nominal atrazine concentration, and transferred into a clean glass bottle with 10 ng of the labeled surrogate compound <sup>13</sup>C<sub>12</sub> PCB-101 to assess the extraction efficiency. Extraction was performed 3 times with a volume of dichloromethane corresponding to approximately 25% of the collected solution. Combined extracts were then reduced to about 50 µl and completed with 50 µl of Tris (4-chlorophenyl) methane (TCPMe, 100 pg µl<sup>-1</sup>) as an internal standard. Analysis was conducted on a gas chromatograph (GC) equipped with a DB-5MS capillary column coupled to a Varian Saturn 2000 ion trap mass spectrometer (MS) by a transfer line kept at 300°C. The injector was operated in splitless mode. Helium was used as the carrier gas (flow rate, 1.0 ml min<sup>-1</sup>). Ionization was performed by electron impact at 70 eV, and the ion trap was operated in MS–MS mode. Atrazine concentrations were calculated based on their response relative to that of <sup>13</sup>C<sub>12</sub> PCB-101 in the same sample. The relative response factor was determined on the basis of a 4-point calibration curve for atrazine, while <sup>13</sup>C<sub>12</sub> PCB-101 and TCPMe were kept at constant concentration (100 µg l<sup>-1</sup>). Atrazine concentrations were corrected based on the recovery of the surrogate compound. Limit of quantification was 0.003 ng l<sup>-1</sup> for atrazine, and analytical precision was 6%.

### Histology and immunohistochemistry

For histological and immunohistochemical studies, 10 fish from each experimental group were euthanized in 100 mg l<sup>-1</sup> of MS222 and 100 mg l<sup>-1</sup> of

sodium bicarbonate, and the whole body was immediately immersed into Bouin's fixative for 24 h, rinsed, and dehydrated in an ascending series of ethanol for embedding in Paraffin (Merck). Following embedment in Paraffin, transversal and longitudinal sections of 6  $\mu\text{m}$  were cut on a Leica RM2255 microtome and collected on glass slides consecutively (all sections were collected) and stained with hematoxylin and eosin (Khodabandeh et al. 2008). All histological slides were analyzed. Histopathological alterations detected in gills of fingerlings were recorded as present or absent and expressed as a percentage of fish affected (prevalence) per experimental group (10 fish each). Observers were aware to which experimental group each slide belonged.

For immunohistochemical studies, the sections were collected on glass slides, which were previously washed in HCl and 90% ethanol (50/50), then rinsed with distilled water, and finally incubated at 37°C for 24 h. Sections were de-paraffinized with xylene and hydrated with a descending series of ethanol (100, 95, 90, 70, and 50%). Sections were pre-incubated for 10 min in phosphate-buffered saline (PBS; pH = 7.3), and then treated with PBS solution containing 2.18 g NaCl for 10 min to mask the free aldehyde groups of the fixative. The sections were then incubated in PBS solution containing milk powder for 20 min, and finally washed in PBS for 1 min (Rajabi & Khodabandeh 2013). Immunolocalization of  $\text{Na}^+, \text{K}^+$ -ATPase was performed through immunofluorescence light microscopy using a mouse monoclonal antibody IgG $\alpha_5$  (Hybridoma Bank, University of Iowa) raised against the  $\alpha$ -subunit of chicken  $\text{Na}^+, \text{K}^+$ -ATPase (Takeyasu et al. 1988). This antibody, diluted in PBS to 20  $\mu\text{g ml}^{-1}$ , was placed on the sections and incubated for 2 h at room temperature in a moist chamber. The slides were rinsed in PBS and were then incubated for 1 h in secondary antibody (fluorescein isothiocyanate conjugated, FITC) under dark conditions. Negative control sections were incubated in BSA-PBS without primary antibody. All slides were rinsed in BS, and were mounted in a medium for fluorescent microscopy (Sigma, ref. 7534) to retard photobleaching. An Olympus digital camera adapted to an Olympus fluorescence microscope was used to obtain images from the tissues. Counting and measuring the dimensions of the ionocytes in gills was performed using images from immunohistochemistry. Ionocytes were counted on longitudinal sections of the gill filament (6 fish group $^{-1}$ ) in random frames of size 217.68  $\times$  161.89  $\mu\text{m}$  (Pelis & McCormick 2001). Measuring the dimensions of the ionocytes was performed using Image Tools (2.0) software (Rajabi & Khodabandeh 2013).

### Electron microscopy

For electron microscopy examination, gill arches were cut and fixed in 50% glutaraldehyde (2.5%) and 50% PBS (0.1 M, pH 7.4) for 12 h. After 12 h, samples were transferred to 0.1 M PBS. Fixed samples were postfixed in diluted osmium tetroxide (in 0.1 M PBS; pH 7.4) for 1 h. Samples were then washed several times with 0.1 M PBS and distilled water and finally dehydrated in an ascending series of ethanol (35, 50, 70, 80, 90, and 100%). For scanning electron microscopy (SEM), following dehydration with ethanol, samples were dried using a freeze-dryer (CHRIST LCG) for 2 h. Samples were sputter-coated (Pelco SC-6) with gold and then examined and photographed with an SEM (KYKY-EM3200; Khoshnood et al. 2011).

### Statistical analysis

Two gills (4 gill arches on either side of the head) of each animal were analyzed for all above methods. Histopathological alterations quantified in the gills were analyzed by means of an ANOVA. For comparison between the surface area of the ionocytes, a Shapiro-Wilks normality test was performed, followed by Levene's homogeneity of variances test. Finally, to determine significant differences between normal data, we used an independent sample *t*-test, and for non-normal data, we performed Mann Whitney *U*-tests. Ionocyte counts were compared with a chi-squared test. All statistical analyses were performed using SPSS (15.0) software.

## RESULTS

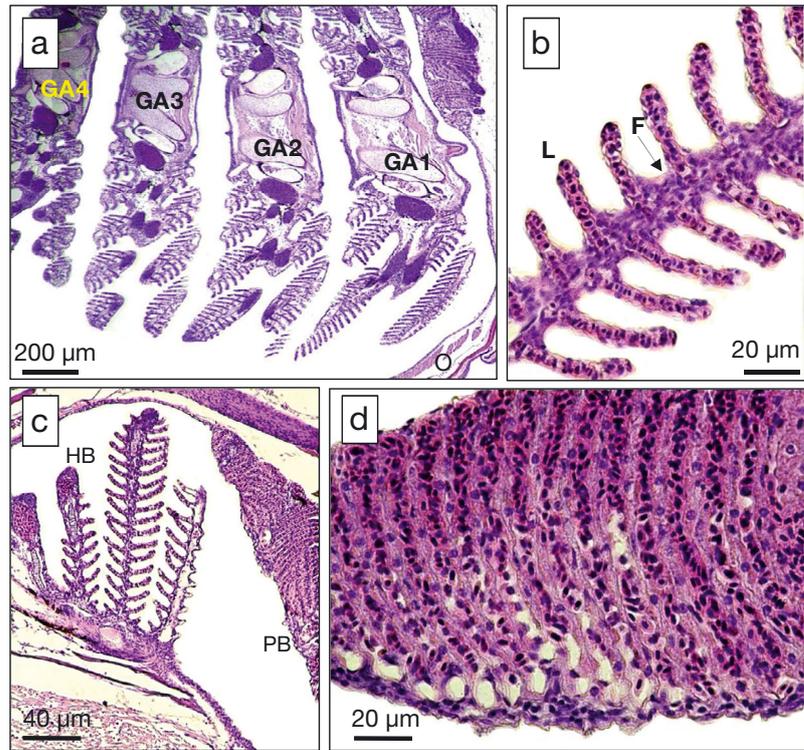
### Morphometric characteristics

The mean ( $\pm$ SE) body weight (BW) and total length (TL) of fingerlings were 2.6  $\pm$  0.01 g and 3.5  $\pm$  0.02 cm. The length and weight of the control and atrazine-exposed groups showed no significant differences ( $p > 0.05$ ) between these 2 experimental groups at the beginning and the end of the experiment (Table 1).

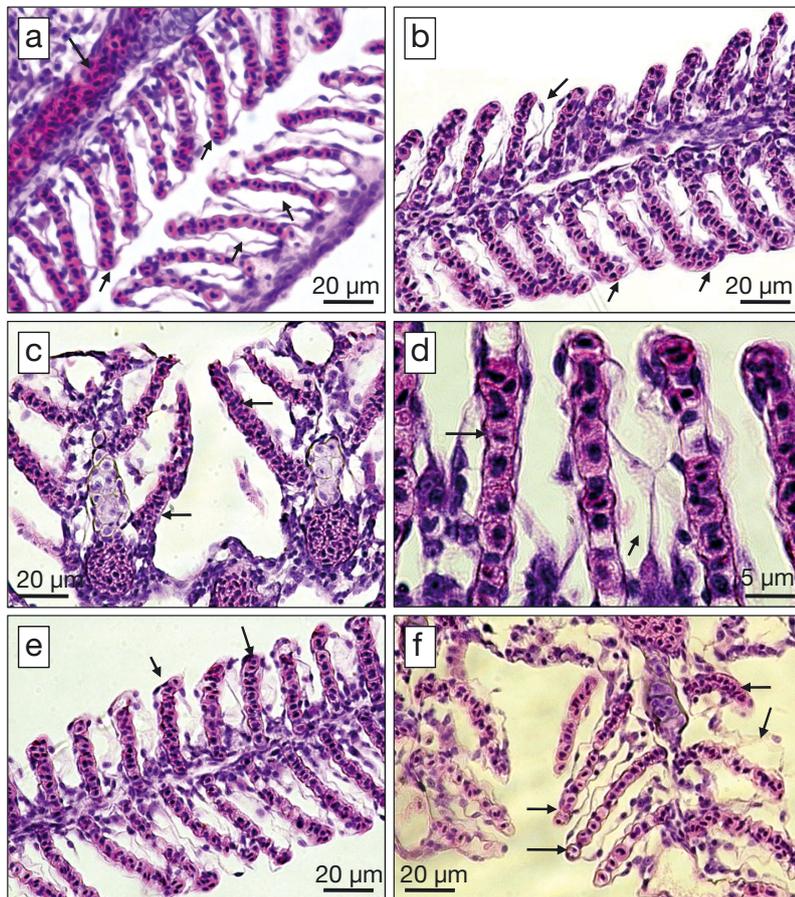
### Histology

Normal gill structure of Caspian kutum fingerlings is shown in Fig. 1. Gills are made up of 3 main parts:

Fig. 1. Normal histological structure of gills in *Rutilus frisii kutum* fingerlings in the control group. (a) Four gill arches (GA) were observed on either side of the head, each bearing 2 rows of filaments. (b) Filaments (F) bearing lamellae (L). (c) On each side of the gill chamber, a hemibranch (HB) was observed composed of few filaments and lamellae. (d) On each side of the head, a pseudobranch (PB in c) was observed composed of many lamella shaped structures



the main gill, which is composed of 4 gill arches on either side of the head (Fig. 1a), each bearing 2 rows of filaments, with the filaments bearing 2 rows of leaf-like lamellae (Fig. 1b); the hemibranch, at the dorso-apical part of the gill chamber, which is composed of 2 to 3 filaments bearing lamellae (Fig. 1c); and the pseudobranch on the apical side of the gill chamber, bearing large numbers of pseudolamellae with many capillaries (Fig. 1d). No recog-



nizable changes were observed in the gills of the control fish (Fig. 1). In contrast, observed changes in the atrazine-exposed group were as follows: detachment of the lamellar epithelium (Fig. 2a,f); thickening of the lamellae (Fig. 2b); blood congestion (Fig. 2a–f); edema in lamellae and filament (Fig. 2b,e); hypertrophy of the pavement cells of the lamellae (Fig. 2d); necrosis (Fig. 2c,f); lamellar fusion (Fig. 2b,d–f), hyperplasia of the filament's epithelium (Fig. 3a); club-shaped lamellae (Fig. 3a,h); detachment of the epithelium of the gill rakers (Fig. 3b); hyperplasia of the lamellar epithelium and lamellar

Fig. 2. Histopathological changes in gills of *Rutilus frisii kutum* fingerlings in the atrazine-exposed group. The most recognizable changes caused by atrazine exposure were (a,f) detachment of the lamellar epithelium; (b) thickening of the lamellae; (b,e) edema in lamellae and filament; (c,f) necrosis; (d) hypertrophy of the pavement cells of the lamellae; (b,d–f) lamellar fusion; and (a–f) blood congestion. In each panel, arrows highlight these alterations

Table 1. Mean ± SE length (cm) and weight (g) of *Rutilus frisii kutum* fingerlings in the experimental groups at the beginning and end of the study

Group	Beginning		End	
	Length	Weight	Length	Weight
Control	3.4 ± 0.01	2.6 ± 0.01	3.5 ± 0.02	2.7 ± 0.02
Atrazine-exposed	3.5 ± 0.02	2.6 ± 0.01	3.6 ± 0.02	2.7 ± 0.01

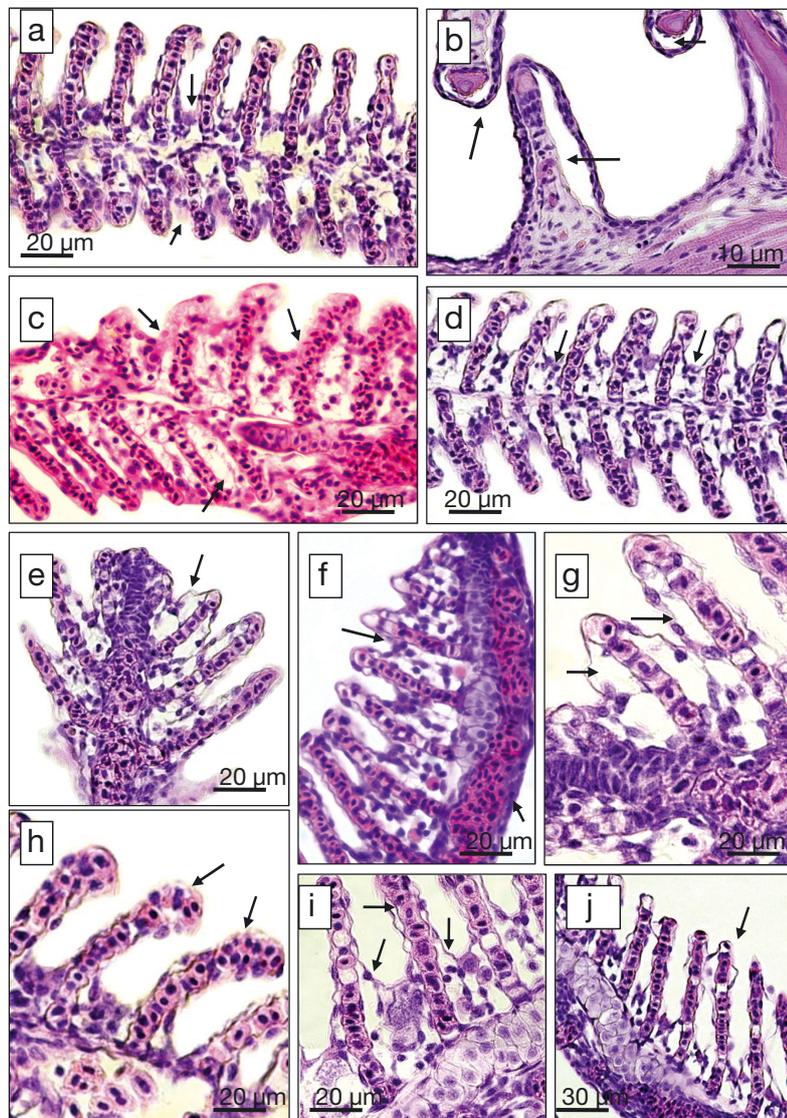
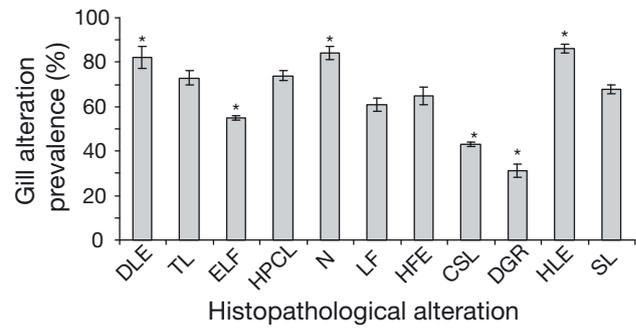


Fig. 3. Additional histopathological changes in gills of *Rutilus frisii kutum* fingerlings in the atrazine-exposed group. The most recognizable changes (arrowed) included (a) hyperplasia of the filament epithelium; (a,h) club-shaped lamellae; (b) detachment of the epithelium of the gill raker; (c) hyperplasia of the lamellar epithelium and lamellar fusion; (d,f,i) edema in the epithelium of the filament and the cells at the base of the lamellae; (e) edema in pavement cells of the lamellae and detachment of this epithelium; (f–j) blood congestion; (h) shrinkage of the lamellae; and (j) necrosis in lamellar epithelium

Fig. 4. Prevalence (%) of gill histopathological alterations in *Rutilus frisii kutum* fingerlings in the atrazine-exposed group. Alterations marked with (\*) were significantly different from other values ( $p < 0.05$ ). Values are mean ± SE. DLE: detachment of the lamellar epithelium; TL: thickening of the lamellae; ELF: edema in lamellae and filament; HPCL: hypertrophy of the pavement cells of the lamellae; N: necrosis; LF: lamellar fusion; HFE: hyperplasia of the filament epithelium; CSL: club-shaped lamellae; DGR: detachment of the epithelium of the gill rakers; HLE: hyperplasia of the lamellar epithelium; SL: shrinkage of the lamellae

fusion (Fig. 3c); edema in the epithelium of the filament and the cells at the basis of the lamellae (Fig. 3d,f,i); edema in pavement cells of the lamellae and lifting up of this epithelium (Fig. 3e); blood congestion (Fig. 3f–j); shrinkage of the lamellae (Fig. 3h); and necrosis in the lamellar epithelium (Fig. 3j).

Results of the quantitative observation of the histopathological alterations in gill tissue of fingerlings showed that the most significant alterations were hyperplasia of the lamellar epithelium, necrosis, and detachment of the lamellar epithelium (Fig. 4). No significant difference was observed in the prevalence of alterations in different gill arches of the studied fish.

### Immunohistochemistry

Immunohistochemical studies showed no differences between the dispersion patterns of the ionocytes within the gill tissue of the atrazine-exposed and control groups. The ionocytes were ob-

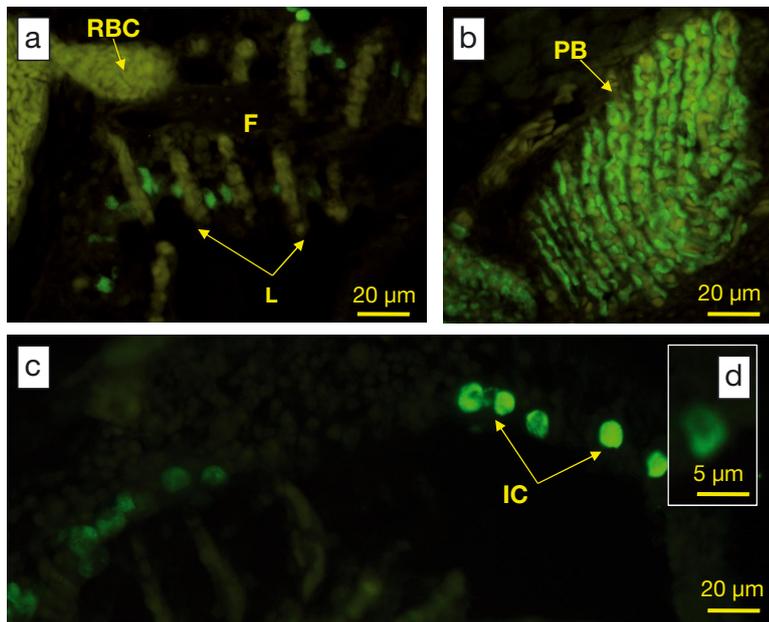


Fig. 5. Immunolocalization of ionocytes in gills of *Rutilus frisii kutum* fingerlings in the atrazine-exposed group. Ionocytes were observed (a,c) on the gill arches, on the gill filaments, and at the basis of the lamellae, (b) in large numbers in the pseudobranch, and (c) at the wall of the gill chamber. (d) Ionocytes were almost round to oval in shape, and the most immunofluorescence was observed at their basolateral membrane. F: filament; IC: ionocyte; L: lamellae; PB: pseudobranch; RBC: red blood cell

served on the gill arches (Fig. 5a,c), wall of the gill chamber (Fig. 5c), on the filament, and at the basis of the lamellae (Fig. 5a,c), and numerous in the pseudobranch (Fig. 5b) in both experimental groups. Ionocytes were round to oval cells with the highest immunofluorescence at the basolateral membranes (Fig. 5d).

More ionocytes were found in fish of the control group than in fish of the atrazine-exposed group, although these differences were not significant ( $p > 0.05$ ; Fig. 6a). The mean surface area of ionocytes was  $52.00 \pm 0.3 \mu\text{m}^2$  and  $57.17 \pm 0.2 \mu\text{m}^2$  in the control and atrazine-exposed groups, respectively. The ionocytes of the atrazine-exposed group were significantly larger than the ionocytes of the control group ( $p < 0.05$ ; Fig. 6b).

### SEM

Observation of the gill tissue following the exposure to atrazine showed several changes: fusion and collapsing of the lamellae (Fig. 7a–c,e), shrinkage and curling of the lamellae at the apical parts of the filaments (Fig. 7a–c), and necrosis of the apical microridges of the pavement cells (Fig. 7d).

### DISCUSSION

The fish gill is a multifunctional organ responsible for respiration, osmoregulation, acid–base balance, and nitrogenous waste excretion. This organ is sensitive to chemicals in water, since the gill filaments and lamellae provide a very large surface area for direct and continuous contact with contaminants in water (Evans et al. 2005). Reviews by Mallatt (1985) and Wood (2001) have provided comprehensive information on structural changes in fish gills in response to toxicant exposure. Gill histopathological changes are, in general, responsive but non-specific to pollutant exposure (Wood 2001). Histopathological alterations observed in our study were not specific to atrazine and have previously been observed following exposure to a wide variety of water contaminants; for example, histopathological alterations in gill tissue were determined previously in rainbow trout *Oncorhynchus mykiss* (Fischer-Scherl et al. 1991), carp *Cyprinus*

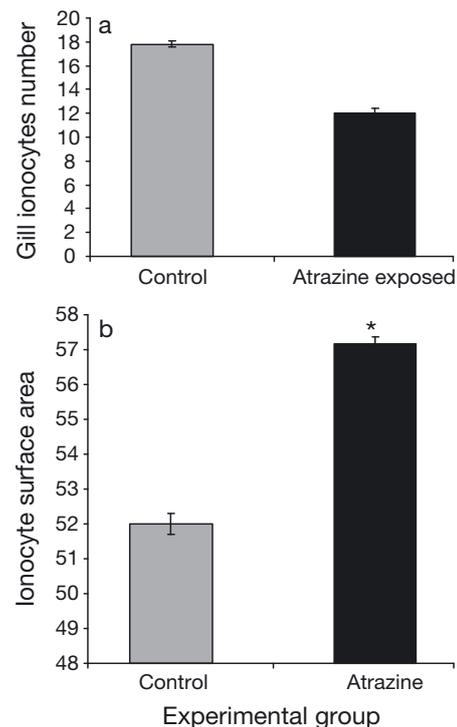


Fig. 6. Comparison of (a) mean number and (b) mean surface area ( $\mu\text{m}^2$ ) of gill ionocytes in *Rutilus frisii kutum* fingerlings in the control and atrazine-exposed groups. Ionocytes were significantly ( $*p < 0.05$ ) larger in the atrazine-exposed group

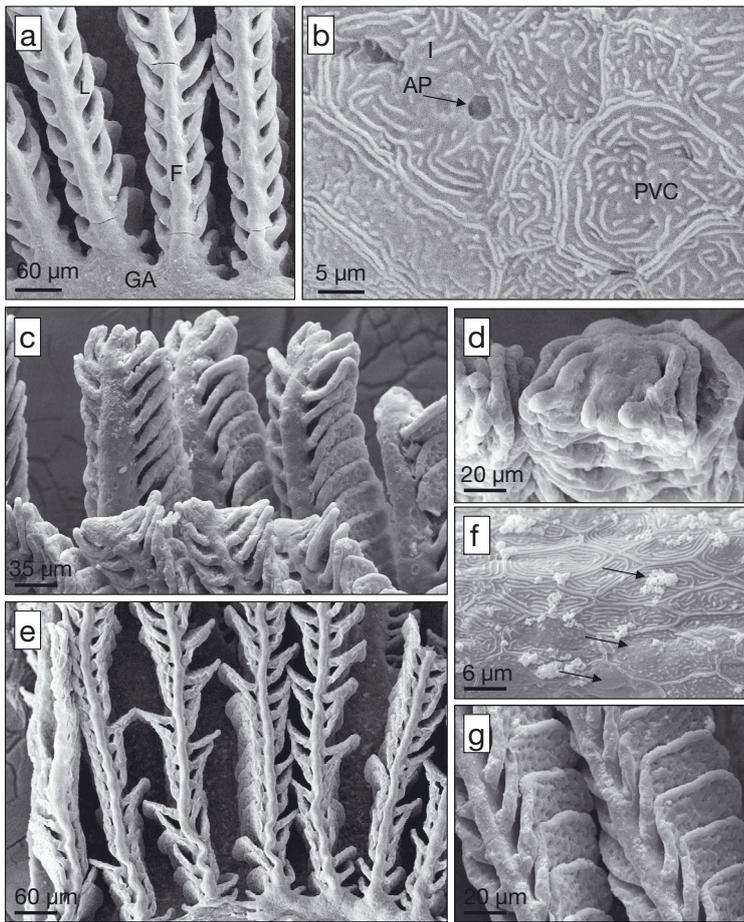


Fig. 7. Ultrastructural alterations in the gills of *Rutilus frisii kutum* fingerlings in control and atrazine-exposed groups using scanning electron microscopy. Normal structure of (a) gill, filaments, and lamellae and (b) pavement cells and ionocytes. Fish in the exposed group showed (c–f) fusion and collapsing of the lamellae; (c–e) shrinkage and curling of the lamellae at the apical parts of the filaments; and (d, f) necrosis of the apical microridges of the pavement cells and mucus aggregation (dark arrows in f). GA: gill arch; F: filament; L: lamellae; I: ionocyte; AP: apical pit; PVC: pavement cell

*nus carpio* (Neskovi et al. 1993), *Gnathonemus petersii* (Alazemi et al. 1996), and *Labeo rohita* (Jayachandran & Pugazhendy 2009) after exposure to water contaminants such as organochlorines, organophosphates, oil pollution, herbicides, and heavy metals.

Among the observed histopathological alterations in the gill tissue of Caspian kutum in our study, thickening of the lamellae, hyperplasia, lamellar fusion, club-shaped lamellae, blood congestion, edema, and necrosis were demonstrated in previous studies to support the fragile lamellae, and help counteract the toxic effects of the herbicide (Yang et al. 2010). Previous studies have suggested that edema in gill tissue after introduction of toxicants is due to increased

permeability of the capillaries (Roberts 1978). Hyperplasia of the lamellae is a defensive mechanism against environmental inflammatory factors that reduces the respiratory surface and also increases the distance between the pollutant and blood. A result of these alterations is the thickening of the epithelium of the lamellae and filaments and ultimately lamellar fusion, all of which were observed in the present study.

Necrosis was observed in the gills of Caspian kutum in the present study. Together with the results of Cengiz (2006), necrosis in epithelial cells is considered a direct response of the gill to environmental pollutants. The same results were observed in rainbow trout following exposure to cypermethrin (Velisek et al. 2006); these observed alterations could interfere with respiration, ion secretion, and reabsorption processes of the gill.

We observed no significant differences in the number of ionocytes, although significant differences were observed in the surface area of these cells between control and atrazine-exposed fish. The most logical reason for the larger surface area of the ionocytes in the atrazine-exposed group would be hypertrophy of these cells which we observed in our histopathological study of the gills. Edema in gill ionocytes has also been reported as one of the effects of atrazine in *Prochilodus lineatus* (Paulino et al. 2012).

## CONCLUSION

Our results showed that the herbicide atrazine is highly toxic to Caspian kutum fingerlings even at sublethal concentrations and acute exposure; severe damage in gill tissue suggests that it could affect osmotic and ionic regulation, which in turn could seriously impair the survival ability of fingerlings experiencing higher salinities during migration from freshwater rivers to the brackish water of the Caspian Sea. Our results also showed that besides the well known negative effects of atrazine on the nervous and reproductive systems, it could seriously affect osmoregulation and respiratory mechanisms in test organisms.

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