HISTOPATHOLOGICAL ALTERATIONS IN THE DIGESTIVE SYSTEM OF *RUTILUS FRISII KUTUM* (KAMENSKY, 1901) FRY AFTER EXPOSURE TO ATRAZINE HERBICIDE

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To investigate the histopathological effects of a most wildly used herbicide, atrazine in Caspian kutum fry, *Rutilus frisii kutum*, fish (3.5 cm TL and 2.6 g BW) were exposed to a sublethal concentration of 12.47 mg/L (½ LC50) for 96h. Acute exposure of *R. frisii kutum* fry to atrazine causes some alterations ½ in the digestive system and the liver of the fry. The most significant alterations were necrosis of intestinal epithelial cells at the apical and basal parts, detaching of epithelial cells from the basement membrane, degeneration of the apical sides of the intestinal folds, hyperplasia in intestinal epithelial cells and hyperplasia and hypertrophy of the goblet cells of the intestine. In hepatic tissue the most significant alterations were dilution of sinusoids, necrosis, vacuolation and increasing the intercellular spaces in hepatocytes, picnotic nuclei of hepatocytes and degeneration of adipose tissue of the liver. Atrazine could affect the nutritional ability and osmoregulation process of the fry by causing histopathological changes in the digestive system even at sublethal concentration and acute exposure.

Keywords: *Caspian kutum*, atrazine, digestive system, liver.

INTRODUCTION

The pollution effects on fish are main scientific issues (Monte-Luna *et al*., 2016; Bănăduc *et al*., 2016; Khoshnood, 2017). The widespread use of chemical agents as pesticides and herbicides, to control the plague and weeds every year, does not necessarily translate to ecological crisis, but there has been considerable discussion in both the scientific literature and the lay press regarding the possibility that environmental chemicals, through their effects on endocrine function, are responsible for a number of reproductive and developmental anomalies in a wide range of wildlife species, from invertebrates through fish, reptiles, birds and mammals, and even including humans (Cooper & Kaviock, 1997).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), which is one of the most widely used herbicides, has been widely applied in agricultural and forestry fields. Due to its relatively high aqueous solubility and high mobility, atrazine can be transported to groundwater by infiltration or to surface waters by water runoff, thus entering aquatic environment easily (Graymore *et al*., 2001),

therefore, it is more frequently detected in groundwater and surface water than any other herbicides in many countries (Ta *et al*., 2006). It has been clear that estuaries and coastal marshes are vulnerable to atrazine contamination because they receive waters carrying agricultural pesticides from upland sources (de Lorenzo *et al*., 2001).

Since atrazine is most commonly found in lakes, rivers and streams, different aquatic species are at particular risk. Various laboratory and ecological applied field studies have shown that atrazine adversely affects multiple biological processes, including growth, metabolism, immune and endocrine system function, in several species of frogs and fish (Srinivas *et al*., 1991; Freeman & Rayburn, 2005; Houck & Sessions, 2006; Forson & Storfer, 2006; Rymuszka *et al*., 2007; Fatima *et al*., 2007; Nieves- Puigdoller *et al*., 2007; Rowe *et al.*, 2008). However, fish are not usually target organisms for pesticides, and specific knowledge about negative effects of pesticides in the field is still considered sparse. Surprisingly, only a few studies have shown that fish, inhabiting natural freshwater ecosystems, may be affected by unintentional spreading of pesticides (Bálint *et al*., 1997; Csillik *et al*., 2000).

Herbicides are often regarded as relatively harmless to fish. Direct effects caused by, for example, the herbicide atrazine are scarce. Fish also can serve as bio-indicators of environmental pollution and can play significant roles in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals resulting from agricultural production via surface run-off or indirectly through food chain of ecosystem (Lakra & Nagpure, 2009).

Early developmental stages are considered to be one of the most sensitive stages in the fish life cycle to the toxic effects of chemical contaminants (Weis & Weis, 1987). Short-term sublethal effects on the growth, behavior or osmotic control may affect the survival of these critical stages and impact population recruitment (Houde 1987, 1989; Sclafani *et al.*, 1997; Alvarez & Fuiman, 2005).

Rutilus frisii kutum (Kamensky, 1901) is one of the native, and commercial fish species of the Caspian Sea. It is a migratory anadromous fish spawning from March to April (Sharyati, 1993; Razavi, 1995) on aquatic weeds and graveled and sandy substrates in rivers and lagoons (Abdoli, 1999). Decline in the stocks and catch of Caspian kutum was caused by overfishing, excessive catches of adults, increased pollution, overexploitation of sands and sediments of the Caspian Sea, and the construction of bridges and dams that alter or blocked the natural spawning grounds (Azari Takami *et al*., 1979; Emadi, 1979). For several years, starting from 1925, artificial breeding raised larvae for release in the 10 most important rivers. According to the Iranian Fisheries Organization's report, more than 150 million juveniles are being released into the Caspian Sea annually (Iranian Fisheries Organization, 2006).

In order to investigate the toxic effects of atrazine herbicide on *Rutilus frisii kutum*, histopathological alterations of the digestive system have been studied. Results of the present study would be useful as basic data for related studies on environmental monitoring on atrazine contamination.

MATERIALS AND METHODS

FISH AND EXPERIMENTAL DESIGN

Caspian kutum, *Rutilus frisii kutum*, fingerlings were obtained from Shahid Ansari Fish Proliferation and Culture Center, Rasht, Iran. Mean total length and mean body weight of fingerlings were 3.5 cm and 2.6 g respectively. Following the determination of 96 h-LC50 of atrazine for the fingerlings (Khoshnood *et al*., 2014), a sub lethal concentration was determined as ½ LC50 (12.47 mg/L). Atrazine was dissolved in distilled water, filtered and added to the aquarium following the method of Pluta (1989).

Fish were exposed to this sub lethal concentration for 96h in triplicate group of 30 fish each (~0.43 g/L biomass) in glass aquaria, in the laboratory conditions. One triplicate group of the fingerlings was held in clean water as the control group. No mortality was observed during the experiments in all experimental groups. The water parameters monitored daily through the experiment for all experimental groups using 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, and the photoperiod was 12h:12h lightness and darkness. Water quality conditions (pH, temperature and O_2) did not differ among treatments, and water did not change during the experiment.

HISTOLOGY

For histological studies, fish were hypothesized and immediately immersed into Bouin's fixative for 24 hours, washed and dehydrated in an ascending series of ethanol for embedding in Paraffin (Merck). Following embedment in Paraffin, transversal and longitudinal sections of 6 µm were cut on a Leica RM2255 microtome and collected on glass slides and stained with Haematoxylin and Eosin (Martoja & Martoja-Pierson, 1967; Khoshnood, 2015a). Histopathological alterations detected in the digestive system of fingerlings were recorded as present or absent and expressed as a percentage of fish affected (prevalence) per experimental group (10 fish each). The slides were studied by the means of knowing that they belong to each experimental group (Khoshnood, 2015b).

CHEMICALS

Two experimental groups for atrazine were investigated in triplicate series of: control group with nominal concentration of 0 ppm (at the beginning of the experiment: t=0), and atrazine exposed group with nominal concentration of 12.47 ppm (at the beginning of the experiment: t=0), both for 96h. The water of both experimental groups was analyzed after 24h of exposure period of 96h for assessing the real concentration of atrazine (t=24).

For analysis of the atrazine concentration in experimental groups, sampled water was transferred into a glass bottle which contains 10ng of $^{13}C_{12}$ PCB-101 to

assess the extraction efficiency. In order to perform extraction, dichloromethane with the volume of about 25% of sample solution was used and the method has been replicated 3 times. A combination of extracts has been reduced to approximately 50 µL and then addition of internal standard: tris (4-chlorophenyl) methane (TCPMe, 100 pg/µL) as internal standard.

Concentration analysis has been conducted using gas chromatography (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 carrier gas was Helium (flow rate, 1.0 mL/min). The electron impact for ionization was 70eV and the ion trap was operated in MS–MS mode. To calculate the atrazine concentrations, sample response relative to the one of $^{13}C_{12}$ PCB-101 in the same sample was considered. Four point calibration curve was considered for relative response factor and $^{13}C_{12}$ PCB-101 and TCPMe were kept at constant concentration of 100 pg/µL. Correction of the atrazine concentrations was calculated on the basis of the recovery of the surrogate compound. The quantification limit was 0.003 ng/L and the precision of analysis was 6%. Limit of quantification was 0.003 ng/L for atrazine and analytical precision was 6% (Khoshnood, 2015b).

RESULTS

CHEMICALS

Measured atrazine concentrations at *t*-0 (Table 1) were within 85–105% of the nominal concentrations. Fish were exposed to nearly constant atrazine concentrations over the bioassay period. Variations of atrazine concentration within each 24-h period were of the same amplitude as the day to day variation at *t*-0. In the control group, atrazine was occasionally detected in trace amounts (<0.01 µg/L).

Table 1

Nominal and measured atrazine concentrations in exposure solution at the beginning (*t*= 0) and the end (*t* = 24) of 24-h laboratory exposure periods

Experimental Group	Nominal Concentrations (ppm)	Measured Concentrations (ppm)	
		t=0	*t*=24
Fingerling	12.47	12.32	12.28

HISTOLOGICAL STRUCTURE OF DIGESTIVE SYSTEM IN R. FRISII KUTUM

Results showed that the primary parts of the digestive system of *R. frisii kutum* are oral cavity and pharynx which lined up by stratified squamous epithelium with numerous goblet cells and taste buds (Fig 1a). Pharynx bears 4 pharyngeal teeth at the lower part and a horny pad at the upper side (Fig 1a).

Esophagus was short with thick muscular layer, and in its epithelium numerous goblet cells and taste buds were observed (Figs. 1a and 1b).

No definite stomach was observed in the digestive system and esophagus has straightly ended up to the intestine (Fig. 2a). At the primary part of the intestine the folding was more than in the ending part, where the lumen got wider and called the rectum (Fig. 2a). The intestine was lined up with a simple columnar epithelium and pear shape goblet cells (Figs. 2b and 2c).

The liver was large and it was observed almost all along to the digestive system except for the oral cavity and pharynx. The most significant cells of the liver were hepatocytes with a central nucleus (Figs. 2a and 2d).

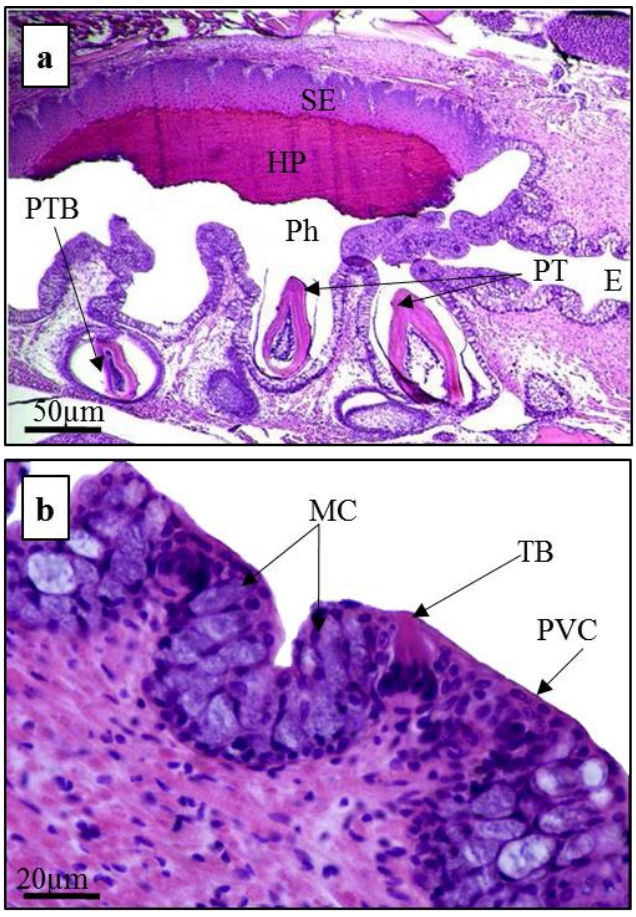

Fig. 1. Normal histological structure of the primary part of the digestive system in *Rutilus frisii kutum* fry. Digestive system begins with oral cavity (not shown), Esophagus (a) and pharynx (a), all lined up by stratified squamus epithelium with numerous goblet cells and taste buds (a). Pharynx bears 4 pharyngeal teeth at the lower part and a horny pad at the upper side (a). Esophagus was short with a thick muscular layer, and in its epithelium numerous goblet cells and taste buds were observed (a and b).

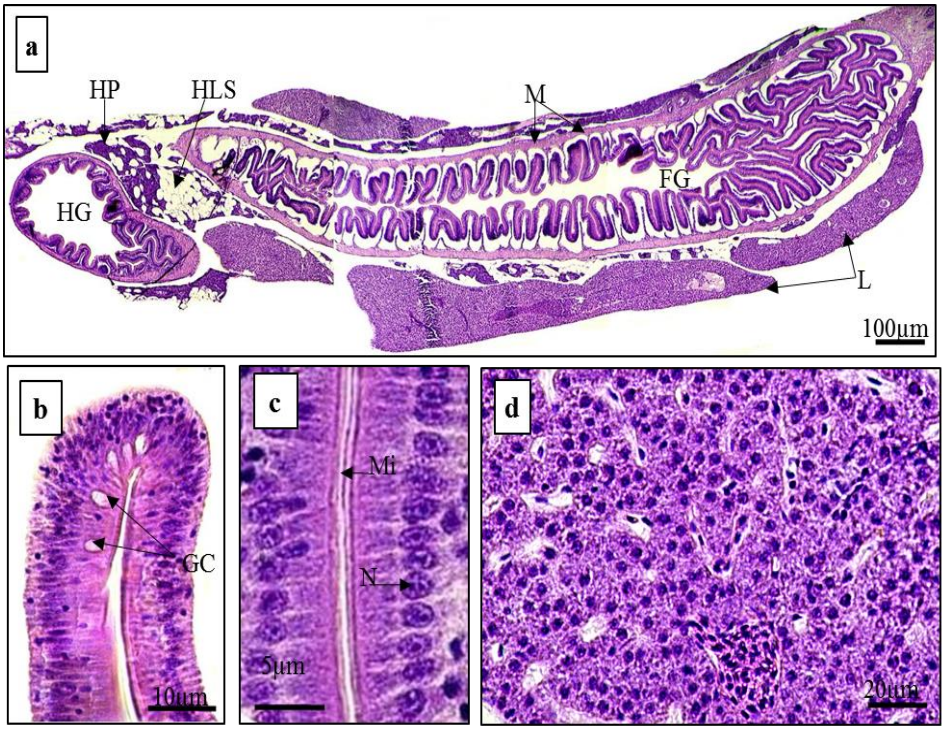

Fig. 2. Normal histological structure of the intestine and liver in *Rutilus frisii kutum* fry**.** Primary part of the intestine with more folding (a) and the ending part, where the lumen got wider and called the rectum (a). Intestine was lined up with a simple columnar epithelium and pear shape goblet cells (b and c). Liver was observed almost all along to the digestive system except for the oral cavity and pharynx (a). The most significant cells of the liver were hepatocytes with a central nucleus (a and d).

HISTOPATHOLOGICAL ALTERATIONS OF THE DIGESTIVE SYSTEM

Results showed that in atrazine exposed fish, the most significant alterations in the digestive system were as follows: necrosis of intestinal epithelial cells at the apical and basal parts (Fig. 3a), detaching of epithelial cells from the basement membrane (Figs. 3b and 3c), degeneration of the apical sides of the intestinal folds (Fig. 3c), hyperplasia in intestinal epithelial cells (Fig. 3d) and hyperplasia and hypertrophy of the goblet cells (Fig. 3e).

In the liver, the main histopathological alterations were dilution of sinusoids (Fig. 4a), necrosis, vacuolation and increasing the intercellular spaces in hepatocytes (Figs. 4b and 4c), picnotic nuclei of hepatocytes (Fig. 4c) and degeneration of adipose tissue of the liver (Fig. 4d).

Histopathological alterations detected in the digestive system 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 (Fig. 5). Results of the quantitative observation of the histopathological alterations in the digestive system of fingerlings showed that the most significant alterations were detaching of epithelial cells, hyperplasia of epithelial cells and necrosis of epithelial cells (Fig. 5).

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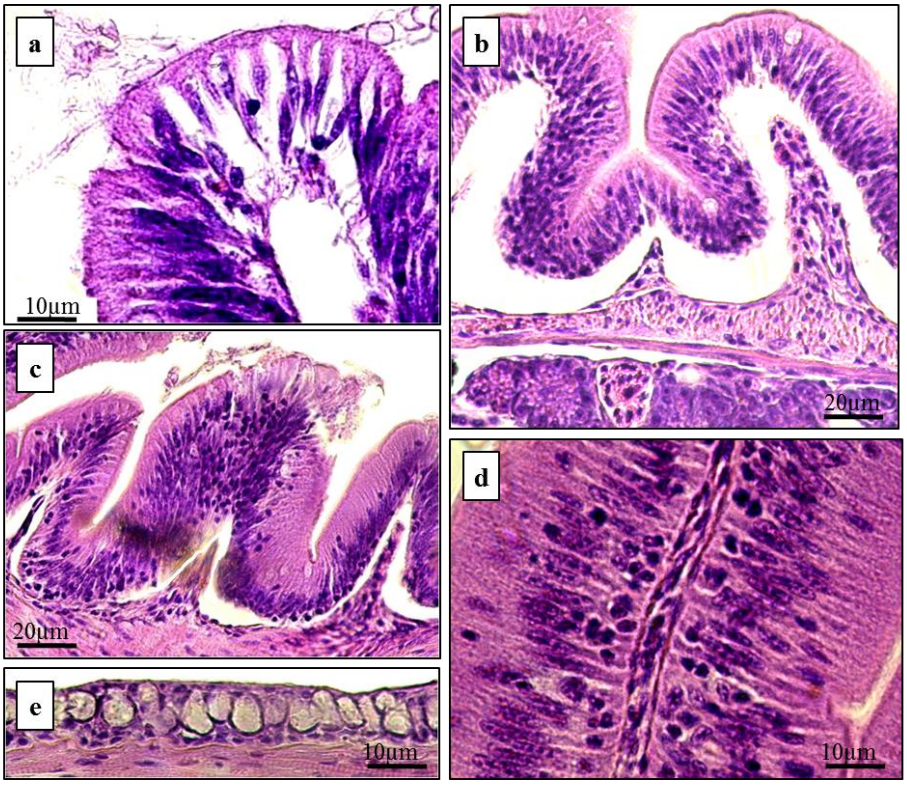

Fig. 3. Histopathological alterations of the digestive system of the *Rutilus frisii kutum* fry after exposure to atrazine herbicide. Necrosis of intestinal epithelial cells at the apical and basal parts (a), detaching of epithelial cells from the basement membrane (b and c), degeneration of the apical sides of the intestinal folds (c), hyperplasia in intestinal epithelial cells (d) and hyperplasia and hypertrophy of the goblet cells (e).

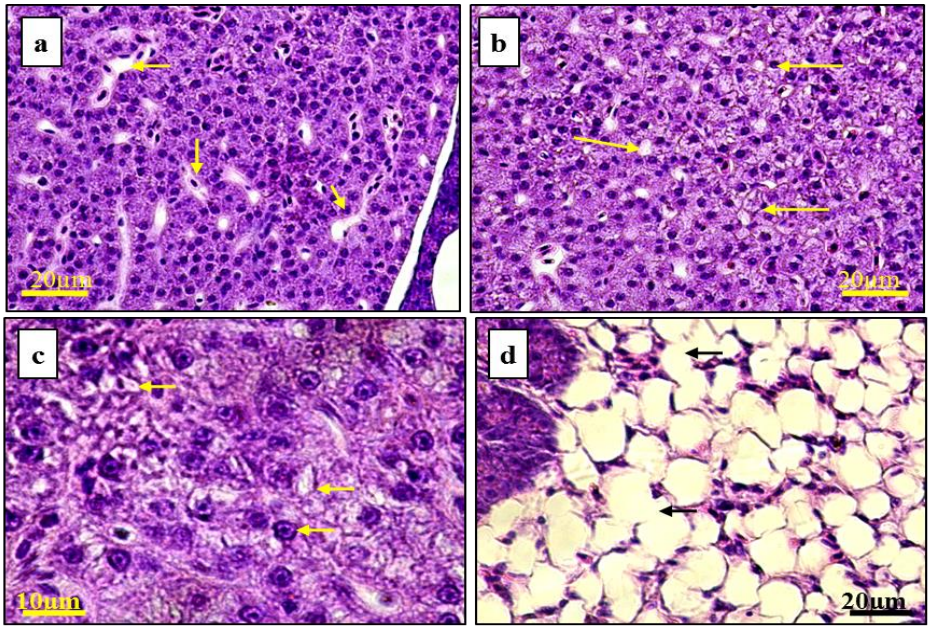

Fig. 4. Histopathological alterations of the liver of the *Rutilus frisii kutum* fry after exposure to atrazine herbicide. Dilution of sinusoids (a), necrosis, vacuolation and increasing the intercellular spaces in hepatocytes (b and c), picnotic nuclei of hepatocytes (c) and degeneration of adipose tissue of the liver (d).

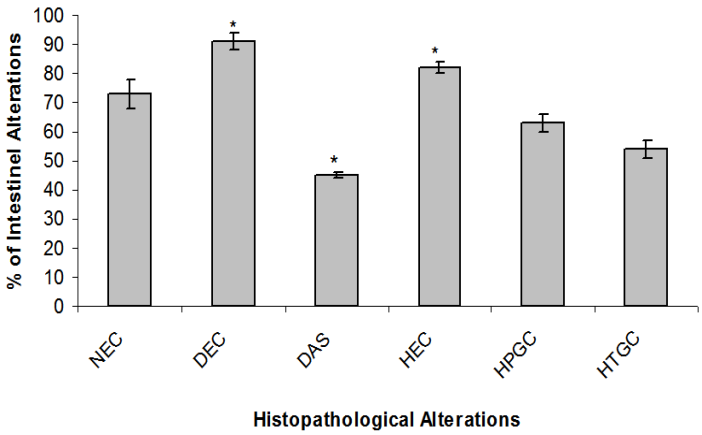

Fig. 5. Prevalence (%) of digestive system histopathological alterations in *Rutilus frisii kutum* fingerlings after exposure to atrazine herbicide. Alterations marked with (*) were significantly different from other values ($p < 0.05$). Values are mean ± SE.

Abbreviations: NEC: Necrosis of Epithelial Cells; DEC: Detaching of Epithelial Cells; DAS: Degeneration of Apical Side; HEC: Hyperplasia of Epithelial Cells; HPGC: Hyperplasia of Goblet cells; HTGC: Hypertrophy of Goblet cells.

DISCUSSION

The digestive system is a multifunctional organ in fish. Besides the main duty of digestion and nutrients uptake, excretion of some wastes, regulation of water and ions, and detoxication in relation with liver are other important functions of the digestive system. Because the digestive system is receiving the surrounding water (through swallowing of food), it could easily get affected by the pollution (Au 2004) and any damages to this organ can cause nutritional and osmoregulatory problems (Sindermann, 1979). Until now different investigations on the effects of xenobiotics on fish digestive system were performed (Chakrabarti & Sinha, 1987; McCarthy & Fuiman, 2008; Senapati *et al*., 2009) but data on the effects of herbicides on fish digestive system are scarce and there are few researches on the effects of atrazine. Different investigations on the effects of herbicides were performed, for example, Senapati *et al*. (2012) showed that exposure of *Anabas testudineus* with Almix herbicide caused the necrosis of the apical parts of the squamous epithelial cells of buccal cavity, pharynx and esophagus, disorder of the columnar epithelial cells of the intestine, increasing in mucus content of the goblet cells and necrosis of the intestinal folds. Another investigation on glyphosate herbicide, on *Channa punctatus* showed necrosis and pathological alterations in the digestive system (Senapati *et al*., 2009). Apical parts of the epithelial cells have a vital role in absorption of nutrients and minerals and it is protected by a mucus layer. This mucus layer also play a role as a lubricating layer for passing foods along the alimentary canal (Sinha & Chakrabarti, 1986; Chakrabarti & Sinha, 1987). Due to these facts, necrosis at the apical parts of the epithelial cells could lead to disorder in nutrient uptake, and further necrosis by eliminating the mucus layer (Senapati *et al*., 2012). Previous studies suggested that alterations in order of the columnar epithelial cell of the intestine and hyperplasia of these cells are protective mechanisms of the intestine against the pollutants (Tuvikene *et al*., 1999). It seems that the increase in number of the mucus cells and their contents observed in the present study, appeared due to the protective duty of mucus layer for the digestive system which was previously seen in different species exposed to pollutants (Au, 2004).

Necrosis of the columnar epithelial cells of the intestine which was observed in the present study could affect the nutritional abilities of the Caspian kutum fry. Previous studies showed that due to the high activity of these cells in transporting ions, water and nutrients, these cells are vulnerable in front of the pollutants and easily become necrotic (Sindermann, 1979; Tuvikene *et al*., 1999; Au, 2004).

The liver is the most important organ for biodeformation of the pollutants, removing of hazardous heavy metals, and storage of some nutrients and metabolism of sexual hormones (Au, 2004). Various investigations were performed on the liver cell and tissue alterations of different fish species in case of exposure to a wide range of pollutants (GlobalTox, 1997; Khoshnood *et al*., 2010). Most of

the pollutants were changed to non-toxic forms by liver special enzyme system, but sometimes this process can make cell or tissue damages in different levels dependent on the concentration and toxic levels of the pollutant in the liver (Au, 2004). Vesiculated hepatocytes, necrosis in adipose tissue of the liver and necrosis in some hepatocytes were the most significant alterations observed in Caspian kutum fry after exposure to atrazine. It has been clear that liver is extremely sensitive to environmental pollution, and due to the natural ability of hepatic cells for concentrating the absorbed pollutants, these cells were faced higher amounts of the hazards compared to other cells of the body (Au, 2004). Generally hepatic alterations are not specific for defining pollutants and besides some of the hepatic alterations have only occurred in specific species, for example, exposure to PAHs, PCBs, DDTs, Chlordane and Dieldrin which cause a wide range of hepatic alterations in English sole, *Pleuronectes vetulus*, like neoplasm hepatocytes, megalocytic hepatocytes, polymorphic nucleus hepatocytes and vacuolated hepatocytes, but in winter flounder, *Pleuronectes americanus*, exposure to PAHs, DDTs or chlordane significantly caused vacuolated and non-neoplasmic increases of the hepatocytes, and non-specific necrosis in hepatocytes (Meyers & Hendricks, 1985; Johnson *et al*., 1992). The histopathological alterations observed in the present study in the liver of the Caspian kutum fry after exposure to atrazine were previously observed in some other fish species exposed to different kinds of pollutants, for example exposure of *Ophiocephalus striatus with* cadmium chloride (Bais & Lpkhande, 2012), exposure of *Salmo trutta* and *Barbatula barbatula* with pesticides, PAHs and ammonium (Gernhöfer *et al.*, 2001), exposure of Nile tilapia, *Oreochromis niloticus* with roundup herbicide (Jiraungkoorskul *et al*., 2002), and exposure of *Heteropneustes fossilis* with cypermetrin (Joshi *et al*., 2007). Comparison between the results of the present study with previous data on the effects of various pollutants on hepatic tissue showed that histopathological alterations of the liver were not specific to pollutant and similar alterations could be observed under the effects of a wide range of pollutants. Results of the present study also showed that it is toxic enough and can produce enormous alterations in the liver of the fry even at sublethal concentration. Results of the previous studies on the toxic effects of atrazine showed that atrazine could have an inhibitory effect on the main hepatic enzymes of the glyconeogenesis (such as hexokinase, glycogen synthase and glucokinase) and lead to lose weight (Curic *et al*., 1999). Histopathological alterations also reported in the hepatic tissue of the zebra fish, *Danio rario*, exposed to atrazine (Yuanxiang *et al*., 2011), include changes in the protein content of hepatocytes too. Chronic exposure to atrazine also caused changes in lipid metabolism and insulin resistance (Lim *et al*., 2009). It is suggested that all these hepatic alterations are dependent on a wide range of cellular biochemical processes in response to oxidative stress, oncogenesis, etc.

CONCLUSIONS

Outcomes of this study revealed that significant degradations in vital fish (Caspian kutum fry) organs of the experimental model such as digestive tract and liver tissues could happen due to acute (short-term) exposure to a sublethal concentration of commercial atrazine herbicide, even though the fish is not a target organism for such substance.

The tissue damages in this case were almost severe so one could conclude that such damages could have resulted in malfunction of the alimentary canal and intoxication duty of the liver and also in nutrition problems and toxicity of the environmental contaminations which finally ended up with mortality at long time (chronic) exposure.

The results also showed that sublethal concentration of atrazine even at acute exposure could affect the liver and make some tissue damage and alterations in this vital organ. Due to the natural responsibility of the liver in intoxication with toxins, drugs, contaminations, etc. it would not be unexpected that probably this organ received a higher concentration of atrazine compared to other internal organs, and for such reason, the tissue alterations were severe in the liver. The severe tissue alterations in the liver could lead to malfunction of this organ for intoxication and influence the whole body of the organism at chronic exposure.

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