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Histopathological changes in the gonads, liver, and kidney of *Glossogobius giuris* exposed to sub-lethal concentration of diazinon

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Abstract

The organophosphorus insecticide, diazinon that is widely used in agricultural field and private industrial premises to control pests, easily accumulates in aquatic ecosystems and exerts toxic effects on aquatic animals. The study aimed to evaluate the acute toxicity (LC_{50}) of diazinon on *Glossogobius giuris* and histopathological alterations of its gonads, liver and kidney due to the effect of this insecticide. *G. giuris* were exposed to different concentrations of diazinon ranging from 0.00025 ppm to 2.048 ppm with parallel untreated control. At the doses of 0.0005 ppm, 0.001 ppm, 0.002 ppm, and 0.004 ppm 40%, 50%, 70%, and 90% mortality of *G. giuris* were observed within 96 hrs, respectively, whereas, 100% mortality was observed at doses above 0.008 ppm. Based on probit analysis, the LC_{50} value of diazinon for 96 hrs of exposure on *G. giuris* was found as 0.001 ppm. During the exposure trial several behavioral alterations including restlessness, sudden and quick movements, loss of equilibrium, increased opercular activities and paralysis were observed in the fish. The histopathological changes observed in the tissues of *G. giuris* indicate that sub lethal concentration as well as higher concentration caused moderate to severe alterations in the liver, kidney as well as gonads. Kidney was found to be the most seriously affected organ compared to gonads and liver. Major histopathological changes like fragmentation of testis and ovary with ruptured wall, karyolysis, hypertrophy, pyknosis and degenerative changes such as necrosis of tubular and haematopoietic cells of kidney, necrotic hepatocytes, pyknosis, hypertrophy, haemorrhage and vacuolation were observed in the liver cells. The present study demonstrated that diazinon is highly toxic especially to *G. giuris* and thus, indiscriminate use of this insecticide in the agriculture and industrial sectors should be strictly controlled.

Key words: toxicity, diazinon, histopathology, *Glossogobius giuris*

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Introduction

Diazinon 60 EC (*O*,*O*- diethyl - *O* - [2 - isopropyl - 4 - methyl - 6 - pyrimidyl] phosphorothioate), developed by Novartis in the early 1950s, is one of the most frequently used non-systemic organophosphate insecticides in the agricultural and household environment worldwide (Cox, 1992). In Bangladesh, diazinon is also widely used to control a variety of sucking and leaf eating insects of rice, fruit trees, sugarcane, corn, tobacco, potatoes and other horticultural plants. It is assumed that a certain percent (about 25%) of the used diazinon is easily washed into surface waters and then enters into ground water. Generally diazinon degrades rapidly but under conditions of low temperature, low moisture, high alkalinity and lack of microbiological degraders, it may remain biologically active in soils for six months or longer (Hamm and Hinton, 2000). Because of indiscriminate use, diazinon may severely affect the

normal physiology, biology, early development, and sometimes may cause sudden death of fishes and other aquatic organisms (Burkepile *et al*., 2000; Rashid *et al.,* 2012). However, farmers use diazinon and such type of other pesticides without considering their immediate and residual impacts on fish and other aquatic organisms. Unfortunately, the production of indigenous fish species especially SIS (small Indigenous species) in natural water bodies decreases very sharply due to various manmade and natural interventions and a number of fish species is going to vulnerable to endangered to critically endangered situation for the last two decades in Bangladesh (IUCN, 2000). Uses of pesticides are considered as one of the most notorious causes of this situation.

The freshwater fish gobi, *Glossogobius giuris* is one of the widely distributed SIS in the freshwater and estuaries, ponds, canals, rivers, *haor*, floodplains, and paddy fields of Bangladesh. This fish is also available as endemic in India, Pakistan, Myanmar and Far-East (Nazrul, 2004). It has a special preference in the diet of people of Bangladesh because of its special taste, low fat and high protein content (Islam and Joadder, 2005). However, similar to other SIS, the abundance of *G. giuris* is also decreasing day by day in natural water bodies. Generally this species use shallow inland waters adjacent to the crop cultivation fields as breeding, feeding and nursing ground (Nazrul, 2004). Contamination of the water of those habitats by the huge amount of insecticides or pesticides applied for crop cultivation is considered as one of the crucial causes for decreasing the abundance of *G. giuris* and that of other SIS. Toxic effects of these pesticides vary with the degree of exposure and the type and concentration.

As diazinon is one of the most frequently used pesticides, therefore, its effects on various physiological aspects of different freshwater species have been studied so far (Rahman *et al*., 2002; Dutta and Meijer, 2003; Ahmed, 2011; Shamoushaki *et al*., 2012; Rashid *et al*., 2012; Rouf and Arain, 2013). However, very limited work has been done on histopathological effects of diazinon on fishes and there is no report about the effect of diazinon on *G. giuris*. To protect this species from being extinct through better management it is important to determine the effects of diazinon on *G. giuris*. Therefore, the present experiment was undertaken to know the toxicity effects of diazinon on the gonads, liver and kidney of *G. giuris*. Side by side median lethal concentration (LC_{50}) of diazinon and behavioural changes of *G. giuris* were also identified.

Materials and Methods

The experimental fish and chemicals

To identify the effect of Diazinon 60 EC on *G. giuris,* experiments were conducted at the Wet Laboratory and Fish Genetics Laboratory of the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh during the period from January to December 2014. Diazinon, ethyl alcohol, formaldehyde, xylene, moltex wax, and eosin of standard grade were collected from the authorized dealer of the chemicals in original sealed container from Mymensingh town, Bangladesh. A total of 200 *G. giuris* were collected from Brahmaputra River flowing by the side of Bangladesh Agricultural University, Mymensingh through fisherman and stocked in the cisterns of Mini Hatchery cum Breeding Complex of the Fisheries Faculty building. Fish were reared in the cisterns and fed with trash fish 2 times daily. The average length and weight of the fish were 10.30±0.360 cm and 16.455±0.346 g, respectively.

Acute toxicity of diazinon on G. giuris and behavioural

To determine the acute toxicity (LC_{50}) on *G. giuris*, fourteen concentrations (0.00025, 0.0005, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, 0.512, 1.024, and 2.048 ppm) of diazinon were used. Nine glass aquaria of size 60×30×30 cm^3 with water holding capacity of 50 L were used in this atudt. Desired concentration of diazinon test solution was prepared by

serial dilution of diazinon from original sealed bottle in 50 L tap water for each aquarium.

After acclimatization in the cisterns ten (10) *G. giuris* with the average length of 24.80 cm and average weight of 141 g were released in each aquarium containing different concentrations (treatment) of diazinon for different times with three replications for each treatment against a control. All tests were done at room temperature. The behavioural and other external changes in the body of *G. giuris* were observed during the exposure periods. Mortality was recorded at 0.5, 1, 3, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hrs of exposure time and dead fishes were removed immediately. The LC_{50} value for *G. giuris* was calculated for 96 hrs of exposure time by probit analysis using the computer programme SPSS version 10.0. A value of $p<0.05$ was considered statistically significant. Temperature, dissolved oxygen and pH of diazinon test solution were measured daily by thermometer, DO meter, and pH meter, respectively and recorded.

Histopathological studies on gonads, liver and kidney

For histopathological investigations, gonads, liver, and kidney samples (1 cm^3) of three *G. giuris* from each treatment were collected at the end of exposure period. The gonads samples were preserved in small plastic vials with Bouin's fluid. The liver and kidney samples were preserved in 10% buffered formalin. The samples were then dehydrated, cleaned and infiltrated in an automatic tissue processor. Alcoholic series of higher concentrations, xylene and paraffin wax were used for gonads, liver and kidney tissue samples in the processor maintaining the appropriate time schedules. The sample were then embedded in paraffin wax and sectioned at a thickness of 5 µm by a microtome. Sections were placed on a water bath at a temperature of 41°C. Suitable sections were selected from the ribbons which were finally picked up over glass slides. Then the prepared slides were placed on a hot plate over night at a temperature of 42°C for the fixation of the sections. Hematoxylene and eosin were used to stain the sections according to the method described by Toman *et al*. (2012). The stained sections were then mounted and later examined under a compound microscope. Five histological slides were randomly chosen for each sample. Photomicrographs of the stained sections were taken by using a photomicroscope and qualitative histopathological changes produced by diazinon were detected.

Results

Acute toxicity and behavioural effects

At 0.512, 1.024, and 2.048 ppm of diazinon all the fish died within 12 hrs of exposure. Similarly, at 0.016, 0.032, 0.064 ppm and 0.002, 0.004, 0.006, 0.008 ppm of diazinon all the fish died within 36 hrs and 72 hrs of exposure, respectively. Therefore, the lower concentrations from the acute toxicity test viz. 0.001, 0.0005, and 0.00025 ppm were used to identify the LC_{50} value of diazinon for *G. giuris*. The effects of different concentrations and exposure time of diazinon on *G. giuris* are presented in Table 1. Mortality of all (100%) *G. giuris* was observed at the 0.512 ppm and above doses within 12 hrs exposure period and from 0.008 to 0.256 ppm within 48 hrs of exposure period. At the doses of 0.004, 0.002, 0.001, 0.0005, and 0.00025 ppm of diazinon, about 90%, 70%, 50%, 40%, and 10% mortality of *G. giuris* were observed within 96 hrs, respectively. All of *G. giuris* were found alive in control treatment. Hence, based on probit analysis the LC_{50} value of diazinon was found to be 0.001 ppm for *G. giuris* for 96 hrs of exposure.

During the study period, the behavioural changes and reactions of *G. giuris* were observed to different concentrations of the diazinon. The first reactions of treated fish were observed within five minutes at the concentrations of 2.048 ppm. Several abnormal behaviours such as restlessness, sudden quick and rolling movement, swimming to the back (at higher doses) were observed. Finally, the affected fish became very weak, settled at the bottom and died. The number of dead *G. giuris* increased with the increasing concentration of diazinon. The most important effect of

the toxicant was observed with lack of equilibrium and spiral swimming pattern of fish. The colour of the fishes also became pale progressively with higher doses at the end of 96 hrs of exposure time. On the other hand, shiny colour and normal behaviour of fishes were observed in the control group.

Table 1. Cumulative mortality of *G. giuris* at diazinon treatments during the experimental period

Concentrations (ppm)	Cumulative Mortality (%)											
	30min	1h	3h	6h	12h	24h	36h	48h	60h	72h	84h	96h
0.00025	-	-	-	-	-	-	-	-	-	10	10	10
0.0005	-	-	-	-	-	-	-	10	20	30	40	40
0.001	-	-	-	-	-	-	-	10	30	40	40	50
0.002	-	-	-	-	-	10	30	40	50	50	60	70
0.004	-	-	-	-	10	20	40	50	50	70	80	90
0.008	-	-	-	30	50	80	90	100				
0.016	-	-	-	20	50	80	90	100				
0.032	-	-	-	30	40	80	80	100				
0.064	-	-	-	50	70	80	80	100				
0.128	-	-	30	60	70	80	100					
0.256	-	30	40	80	90	100						
0.512	20	30	60	80	100							
1.024	40	60	70	80	100							
2.048	50	70	80	100								
Control	-	-	-	-	-	-	-	-	-	-	-	-

The detail water quality parameters viz. temperature, dissolved oxygen and pH were also observed. The mean water temperature, dissolved oxygen and pH of different treatments including control during the whole study period were 29.14±0.17^{o}C, 4.31±0.09 ppm, and 7.56±0.79, respectively. There was little variation in the water quality parameters in the diazinon test media. The temperature and pH of the test media were found slightly increased with increase concentrations of diazinon, whereas, the average dissolved oxygen in the test media was higher at lower concentrations.

Histopathological effects of diazinon on the gonads, liver and kidney of G. giuris

The histopathological changes observed in the tissues *of G. giuris* in the present study indicate that sub lethal concentration as well as higher concentration caused moderate to severe alterations in the liver, kidney as well as in the gonads, which are the important organs performing vital functions like detoxification, respiration, reproduction, acid-base balance, excretion etc. In the histological observation of gonads, structure and systematic arrangement of cells of both testis and ovary were found normal where no diazinon was used (Figure 1A, 2A). At an exposure to 0.001 ppm diazinon, mild fragmentation of testis cell with ruptured wall and karyolysis were found (Figure 1B) and at the higher doses i.e., 0.032 and 0.064 ppm of diazinon, severe fragmentation of testis cell with ruptured wall and karyolysis were observed (Figure 1C and 1D). Same pathological signs were seen in the ovary of *G. giuris*. At the dose of 0.001 ppm diazinon, mild fragmented ovary with ruptured wall, abnormal shape, necrosis and karyolysis were found (Figure 2B). More fragmentation, ruptured wall, and karyolysis of ovary were observed when exposed to 0.032 and 0.064 ppm diazinon (Figure 2C, 2D).

The organ most associated with the detoxification and biomarker process is liver and due to its function,

position and blood supply, it is also one of the organs most affected by contaminants in the water. The liver of *G. giuris* exposed to both low and high doses of diazinon have been found to be affected.

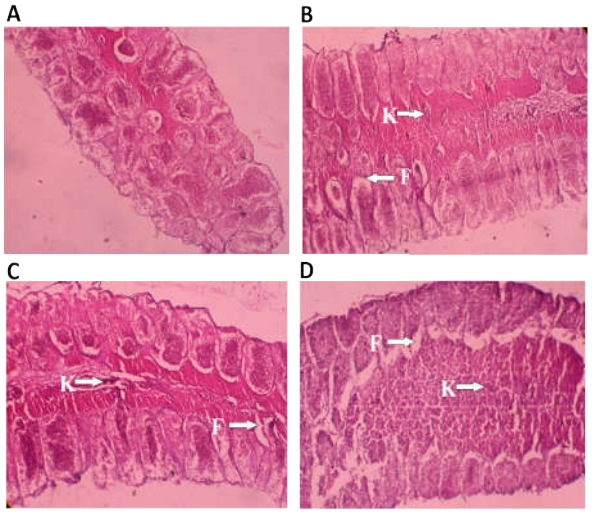

Figure 1. Photomicrograph of testis section of *G. giuris* (stain: H&E; magnification: ×400); control group (A): normal structure; four days exposure to 0.001 ppm of diazinon (B): fragmentation (F), ruptured wall and karyolysis (K); three days exposure to 0.032 ppm of diazinon (C): fragmentation (F), ruptured wall and karyolysis (K); two days exposure to 0.064 ppm of diazinon (D): severe fragmentation (F), ruptured wall and karyolysis (K).

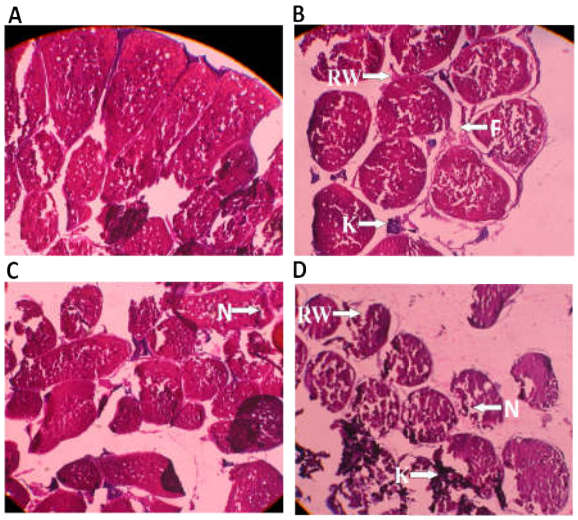

Figure 2. Photomicrograph of ovary section of *G. giuris* (stain: H&E; magnification: ×400); control group (A): normal structure; four days exposure to 0.001 ppm of diazinon (B): ruptured wall (RW), fragmentation (F), karyolysis (K); three days exposure to 0.032 ppm of diazinon (C): ruptured wall (RW), karyolysis (K), necrosis (N); two days exposure to 0.064 ppm of diazinon (D): ruptured wall (RW), karyolysis (K), and necrosis (N).

The hepatocytes and other cells of the liver in control groups of fishes were normal and systematically arranged (Figure 3A). At the dose of 0.001 ppm, hypertrophy of hepatocytes, mild necrosis and minor vacuolation were found (Figure 3C). With the increase of diazinon concentration to 0.032 ppm, severe necrotic hepatocytes, pyknosis, hypertrophy, haemorrhage, and vacuolation were observed (Figure 3C). But at the concentration of 0.064 ppm the same kind of abnormalities were observed as that exposed to 0.032 ppm but with more severe intensity (Figure 3D). The kidney is a vital organ of body and proper kidney function is very much necessary to maintain the homeostasis. With severe intoxicated conditions, the degenerative process leads to tissue necrosis in the kidney. Haematopoietic cells of kidney were found normal and systematically arranged in the control groups of *G. giuris* (Figure 4A). Mild necrosis, pyknosis and tubular degeneration were seen in the kidney tissue after 4 days exposure to 0.001 ppm of diazinon (Figure 4B). Severe necrosis, vacuolation, pyknosis and tubular degeneration were observed in the kidney tissue upon exposure to 0.032 and 0.064 ppm (Figure 4C, 4D).

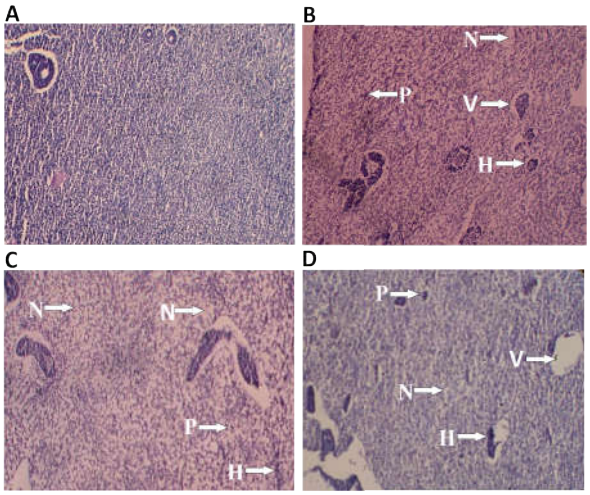

Figure 3. Photomicrograph of liver section of *G. giuris* (stain: H&E; magnification: ×400); control group (A): normal structure; four days exposure to 0.001 ppm of diazinon (B): necrosis (N), pyknosis (P), vacuolation (V), and hemorrhage (H); three days exposure to 0.032 ppm of diazinon (C): necrosis (N), pyknosis (P), and hemorrhage (H); two days exposure to 0.064 ppm of diazinon (D): necrosis (N), pyknosis (P), vacuolation (V), and hemorrhage (H).

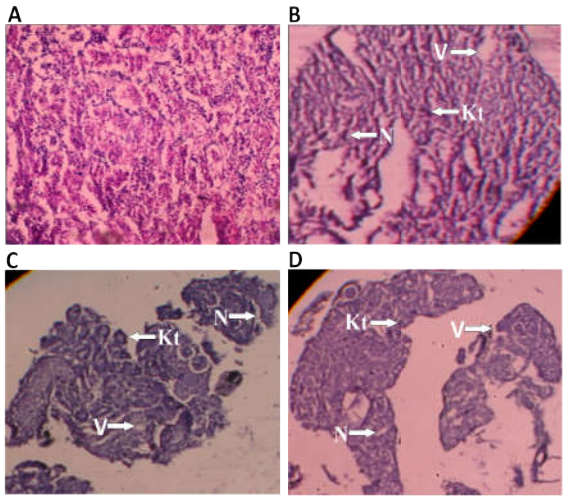

Figure 4. Photomicrograph of kidney section of *G. giuris* (stain: H&E; magnification: ×400); control group (A): normal structure; Four days exposure to 0.001 ppm of diazinon (B): necrosis (N), and pyknosis (P); three days exposure to 0.032 ppm of diazinon (C): necrosis (N), tubular degeneration (Kt), and vacuolation (V); two days exposure to 0.064 ppm of diazinon (D): necrosis (N), tubular degeneration (Kt), and vacuolation (V).

Discussion

To determine the LC_{50} of diazinon on *G. giuris* 14 different doses were used. The highest and most rapid mortality was observed in fish exposed to the highest concentration of diazinon tested. The LC_{50} value of *G. giuris* for 96 hrs was found to be as low as 0.001 ppm. Hence, diazinon is considered a severely toxic substance to *G. giuris* as this fish is very sensitive to the presence of any kind of toxic chemicals. Rahman *et al*. (2002) reported LC_{50} values of diazinon for the air breathing fishes, *Anabas testudineus* and *Channa punctatus* were 6.55 and 3.09 ppm for 96 hrs of exposure, respectively. These values are very high compared to the value of diazinon obtained in the present study for *G. giuris* because air breathing fish have a high tolerance level for diazinon. On the other hand, in case of large sized fishes like *Cirrhinus mrigala* and *Cyprinus carpio* the LC_{50} values of diazinon for 96 hrs were 5.8 ppm, 8.15 ppm, and 9.76 ppm, respectively (Rauf and Arain, 2013; Ahmed, 2011). From these studies a similarity was observed in the LC_{50} values obtained for different larger sized species and these values are significantly higher compared to *G. giuris*. On the other hand, Hossain and Halder (1997) found that the median lethal concentration (LC_{50}) of Talstar 2.5 EC on *Labeo rohita* fry was 0.0014 ppm for 48 hrs which is very much similar to the findings of the present study (LC_{50} of diazinon on *G. giuris*) although the exposure time was less for *Labeo rohita*. Observing of similar LC_{50} values for two small fish i.e., *Labeo rohita* fry and *G. giuris* suggested that SIS fish species and/or large fish species in their early stages are very vulnerable to the exposure of diazinon compared to larger ones. Dutta and Meijer (2003) reported that the age of fish, size, and duration of exposure affect the toxicity potential of diazinon in various fish species and the degree of its sensitivity varied even among the fish of the same genus and family. Rahman *et al.* (2002) also reported that the LC_{50} value for 96 hrs of exposure to diazinon for the small fish *Barbodes gonionotus* to be 2.72 ppm which is much higher than the present findings. Phylogenetic difference between the species might be the reason for large variation in the LC_{50} value. The difference in the toxicity of diazinon may be attributed to differences in susceptibility and tolerance regarding absorption, biotransformation, and excretion of diazinon.

Behavioural changes in fish are important indicators of potential toxic effects of pesticide exposure. In the present study, several abnormal behaviours such as restlessness, loss of equilibrium, increased opercular activities, surface to bottom movement, sudden quick movement, and resting at the bottom were observed. Similar behavioural patterns were noticed by Rahman *et al*. (2002) on *Anabas testudineus*, *Channa punctatus*, and *Barbodes gonionotus*, and Haque *et al.* (1993) on *Puntius gonionotus*. They reported that due to the toxicity effects of diazinon, in all cases the fish gradually lost equillibrium, became paralyzed and finally settled down to the bottom of the aquarium and remained at the same place till death. The most important effect of diazinon that was found in the present study may be the alterations of the nervous and brain systems, which was obvious with lack of equilibrium and spiral swimming pattern of *G. giuris* to

that found by Shamoushaki *et al.* (2012) on *Rutilus frisii kutum* due to the effect of diazinon.

Histopathological observation is considered to be a sensitive bio-monitoring tool in toxicant impact assessment to indicate the effect of toxicants on fish in pesticides polluted aquatic ecosystems (Marchand *et al.,* 2009). Pesticides in polluted aquatic ecosystem are accumulated mainly in the metabolically active tissues of fish such as liver, kidney, gonads and gill (Oruce and Usta, 2007) and cause histopathological damage to those organs. *G. giuris* was exposed to different concentrations of diazinon and its effect on some internal organs of this fish for some selected concentrations has been observed. In the gonad (testis and ovary) samples of *G. giuris* under control, normal shape and arrangement of gonadal materials and histopathological states were observed. At 0.001 ppm of diazinon both testis and ovary of the *G. giuris* were found somewhat fragmented with ruptured wall. At 0.032 and 0.064 ppm of diazinon, karyolysis and fragmented ovary and testis with abnormal shape and arrangement were observed which agreed with the findings of Hossain *et al.* (2002). Sivarajah *et al.* (1978) also reported similar type of fragmentation and karyolysis in the ovaries of *Salmo gairdneri* and *Cyprinus carpio* when exposed to the organochlorine pesticide, Aroclor. The liver of *G. giuris* in the control was normal without necrosis, hepatocytes and other cells were normal and systematically arranged. Tissue sample of the liver from fishes treated with 0.004, 0.032, and 0.064 ppm of diazinon showed marked degenerative changes, severe necrosis, pyknosis, haemorrhage and vacuolation in all the tested fish which is in agreement with the findings of Rahman *et al.* (2002) for *Anabas testudineus*, *Channa punctatus*, and *Barbodes gonionotus*; and Omitoyin *et al.* (1999) for *Sarotherrodon galilaeus*. Dubala and Shah (1979) exposed *Channa punctatus* to 1.0 ppm malathion and reported either damage or disintegration in about one third of hepatic cells and acute shrinkage of nuclear materials. Kabir and Begum (1978) reported cytoplasmic degeneration, pyknotic nuclei in liver tissues; vacuolation in hepatic cells and rupture of blood vessels; degenerative hepatic cells and necrotic nuclei in *Heteropneustes fossilis* exposed for 25 days to 5, 10, and 20 ppm diazinon, respectively. Such qualitative changes of liver tissues reported by Dubala and Shah (1979) and Kabir and Begum (1978) were similar to those of the present study. Kidney tubules and hematopoietic cells of kidney were systematically and regularly arranged in the control group of the tested fish. At the dose of 0.001 ppm mild pyknosis and mild degenerative changes viz. necrosis of tubular and hematopoietic cells were observed in *G. giuris* as also observed by Hossain *et al.* (2002) and Omitoyin *et al.* (2006). Treatments with of 0.032 and 0.064 ppm recorded severe necrosis, pyknosis, hemorrhages and tubular degeneration in the kidney of *G. giuris*. Similar histological changes were also reported by Rahman *et al.* (2002) in *Anabas testudineus, Channa punctatus*, and *Barbodes gonionotus* exposed to diazinon for several days. It is, therefore, found that exposure to chronic sub-lethal concentrations of diazinon resulted in significant behavioural changes and histopathological alterations in some most important internal organs of *G. giuris* which may be potentially disruptive for the survivability of *G. giuris* in wild environments.

Conclusion

The result of the present study revealed that diazinon is toxic to fish and causes histopathological changes in fish vital organs. The recorded 96 hrs LC_{50} value for *G. giuris* is very lower which indicate that *G. giuris* is quite susceptible to diazinon and its mortality increases proportionally at higher doses. In case of histopathological study, it can be said that at higher doses some important organs of *G. giuris* viz. gonads, liver, and kidney were severely damaged that ultimately causes the death of the fish. Therefore, strict biosecurity should be taken into consideration when diazinon is used by the farmers in agriculture fields particularly surrounding aquatic environment.

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