

## Histopathological and haematological response of male rainbow trout (*Oncorhynchus mykiss*) subjected to butachlor

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**ABSTRACT:** This study was aimed at assessing the histopathological and haematological effects of a widely used herbicide on rice paddy fields, i.e. butachlor, on rainbow trout. Fish were exposed to butachlor at a concentration of 0.39 mg/l, for a period of 10 days. Haematologically, fish showed a significant decrease in erythrocyte count, haemoglobin, white blood cells and lymphocytes and a significant increase in neutrophils compared to controls ( $P < 0.05$ ). Histopathological observations of prepared sections of the treatment group also revealed pathological lesions of varying severity in studied organs, including liver (hyperaemia and haemorrhage, bile duct hyperplasia, dilated sinuses, interstitial oedema, monocellular necrosis, nuclear degeneration and hypertrophy in hepatocytes), gills (hyperplasia and hyperplasia of lamellar epithelium, fusion of lamellae, rod-like structures of secondary gill lamellae, cystic-like lesions) and kidneys (vacuolar degeneration of tubular epithelium, desquamation of epithelium and necrosis of tubular epithelium). It is concluded that butachlor caused changes in certain haematological parameters and histopathologically, exerted destructive effects on the gills, liver and kidneys of rainbow trout.

**Keywords:** erythrocyte profile; leukocyte profile; butachlor; acute exposure; liver; gill; kidney

Butachlor ( $C_{17}H_{26}ClNO_2$ ) is a widely used herbicide on rice paddy fields and Asian farmers expend more than one billion pounds on this chemical annually (Ateeq et al. 2002).

As rice fields are mainly located alongside rivers, this herbicide is often released into rivers and can affect its inhabitants. Butachlor is one of widely used herbicides in the northern region of Iran and its concentration in the Shahid Beheshti sturgeon fish hatchery has already been reported to be 0.67 ppb (Arshad et al. 2006).

Butachlor is stable in the soil of farmlands and water systems. The remnants of the pesticide can enter ground waters used for human consumption and thus can be potentially hazardous for human health (Yu et al. 2003). The severe effects of the herbicide on many animals have been reported and include retardation of growth and reproduction in earthworms (*Eisenia fetida*) (Muthukaruppan and Gunasekaran 2010), damage to epithelial tissue in *E. fetida* (Muthukaruppan et al. 2005), neurotoxicity

in land snails (Rajyalakshmi et al. 1996), genotoxicity in toads, frog tadpoles and fishes (Ateeq et al. 2006; Yin et al. 2008; Guo et al. 2010), while it acts as an indirect mutagen in hamsters and rats (Hsu et al. 2005). The 96h  $LC_{50}$  for the acute toxicity of butachlor in fish varies from 0.14 to 0.52 mg/l and its rate for rainbow trout (*Oncorhynchus mykiss*) has been reported to be 0.52 mg/l, showing its high toxicity in fish (Tomlin 1994).

Histopathological changes of organs such as the gills, kidneys and liver, have been described as biomarkers in the evaluation of the health of fish exposed to pollutants (Hesser 1960; Schwaiger et al. 1997). They are responsible for vital functions and can alter haematological parameters because of changes in their activity in response to various stress factors.

Following an increase in the numbers of rainbow trout farms situated close to rivers in the northern region of Iran, this research was conducted to study the haematological and histopathological effects of

acute exposure to the butachlor herbicide (75% of 96h LC<sub>50</sub>) on liver, gill and kidney tissues of rainbow trout (*Oncorhynchus mykiss*). The results of the present study could contribute to a better understanding of the effects of this widely used herbicide and may facilitate assessment of its impact on the environment for managerial purposes.

## MATERIAL AND METHODS

This study was performed on 40 male rainbow trout with an average weight of  $403 \pm 77.9$  g obtained from a native farm from the Mazandaran province (Northern Iran). The specimens were transferred to the laboratory and acclimated to the laboratory conditions for a week. Equal numbers were then randomly divided into two 1000 l tanks (20 fish each), one which served as the control group, while the other was administered butachlor treatment. The fish in each tank were treated in replicate.

The 96h LC<sub>50</sub> for acute butachlor toxicity in rainbow trout has been reported to be 0.52 mg/l (Tomlin 1994). Hence, this study was performed using 75% of the above mentioned concentration, i.e. 0.39 mg/l, to induce acute butachlor toxicity (Purity 60%, H.P.C., Iran). To ensure consistency, we validated the concentration of butachlor using a HPLC system (Junghans et al. 2003; Del Buono et al. 2005).

During the entire experiment the water temperature (°C), pH, oxygen (mg/l) ammonia (ng/l) and nitrite (µg/l) levels were  $16.2 \pm 1.2$ ,  $7.9 \pm 0.1$ ,  $7.5$  to  $9$ ,  $22 \pm 3.1$  and  $32.2 \pm 0.7$ , respectively. The fish were not fed during the duration of the experiment. The time of exposure was 10 days. Fifty percent of the water of each tank was replaced daily with fresh water containing the experimental concentration of butachlor.

On Days 1, 5, and 10 of the experiment fish were sampled from each group ( $n = 15$ ) and were anaesthetised immediately using MS-222 (100 mg/l). Blood samples were taken from the caudal vasculature using a heparinised syringe and transferred to tubes containing 0.01 ml sodium heparin solution (5000 IU) (Rotexmedica, Germany).

Haematological indices including white blood cells (WBCs), differential leukocyte count, erythrocyte count (RBC), haemoglobin (Hb), packed cell volume (PCV), mean erythrocyte haemoglobin (MCH), mean erythrocyte volume (MCV) and mean colour concentration (MCHC) were measured. The procedures were based on unified methods for haematological examination of fish (Svobodova et al. 1991).

At the end of the experiment liver, gill and kidney tissue samples were taken for histopathological studies from five fish of both treated and control groups.

The samples were fixed in Bouin's solution and then transferred into 70% alcohol after 48 h. The histological sections were prepared based on Hewitson et al. (2010). Six µm semi-thin sections were made and then stained with hematoxylin-eosin. The mounted slides were observed and photographed using a Leica microscope equipped with a digital Dino camera. Data were statistically analysed using the SPSS package (SPSS 1998). One-way ANOVA test was used for analyses of variance.  $P < 0.05$  was set as the criterion for statistical significance. All data are expressed as mean  $\pm$  SE.

## RESULTS

Results of haematological profiling are shown in Tables 1 and 2. Exposure of male rainbow trout to

Table 1. Differential leukocyte count in rainbow trout after acute exposure to butachlor ( $n = 15$ )

Treatment	Time (day)					
	1		5		10	
	Ctrl	B	Ctrl	B	Ctrl	B
WBC ( $\times 10^3$ /ml)	$77.33 \pm 4.04$	$58 \pm 7.54^*$	$70.06 \pm 1.36$	$53.33 \pm 7.63^*$	$64.5 \pm 8.78$	$35 \pm 10.0^*$
Lymphocytes (%)	$63.66 \pm 4.7$	$59 \pm 4.58$	$61.33 \pm 3.78$	$56.66 \pm 3.2$	$58.6 \pm 3.05$	$43.66 \pm 8.5^*$
Neutrophils (%)	$34 \pm 5.29$	$39.33 \pm 6.02$	$36.33 \pm 4.93$	$36.0 \pm 7.54$	$38.6 \pm 2.51$	$54 \pm 7^*$
Eosinophils (%)	$1.33 \pm 1.15$	$1 \pm 1$	$1 \pm 1$	$2 \pm 0$	$0.66 \pm 0.58$	$1.66 \pm 1.15$
Monocytes (%)	$1 \pm 1$	$0.66 \pm 0.58$	$1.66 \pm 0.58$	$2 \pm 1$	$2 \pm 1$	$1 \pm 1$

\*indicates significant differences in values between the control (Ctrl) group and the group treated with butachlor (B), at ( $P < 0.05$ ); data are expressed as mean  $\pm$  SD

Table 2. Derived haematological parameters in rainbow trout after acute exposure to butachlor ( $n = 15$ )

Treatment	Time (day)					
	1		5		10	
	Ctrl	B	Ctrl	B	Ctrl	B
RBC ( $10^6/\text{mm}^3$ )	$1.30 \pm 0.16$	$1.26 \pm 0.10$	$1.28 \pm 0.12$	$1.13 \pm 0.17$	$1.27 \pm 0.06$	$1.01 \pm 0.12^*$
Hb (g/dl)	$14.13 \pm 0.45$	$13.0 \pm 0.55$	$13.95 \pm 0.25$	$13.1 \pm 0.91$	$14.2 \pm 0.9$	$12.2 \pm 0.95^*$
PCV (%)	$34.0 \pm 3.60$	$27.50 \pm 5.5$	$32.16 \pm 1.25$	$28.0 \pm 4.0$	$33.5 \pm 2.50$	$27.0 \pm 1.0$
MCV (fl)	$266.23 \pm 64.5$	$217.2 \pm 29.7$	$253.2 \pm 34.4$	$247.98 \pm 1.9$	$262.87 \pm 7.7$	$268.71 \pm 22.4$
MCH (Pg)	$109.9 \pm 18.1$	$103.96 \pm 5.31$	$109.6 \pm 12.7$	$117.5 \pm 18.2$	$111.46 \pm 2.12$	$122.16 \pm 21.6$
MCHC (%)	$41.78 \pm 3.0$	$48.73 \pm 9.6$	$43.39 \pm 0.91$	$47.3 \pm 6.98$	$42.41 \pm 0.47$	$45.27 \pm 5.2$

\*indicates significant differences in values between the control (Ctrl) group and the group treated with butachlor (B), at ( $P < 0.05$ ); data are expressed as mean  $\pm$  SD

a toxic concentration of butachlor resulted in significantly decreased white blood cell counts in the treatment group compared to the control ( $P < 0.05$ ).

Lymphocyte values were significantly lower in the exposed fish; in contrast, the level of neutrophils was higher in the treated group than in the control group ( $P < 0.05$ ). Treatment resulted in significantly lower values ( $P < 0.05$ ) of RBC and Hb compared to those of the control group. There was no significant effect on eosinophils, monocytes, PCV, MCHC, MCH and MCV ( $P > 0.05$ ).

Histologically, no changes were observed in the liver (Figure 1c), kidney (Figure 2c) and gills (Figure 3c) of the control fish. Under high concentration of butachlor sinusoids of liver were dilated by red blood cells. Hyperaemia and haemorrhage were frequently seen in the liver. Bile duct hyperplasia was also observed (Figure 1a). Monocellular necrosis, hypertrophic hepatocytes and nuclear degeneration of hepatocytes were evident. Interstitial oedema was noted in the liver (Figure 1b).

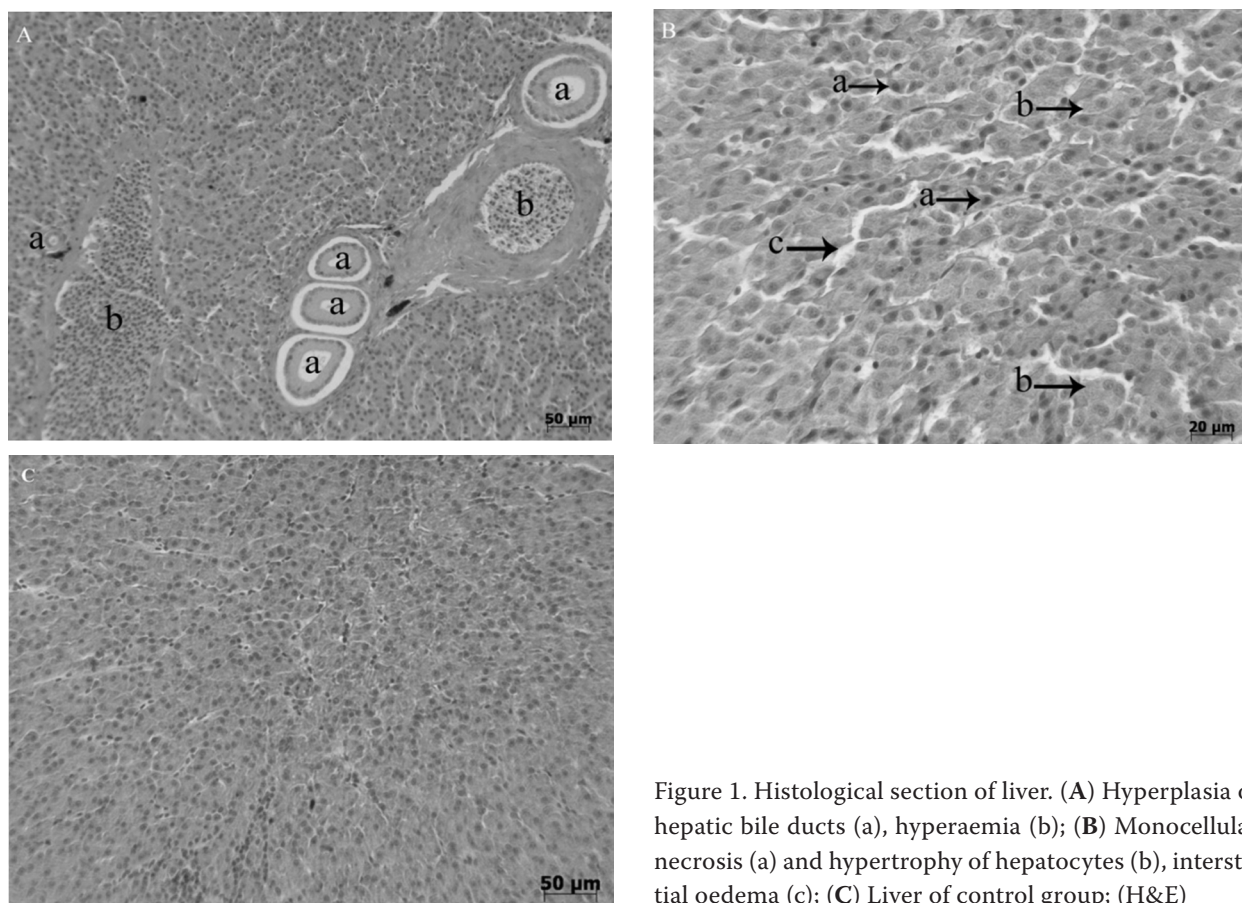


Figure 1. Histological section of liver. (A) Hyperplasia of hepatic bile ducts (a), hyperaemia (b); (B) Monocellular necrosis (a) and hypertrophy of hepatocytes (b), interstitial oedema (c); (C) Liver of control group; (H&E)



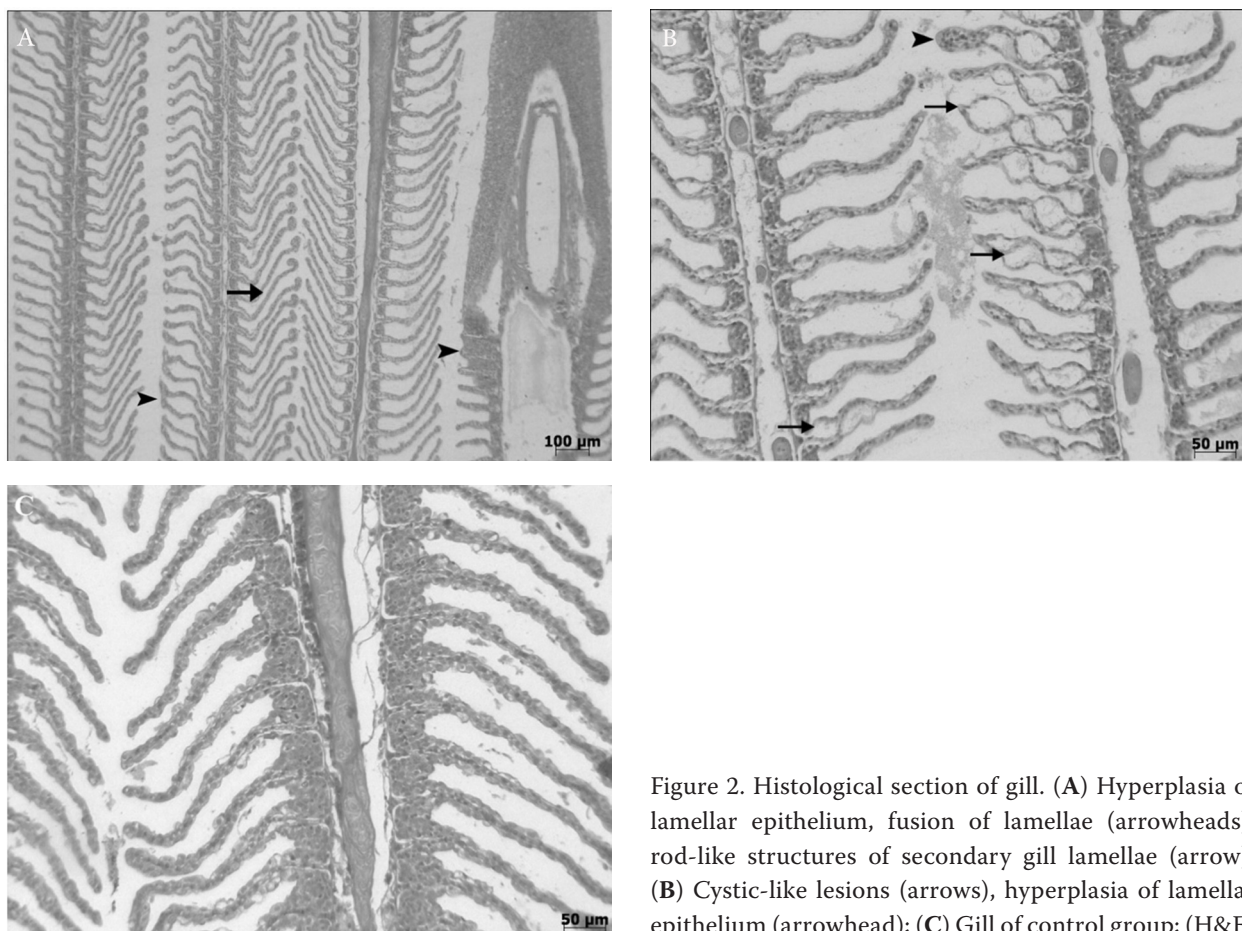


Figure 2. Histological section of gill. (A) Hyperplasia of lamellar epithelium, fusion of lamellae (arrowheads), rod-like structures of secondary gill lamellae (arrow); (B) Cystic-like lesions (arrows), hyperplasia of lamellar epithelium (arrowhead); (C) Gill of control group; (H&E)

Hypertrophy and hyperplasia of lamellar epithelium, fusion of lamellae, rod-like structures of secondary gill lamellae (Figure 2a) and cystic-like lesions (Figure 2b) constituted the histological lesions in the gills. In the kidneys, histopathology indicated vacuolar degeneration of tubular epithelium especially in proximal tubules, desquamation of epithelium (Figure 3a) and necrosis of tubular epithelium (Figure 3b).

## DISCUSSION

Herbicides such as butachlor are recognised as persistent organic pollutants (POPs) They persist in the environment and dissolve in fat and thus become biomagnified and bioaccumulated. As they increase in concentration along the food chain, evaluation of the effects of these pollutants on wildlife is important to reduce their hazard (FDA 1994). Histopathological alterations of vital organs (Schwaiger et al. 1997) and haematological indices (Hesser 1960) have been considered valuable for assessing the effects of pollutants in fish. Ada et al.

(2011) evaluated the effect of different concentrations of butachlor on *Oreochromis niloticus* juveniles and showed that the highest concentration of butachlor (1.8 mg/l) elicited an increase in RBC, Hb, MCHC, WBC and erythrocyte sedimentation rate whereas the PCV was lower in the treated group. However, MCH and MCV did not show any significant reduction compared to the control. Also, they noted that in their experiment the effects of butachlor on haematological parameters did not increase linearly with toxin concentration. Thus, differences may be explained by differences in herbicide concentration and time of exposure or fish species.

The decrease in RBC and Hb levels in our study, obvious signs of anaemia, could be explained as a compensatory response to reduce the oxygen carrying capacity. This would serve to maintain gas transfers and indicates a change in the water blood barrier for gas exchange in the gill lamellae (Jee et al. 2005).

The significant decrease in the Hb and RBC values may also be due to either an increase in the rate at which they are destroyed or to a decrease in the rate

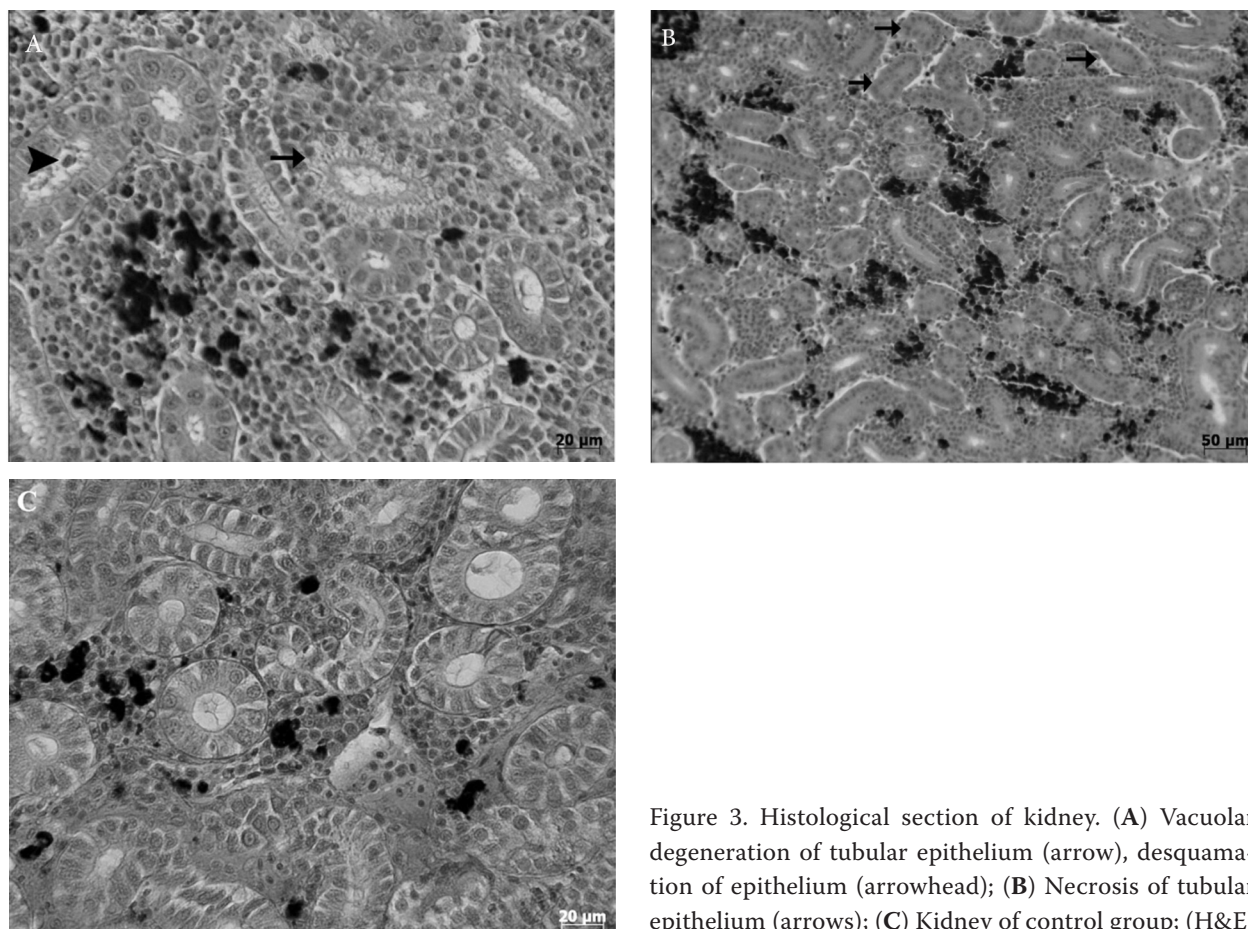


Figure 3. Histological section of kidney. (A) Vacuolar degeneration of tubular epithelium (arrow), desquamation of epithelium (arrowhead); (B) Necrosis of tubular epithelium (arrows); (C) Kidney of control group; (H&E)

of synthesis (Reddy and Bashamohideen 1989). Also, a reduction in lymphocyte values could be caused by the effects of butachlor as an (anti)androgenic endocrine disruptor, because androgens play a role in haematological homeostasis by mediating lymphocyte proliferation (Milla et al. 2011). Conversely, butachlor exposure resulted in an enhancing effect on neutrophil values in the fish. This may be due to the ability of butachlor to induce an immune response. It has been shown that the neutrophil and macrophage activator gene *IL-1b* is significantly induced by butachlor in embryonic zebrafish (*Danio rerio*) (Tu et al. 2013). Reduction of WBC may be a consequence of axenoandrogens, as butachlor appears to target leukocytes (Milla et al. 2011).

Fish are subjected to pesticides in their environment and thus suffer from histological alterations of vital organs (Anbu and Ramaswamy 1991). Butachlor is said to be a contact poison meaning that the gills, liver and kidneys are most affected by this chemical.

Observed histopathological alterations in the liver are common and these structural changes may cause obstruction of circulation and digestive sys-

tems (Boran et al. 2012). It has been demonstrated that butachlor acts as a spindle fibre inhibitor and may therefore lead to liver cells with abnormal sets of chromosomes (Ateeq et al. 2002). Additionally, high concentrations of butachlor have been shown to destroy cell structure (Tilak et al. 2007).

The observed pathological changes after butachlor treatment in the gills of rainbow trout are in agreement with reports in other fish species treated with different pollutants (Cengiz and Unlu 2003; Altinok and Capkin 2007; Velmurugan et al. 2009; Ahmed et al. 2013). This is perhaps because of the direct contact of gills with the toxin. However, cystic-like structure lesions are rare in toxicological studies, while other gill lesions are more common. This may stem from the function of butachlor as a peroxisome proliferator (PP) compound associated with increased cell proliferation (Coleman et al. 2000; Ou et al. 2000). Damage to gills upon exposure to pesticides can lead to respiratory distress (Magare and Patil 2000). Therefore, the findings of the present study indicate that butachlor could distort the normal respiratory function of rainbow trout and may lead to mortality.

Contact with pesticides affects the renal tubules in fish and results in abnormal metabolism (Yokote 1982). Our results show that the kidneys of rainbow trout can be damaged by toxic concentrations of butachlor. However, in contrast to our study, Guo et al. (2010) found that acute exposure to butachlor induced a marked dysfunction in gills, but not the liver and kidneys of flounder (*Paralichthys olivaceus*).

Detrimental histopathological effects can have a negative impact on the growth performance of reared fish and can lead to a decrease in growth and economic losses. However, the observed histopathological alterations found in this study were a result of increased activities of the gills, liver and kidneys and seem to be reversible under suitable conditions.

The negative effects of this pesticide on the reproduction of the migratory kutum fish (*Rutilus frisii kutum*) has been confirmed (Lasheidani et al. 2008). Based on the coincidence of the use of butachlor in rice paddy fields with the reproductive season of fish in the rivers of Northern Iran, it can be assumed that this pesticide negatively impacts on fish breeding resulting in a decline in their offspring. Therefore, the monitoring of pesticides in the rivers of this region, especially during spawning season, is necessary.

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