Toxicology Reports 4 (2017) 348–357

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Comparison of waterborne and intraperitoneal exposure to fipronil in the Caspian white fish (*Rutilus frisii)* on acute toxicity and histopathology

Rashid Alijani Ardeshir[a], Hossein Zolgharnein[a], Abdolali Movahedinia[a], Negin Salamat[a], Ebrahim Zabihi[b,*]

[a] Department of Marine Biology, Faculty of Marine Sciences, Khorramshahr University of Marine Science and Technology, P.O. Box 669, Khorramshahr, Iran
[b] Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

ARTICLE INFO

Chemical compounds studied in this article:
Fipronil (PubChem CID: 15278226)
Phenoxyethanol (PubChem CID: 17848643)
Haematoxylin (PubChem CID: 442514)
Eosin (PubChem CID: 11048)
Picric acid (PubChem CID: 6954)
Formaldehyde (PubChem CID: 712)
Acetic acid (PubChem CID: 176)
Ethanol (PubChem CID: 702)
M-xylene (PubChem CID: 7929)

Keywords:
Fipronil
Caspian white fish
Acute toxicity
Administration route

ABSTRACT

Fipronil is an effective insecticide widely used in agriculture with potential ecotoxicological consequences. The median lethal dose (LD_{50}) and concentration (LC_{50}) of fipronil in 16.3 g Caspian white fish, *Rutilus frisii kutum* fingerlings were determined. To determine the LD_{50}, a total of 133 fish were assigned to 19 tanks (7 fish/tank) including one control and 6 treatment groups (300, 450, 550, 650, 750, 850 mg/kg). Fish were injected intraperitoneally and monitored at 96 h. The LD_{50} of fipronil was 632 mg/kg suggesting it was slightly toxic to the Caspian white fish. To determine LC_{50}, 114 fish were assigned to 19 tanks (6 fish/tank) including one control and 6 treatment groups (300, 400, 500, 600, 700, 800 µg/L). The LC_{50} of fipronil was 572 µg/L, which was highly toxic to the fish. The degree of tissue change (DTC) in vital organs from moribund fish exposed via waterborne exposure showed severe damage (DTC: 71 ± 52 for 700 µg/L) in the gill, including aneurisms, extensive fusion and necrosis. The fish exposed through the intraperitoneal route seemed to have severe lesions (DTC: 66 ± 50 for 750 mg/kg) in the kidney, involving hemorrhage, tubular degeneration and necrosis. The liver had no significant differences in DTC values between the two routes and showed pyknosis and sinusoid dilation. Hematoxylin and eosin staining did not show any histological alterations in the brain but nissl staining showed some alterations in distribution of purkinje cells. Generally, this study showed that the route of exposure to fipronil not only affects its acute toxicity but also determines the main target organs of toxicity and histopathological alterations in Caspian white fish.

1. Introduction

Fipronil is a relatively new insecticide with a wide range of uses in agriculture. Fipronil toxicity results from its ability to block gamma-aminobutyric acid-gated chloride channels of neurons in the central nervous system [1]. The increasing use of this pesticide has raised concerns for its harmful effects on human health and the environment [2]. In addition to insects, fipronil has toxic effects on non-target organisms, such as aquatic invertebrates [3], fish [4], some reptiles [5], birds [6] and mammals [7]; and the acute toxicity of fipronil has been determined for these animals.

Median lethal concentration (LC_{50}) and dose (LD_{50}) have been widely used to determine acute toxicity in aquatic and terrestrial animals, respectively. Waterborne administration has advantages such as simulating environmental exposure, involving no anesthesia and less handling of fish and relatively higher absorption rate constant for contaminants. Although waterborne exposure is a common route of toxicant absorption in the aquatic environment, LD_{50} have also been determined in these animals, especially in fish. Compared to waterborne (w.b.) exposure, evaluating intraperitoneal (i.p.) exposure to fipronil in fish has also some advantages. Although both LD_{50} and LC_{50} estimate expressed toxicity, LD_{50} can be a closer estimate of inherent toxicity and is determined based on a whole-body dose (mg/kg) and not water concentration (mg/L) (Hodson, 1988). Moreover, toxicological studies such as detoxification mechanisms in fish, based on LD_{50}, can be more accurately extrapolated to terrestrial mammals. Participants in the Collaborative Workshop on Aquatic Models and 21st Century Toxicology, held at North Carolina State University on May 5–6, 2014, agreed that small fish models can be used as biological model in toxicology and have advantages over mammalian models if standardized protocols are prepared and used [8]. They also recognized the need for extensive studies on fish toxicology and non-water exposure of fish to toxicants. The other reason for determination of the LD_{50} of fipronil in fish is related to its low/moderate water solubility [9] which makes it

* Corresponding author.
E-mail address: e.zabihi@mubabol.ac.ir (E. Zabihi).

http://dx.doi.org/10.1016/j.toxrep.2017.06.010
Received 6 March 2017; Received in revised form 22 June 2017; Accepted 22 June 2017
Available online 23 June 2017

difficult to determine the fipronil dose response relationship. In addition, photolysis can transform fipronil into its metabolites (fipronil-desulfinyl and fipronil-sulfone), which are more toxic than the parent compound for fish [10,11]. Therefore, measurements of fipronil's effects on fish should be considered along with its metabolites. On the other hand, measurement of LC_{50} for larger fish needs larger amounts of fipronil and proper water in a non-static system. Consequently, it is not a good option economically and environmentally. Thus, measurement of LD_{50} of fipronil in fish is necessary to be used for future research, and this study is the first time.

In spite of the advantages and disadvantages cited above, this study was designed to compare the acute toxicities of fipronil through both w.b. and i.p. exposure and to determine the main target of toxicity in Caspian white fish. Previous studies have shown that histopathological studies are a precise and rapid way to show the direct effect of toxicants on target organs [12–14] and similar tests were selected for this study.

Fish are the most important aquatic food and as such can contaminate human populations. In the area south of the Caspian Sea, fipronil is mostly used in rice fields against striped rice stemborer. The streams containing fipronil from the farms enter the Caspian Sea (salinity ≈ 13 ppt) and might affect aquatic life. Caspian white fish (*Rutilus frisii kutum*), belonging to the cyprinidae family, is the most popularly consumed fish in this region and cultured extensively. Thus, both as a model and to provide information concerning the implications of fipronil use, the median lethal dose and concentration of fipronil in fish was studied.

2. Materials and methods

2.1. Fish

Two hundred and fifty Caspian white fish fingerlings (mean body weight: 16 ± 3 g) were obtained from the Shahid Rajai Fish Proliferation and Culture Center (Sari, Mazandaran Province, Iran). The fish were randomly divided into groups without determination of the male: female ratio. Fish were acclimated for 2 wk prior to the test, and fed commercial fish food until the day before fipronil exposure.

2.2. Determination of 96 h LD_{50} value for fipronil

2.2.1. Fish environment and handling

Nineteen plastic tanks (1000 L capacity) including a negative control tank (no replicate) and treatment tanks with water-shower aeration and a semi-static system were used. Non-chlorinated well water with characteristics showed in Table 1 was used. The photoperiod was 13 h light and 11 h dark. The volume of water used in each tank was about 200 L.

2.2.2. Preparation and injection of fipronil solution

Fipronil (98% purity, 50:50 racemic mixture) was purchased from the Moshkfam Fars Chemical Company (Shiraz, Iran). Stock solutions of fipronil were prepared in 6 amber glass vials containing 5cc sunflower oil and 108, 162, 198, 234, 270, 306 mg fipronil and one glass vial containing only 5cc sunflower oil. To dissolve fipronil in the oil, the stocks were vortexed for 30 min. Before the injection, the fish were anesthetized using phenoxyethanol, and weighed. For each treatment, 0.25 ± 0.05cc of the standard solution was i.p. injected into the fish using an insulin syringe based on the weight of each fish.

Table 1
Characteristics of groundwater used for the experiment.

Parameter	Value
pH	6.9 ± 0.3
Dissolved oxygen (mg/L)	7.8 ± 0.5
Temperature (° C)	18.1 ± 0.8
Total hardness (mg/L)	394 ± 7
Total dissolved solid (mg/L)	440 ± 20
EC (μs/cm)	860 ± 20
Nitrate (mg/L)	0.9 ± 0.3
Nitrite (mg/L)	0.009 ± 0.003
Bicarbonate (mg/L)	410 ± 10

2.2.3. Experimental design for LD_{50}

There were 6 treatment groups with three replicates and 7 fish in each group. After some experimental tests for estimation of lethal dose range, fipronil was i.p. injected into the fish at 300, 450, 550, 650, 750, 850 mg/kg of fish weight. The fish were monitored for 96 h (4 d) for any mortality and then sacrificed for histopathological tests.

2.3. Determination of 96 h LC_{50} value for fipronil

2.3.1. Experimental design

There were 6 treatment groups with three replicates and 6 fish for each group. Nineteen plastic tanks (20 L capacity) including a negative control tank (no replicate) and treatment tanks with air pump aeration and static system were used for determining the LC_{50}. Oxygen dissolved concentration and pH were maintained around 8 mg/L and 7.5, respectively. After acclimation, 6 fish were randomly transferred into each tank containing 15 L of non-chlorinated well water and 4.5, 6, 7.5, 9, 10.5 and 12 mg fipronil (without solvent) for 96 h and the number of dead fish were recorded daily. Moreover, to record any changes in behavior, fish were observed for about 1 h once daily.

2.4. Histopathological tests

After 96 h of exposure, three moribund fish from the 450, 550, 650 and750 mg/kg, and 400, 500, 600 and 700 μg/L (the treatment groups which had enough moribund fish) fipronil exposed tanks and three fish from the control tank were sacrificed by decapitation, dissected, and the gills, livers, kidneys and brains were fixed in Bouin's solution for 48 h. The tissue were rinsed in a graded series of ethanol to be dehydrated, cleared in xylene, embedded in paraffin, sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H & E). Nissl staining was also done for the brain tissue according to Parent et al. [15]. Three random sections per fish tissue were observed under the light microscope (Olympus Co, Tokyo, Japan) and photographed using a Microscope Camera Eyepiece (Dino-Lit Premier AM7023; AnMo Electronics Corporation, Taiwan). The histological alterations for each organ studied were assessed semi-quantitatively for the degree of tissue change (DTC), according to the procedures of Poleksic and Mitrovic-Tutundzic [16]. The alterations were classified into three stages, including stage I (without alteration, i.e., normal functioning of the tissue), stage II (some to severe damage), and stage III (very severe and irreparable damage). DTC was calculated using the following formula: DTC = (1 X SI) + (10 X SII) + (100 X SIII) where SI, SII and SIII is equal to the summation of alterations in each stage. Then, $0 \leq DTC \leq 10$ indicates normal functioning of the organ; $11 \leq DTC \leq 20$ indicates slight damage to the organ; $21 \leq DTC \leq 50$ indicates moderate damage to the organ; $50 \leq DTC \leq 100$ indicates severe lesions and $100 < DTC$ indicates irreversible damage to the organ.

2.5. Statistical methods

Data analysis was done using MedCalc (ver. 16.8.4) statistical software (Microsoft Partner, Korea). The acute toxic effect of fipronil on the Caspian white fish was determined by the use of Finney's probit analysis. A 95% confidence interval was calculated for the analysis. Sigma Plot ver. 11 software (Systat Software, Inc., CA, USA) was used for statistical analysis. The Mann–Whitney test was used for comparison of DTC results. The significance level was set at $P < 0.05$.

3. Results

3.1. Clinical signs

About 12 h after i.p. injection, fish showed clinical signs of semi-circular swimming behavior (this behavior was not observed in the control group). With high doses, the fish showed muscle shivering. In some cases, darkening and swelling on the dorsal side were also observed. The dead fish showed erected pectoral fins, larger livers (increased hepatosomatic index (HSI) without significant alteration in body weight, data not shown) and color changes of the gall bladder bile along with more reddish kidneys.

With waterborne exposure, fish showed semi-circular swimming behavior and erected pectoral fins. Moreover, some fish showed hemorrhaging in the eye (hyphema) and muscle shivering.

3.2. Mortality

No mortalities were recorded during the acclimation period (except for the first day of the acclimation with 4 mortalities) and in the control group. During the exposure period, the fish were considered dead if they did not have any movement. Most mortalities happened on the first day (Table 2) in the treatment groups. No mortality was observed in the negative controls. The LD_{50} was calculated as 632 mg fipronil/kg fish (95% CI = 585–682) (Table 3). The cumulative mortality by treatment group was 100% at 850 mg/kg, 57.1% at 750 mg/kg, 52% at 650 mg/kg, 33.3% at 550 mg/kg, 23.8% at 450 mg/kg and no mortality at 300 mg/kg fipronil.

With w.b. exposure most mortalities in the treatment groups took place after 48 h (Table 4). LC_{50} was calculated as 572 μg/L (95% CI = 530–615) (Table 5). The cumulative mortality was recorded as 100% at 800 μg/L, 72.2% at 700 μg/L, 66.6% at 600 μg/L, 22.2% at 500 μg/L and 16.6% at 400 μg/L (Figs. 1 and 2).

3.3. Histological changes

Microscopic observations of 9 sections from the control fish showed that the gills, livers, kidneys and brains were normal (Fig. 3A). In fish exposed in water, the gills showed hypertrophy in the secondary and primary lamella, hyperplasia, aneurysm, extensive fusion, deletion and necrosis (Fig. 3 D-F) (Table 6). The mean DTC values calculated for the 63 exposed gill sections showed that histopathological alterations of the gills in i.p. exposure (DTC: 3.1 ± 0.9, 2.3 ± 1.4, 1.2 ± 1 and 1.2 ± 1.1 for 750, 650, 550 and 450 mg/kg, respectively) (Fig. 3B and C) was significantly less than that in w.b. exposure (DTC: 71 ± 52, 60 ± 49, 14.4 ± 1.6 and 14 ± 4 for 700, 600, 500 and 400 μg/L, respectively) ($P < 0.05$) (Fig. 7). There was either a dose (concentration) response relationship between fipronil and gill histological alterations or significant differences in gill DTC values between the two routes (Fig. 7). The livers of fish exposed to fipronil via the i.p. route showed structural alteration in the exocrine pancreatic duct, pyknotic nucleus and sinusoid dilation (Fig. 4C and D). There was no significant difference ($P \geq 0.05$) in DTC values of the livers between the i.p. route (DTC: 10 ± 5, 9.5 ± 5.1, 7.7 ± 4.1 and 8.8 ± 4.7 for 750, 650, 550 and 450 mg/kg, respectively) and w.b. exposure (DTC: 7 ± 5, 7.6 ± 4.9, 7 ± 5 and 6.7 ± 4.7 for 700, 600, 500 and 400 μg/L, respectively) to fipronil nor any dose (concentration) relationship between fipronil and liver histological alterations. However, liver DTC values with the i.p. route were higher than that with w.b. exposure (Fig. 7). With the i.p. exposure, the kidney showed a higher degree of

Table 2
Daily numbers of dead Caspian white fish after intraperitoneal injection of different doses of fipronil.

Number of dead fish by day					
Dose (mg/kg)	Day 1	Day 2	Day 3	Day 4	Decapitation on day 5
300	0	0	0	0	21
450	5	0	0	0	16
550	7	0	0	0	14
650	8	0	0	3	10
750	9	0	0	3	9
850	15	0	3	3	0
Total	44	0	3	9	70

Table 3
Dose-response values after intraperitoneal injection into Caspian white fish (degree of freedom was 1, $P < 0.05$).

Probability	Dose (mg/kg)	95% Confidence interval	
LD_{10}	408	298	472.8
LD_{20}	485	403.6	537.6
LD_{50}	632.4	585.4	681.5
LD_{80}	779.9	724.6	867.9
LD_{90}	857	788.6	974.1
LD_{99}	1040	934.1	1230

Table 4
Daily numbers of dead Caspian white fish after waterborne exposure to fipronil.

Number of dead fish by day					
Concentration (μg/L)	Day 1	Day 2	Day 3	Day 4	Decapitation on day 5
300	0	0	0	0	18
400	0	0	0	3	15
500	0	0	0	4	14
600	0	0	6	6	6
700	0	7	0	6	5
800	12	6	0	0	0
Total	12	13	6	19	58

Table 5
Dose − response values after waterborne exposure in the Caspian white fish (degree of freedom was 1, $P < 0.05$).

Probability	Dose	95% Confidence interval	
LC_{10}	401.4	313.9	454.6
LC_{20}	460	393.1	504.8
LC_{50}	572.1	530.1	615.1
LC_{80}	684.3	638.4	754.3
LC_{90}	742.9	688.1	833.8
LC_{99}	882.1	800.2	1030

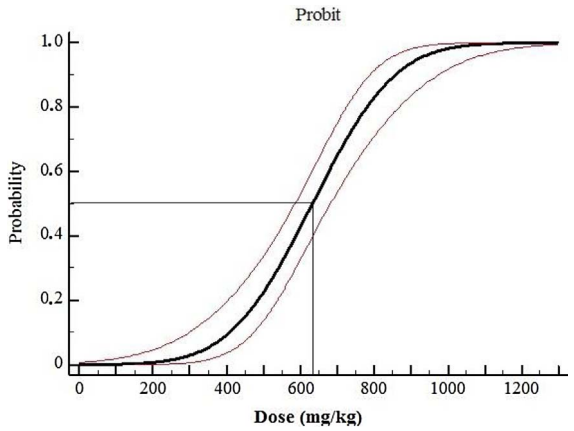

Fig. 1. Fipronil 96-h predicted mortality − dose response curve for Caspian white fish fingerlings based on parameter estimates from the probit analysis. Fish were injected intraperitoneally with different doses of fipronil. LD_{50} was calculated as 632 mg/kg. Confidence interval (95%) curves are also shown.

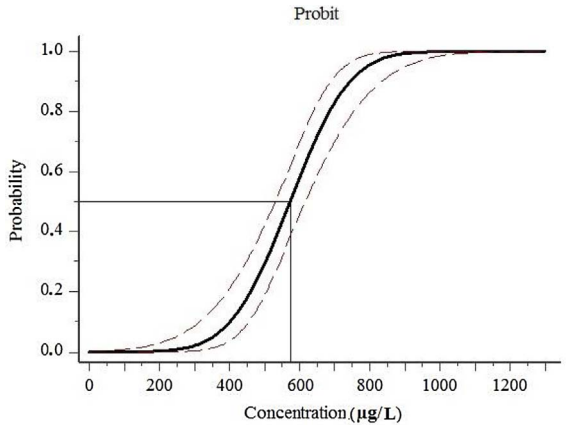

Fig. 2. Fipronil 96-h predicted mortality − concentration response curve for Caspian white fish fingerlings based on parameter estimates from the probit analysis. Fish were exposed to waterborne fipronil. LC_{50} was calculated as 572 µg/L. Confidence interval (95%) curves are also shown.

damage, including hemorrhaging, degeneration and necrosis of the tubule (Fig. 5C-G); and these changes with the i.p. exposure (DTC: 67 ± 50, 66 ± 48, 16.2 ± 4.4 and 17 ± 3 for 750, 650, 550 and 450 mg/kg, respectively) were significantly higher than that with the w.b. exposure (DTC: 13 ± 8, 12.8 ± 8, 7.4 ± 5 and 7 ± 6 for 700, 600, 500 and 400, respectively) ($P < 0.05$). Moreover, there was a dose response relationship between fipronil and kidney DTC values with the i.p. route (Fig. 7). The brain showed no damage with H & E staining, and nissl bodies, perikaryon, nerve fibers and granule cells were normal (Fig. 6). However, nissel staining showed that some alterations in the distribution of purkinje cells at the cerebellum (Fig. 6). There was no dose (concentration) response relationship between fipronil and brain DTC values or significant difference in brain DTC values between the two routes (Fig. 7).

4. Discussion

4.1. Acute toxicity

To the best of our knowledge, this is the first study conducted to determine the i.p. LD_{50} of fipronil and to compare its acute toxicity and histopathological effects in fish after administration through different routes of exposure. Trophic (feeding), w.b. exposure, and i.p. injection are the common routes of xenobiotic administration with fish. The preferred route of administration depends on the purpose of the study and the physicochemical properties of the toxicants. Determination of the LD_{50} for chemicals in fish is a simpler, faster and less expensive alternative than LC_{50} [17].

According to the US Environmental Protection Agency [18], LD_{50} measured in this study for fipronil (632 mg/kg) would have a slight toxicity (501–2000 mg/kg) while the measured LC_{50} (572 µg/L) would have a high toxicity (0.1–1 mg/L). The toxicity of fipronil to many aquatic organisms varies from highly toxic to very highly toxic with w.b. exposure. Previous studies of the LC_{50} determination with different species of fish showed that fipronil had a high toxicity (100–1000 µg/L) in rainbow trout (*Oncorhynchus mykiss*, 246 µg/L), Japanese carp (*Cyprinus carpio*, 340 µg/L), sheephead minnow (*Cyprinodon variegatus*, 130 µg/L) and very high toxicity (< 100 µg/L) in bluegill sunfish (Lepomis macrochirus, 83 µg/L) and Nile tilapia (*Oreochromis niloticus*, 42 µg/L) [2]. Comparison of the LC_{50} previously measured for fipronil in different species of fish with those in this study showed some differences that may be related to factors such as species and weight of tested fish, the water quality characteristics and the purity of the fipronil.

In addition to the dose and duration of exposure, the route of administration can affect the degree of the toxicity [19,20]. Previous studies conducted on toxicokinetics of fipronil in rainbow trout [21] and green frogs [22] after w.b. exposure showed that it could be easily absorbed and distributed to most organs. After i.p. exposure, fipronil is absorbed into the bloodstream, transferred directly to the liver with potential detoxification [23], carried to the heart by non-oxygenated blood, and pumped to the gills. Finally, the oxygenated blood, containing fipronil and its metabolites is circulated in the whole body. With w.b. exposure, fipronil absorption from water is efficient due to the counter-current system in the gills [24] and its local concentration in the gill epithelial layer is much higher than with i.p. exposure. In fact, insecticides can induce contraction of gill pillar cells, widening of blood spaces, and consequently, lowering circulation at the gill level. This process might decrease relative fipronil distribution to the gill epithelium after i.p. administration compared to the w.b. route [25,26]. With the w.b. route, the oxygenated blood containing fipronil is distributed to other organs before potential detoxification in the liver, and occurrence of aneurisms intensifies this phenomenon. However, the processes cited above should be considered along with the elimination rate of fipronil from the fish body (half-life ~14 h with waterborne exposure) [21].

Most mortalities with the i.p. exposure were recorded on the first day whereas this happened on the last day for waterborne exposure (Tables 2 and 4). This can be explained by the fact that with the i.p. route the abrupt introduction of fipronil into the peritoneal cavity is done using one large dose while w.b, exposure results in a lower but constant absorption of fipronil from the water [27,20].

4.2. Histopathology

Histological alterations reported in this study, and generally in all histopathological studies, are explained in terms of two types of structural changes. Some alterations result from direct toxic effects, and others are defense strategies against toxicants to decrease their effects [28].

4.2.1. Gills

Some of the gill histological changes observed in this study such as lifting, fusion, hyperplasia and hypertrophy generate obstacles to prevent toxicants entering into the blood [29]. Aneurysms, as a circulatory disturbance, occur when pillar cells are damaged, leading to increased blood flow into the lamella [30]. Qureshi et al. [31] reported that common carp exposed to sub-acute concentrations of fipronil (400 µg/L) showed disruption of primary lamellae, atrophy of secondary lamellae, lamellar degeneration, and epithelial necrosis. Ghisi et al. [32] observed aneurisms, hyperplasia and lamellar fusion in the gills of *Rhamdia quelen* after 60 days (0.23 µg/L fipronil). Moreover, a previous study on pesticide exposure showed that aneurysms were a common cause of damage in gills [33]. The lifting and swelling observed in this study can be related to alterations in gill sodium − potassium ATPase [34]. Gupta et al. [35] reported that fipronil has inhibitory effects on the ATPase activity in the gill of *Cyprinus carpio* fry. This inhibitory effect of fipronil may disturb the osmoregulatory capacity of fish [36] and consequently, lead to fish deaths [37]. The necrosis of the gills observed in this study (700 µg/L) can take place as a result of severe oxidative stress and lipid peroxidation [38]. Previous studies showed that fipronil and pesticides generally are usually associated with oxidative stress [38,39]. Comparison of histopathological effects of fipronil between the two routes showed that this insecticide lead to more damage of the gills with the w.b. route in comparison with i.p. injection, which showed little damage. DTC calculation for gills showed significance differences ($P < 0.05$) between the two routes and dose (concentration) response relationships. The gills with the role of respiration, osmoregulation and acid-base regulation are an important organ in fish. The DTC values measured in the fish gills exposed via the w.b. route (700 µg/L) showed sever lesion ($50 \leq DTC \leq 100$), and this high rate of damage in the gills can be considered as another important factor contributing to the high toxicity of fipronil with this route.

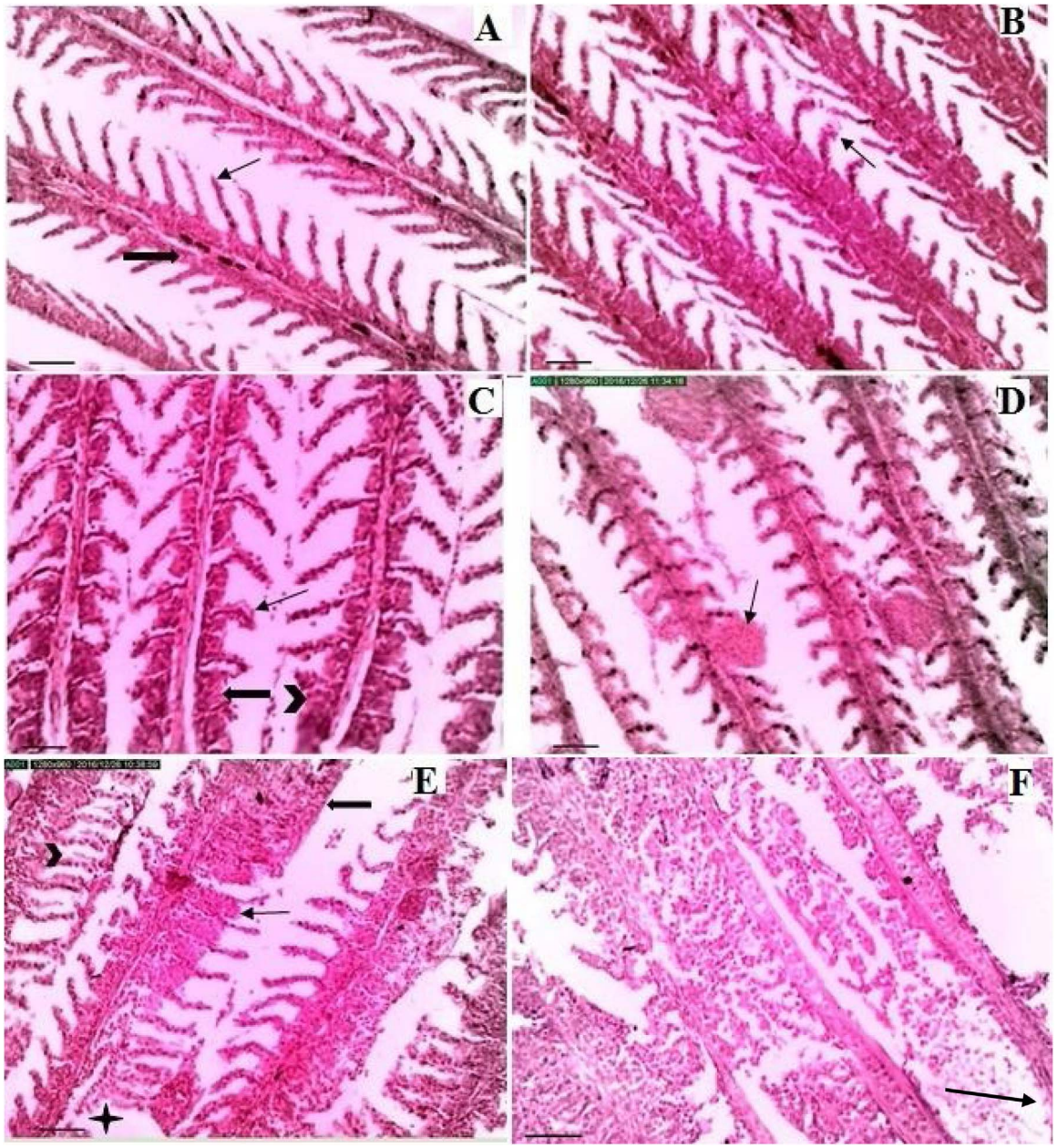

Fig. 3. Histological alterations in the Caspian white fish gills with intraperitoneal (B and C) and waterborne (D, E and F) exposure to fipronil (750 mg/kg and 700 μg/L). A: Normal histological structure, primary (thick arrow) and secondary (narrow arrow) lamellae. B and C: Hypertrophy in the secondary (narrow arrow) and primary (thick arrow) lamella, hyperplasia and fusion between secondary lamellas (arrowhead). D: Aneurysm (arrow). E: Extensive fusion and mucus secretion (thick arrow), hyperplasia (narrow arrow), lifting (arrowhead) and deletion (star), F: Necrosis (arrow). (H & E, x 725, scale bar: 0.05 mm).

4.2.2. Liver

Livers are the most important organ for detoxification and biotransformation of toxicants [40]. The DTC value measured for the livers of fish exposed to fipronil via the two routes showed that the normal function of this organ was maintained ($0 \leq DTC \leq 10$). Pyknosis was the most obvious damage in the liver and appeared with both routes of exposure. This damage is the initial step toward necrosis or apoptosis, and it seems that livers will show more damages after chronic exposure to fipronil, as reported by Mossa et al. [41] for the livers of male albino rats after sub-chronic exposure (45 d) to fipronil. Moreover, Ali et al. [42] examined fipronil exposure effect on Japanes quail in a 15-day gavage administration. The liver histopathological observations showed fatty degeneration, focal aggregations of lymphocytes and necrosis of few hepatic cells.

4.2.3. Kidney

Fish kidneys are an important organ for the excretion of toxicants, homeostasis and often is one of the first organs to be affected by toxicants [43]. In this study, degeneration of the tubule and hemorrhaging were the most frequent damages in the kidney with i.p. exposure. Necrosis and edema in fish kidney exposed to fipronil were also reported in a previous study [31]. Badgujar et al. [39] evaluated effect of different doses of fipronil (2.5, 5 and 10 mg/kg) on kidney of mice administered via oral exposure for 28 days. The kidney showed dilation of collecting tubules, congestion and severe degenerative changes along with necrosis of tubular lining cells. Concentrating fipronil and its metabolites due to reabsorption in the renal tubules can result in more histological alterations. The DTC values of the kidneys showed significant differences ($P < 0.05$) between the two routes of exposure, and the damages were greater with the i.p. route. Moreover, a dose response relationship was only observed with the i.p. route (Fig. 7).

Table 6
The frequency (F.) of histopathological alterations in Caspian white fish after 96 h exposure to 450, 550, 650 and 750 mg/kg, and 400, 500, 600 and 700 μg/L fipronil. Absent: (F = 0), rare: (F = 1), low frequency: (F = 2), frequent: (F = 3), very frequent: (F = 4).

Tissue	Histological alterations	i.p. route				w.b. route				Control	Stage
		F. (0–4)				F. (0–4)				F. (0–4)	
		450	550	650	750	400	500	600	700		
Gill	Epithelial lifting	2	1	2	2	4	4	4	4	1	I
	Hypertrophy	1	1	2	2	3	3	4	4	1	I
	Hyperplasia	1	1	1	2	3	3	3	4	0	I
	Deletion	0	0	0	0	3	3	3	3	0	I
	Aneurysm	0	0	0	0	3	3	4	3	0	II
	Necrosis	0	0	0	0	0	0	1	2	0	III
	Lamellar fusion	1	1	1	1	4	4	4	4	0	I
Liver	Pyknosis	4	4	4	4	3	3	4	3	0	II
	Structural alteration	2	1	3	3	1	1	1	2	1	I
	Congestion of blood vessels	3	3	3	3	2	2	2	2	1	I
	Sinusoid dilation	2	2	3	3	1	1	1	1	0	I
Kidney	Thrombosis	3	3	3	3	2	2	2	2	1	I
	Hemorrhage	2	3	3	3	0	0	0	0	0	II
	Congestion, hemolysis, and edema of blood vessels	2	1	2	2	1	2	1	1	0	I
	Degeneration of hematopoietic tissue	1	1	1	1	1	1	2	2	0	II
	Thyroidisation	2	1	2	2	1	1	1	1	0	I
	Degeneration of the tubule	4	4	4	3	1	1	1	1	0	II
	Necrosis of the tubule	0	0	2	2	0	0	0	0	0	III
Brain	Alteration in distributions of purkinge cells	1	1	1	4	1	1	1	4	0	I

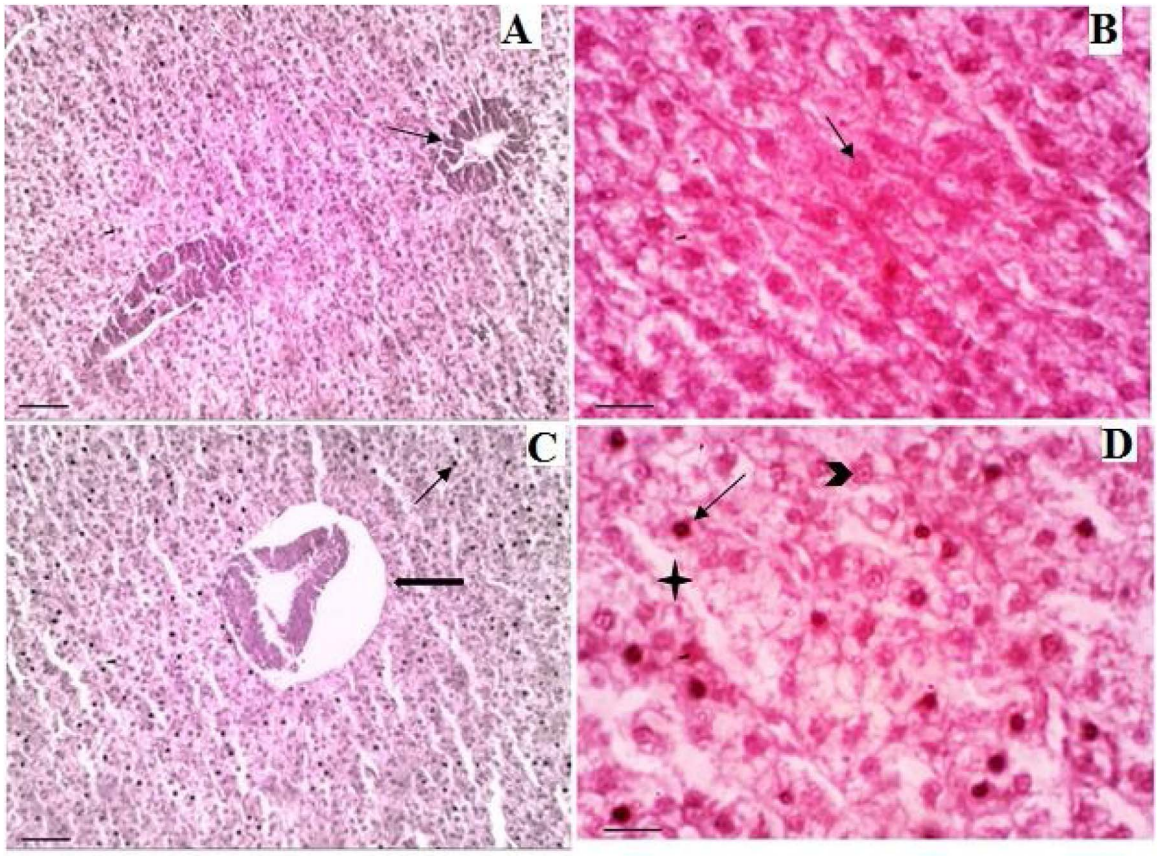

Fig. 4. Histological alterations in the Caspian white fish livers with intraperitoneal (C and D) exposure to fipronil (750 mg/kg). A (x 725): Normal histological structure, exocrine pancreatic duct (arrow). B (x 2900): Normal nucleus. C (x 725) and D (x 2900): Structural alterations in exocrine pancreatic duct (thick arrow), pyknosis (narrow arrow), normal nucleus (arrowhead) and sinusoid dilation (star). (H & E, scale bar: 0.05 mm (A and C) and 0.02 mm (B and D).

Slight toxicity of fipronil with i.p. and, consequently, injection of higher doses of this insecticide into the fish, in comparison with the w.b. route, can result in DTC value (66 ± 50) implying severe damage (50 ≤ DTC ≤ 100) (750 mg/kg).

4.2.4. Brain

Although fish brains are not often considered as a vital organ in most studies, compared to the three previous organs, it may be a target organ for fipronil. There had been no reports about the histological effects of fipronil on fish brains. Badgujar et al. [39] reported that

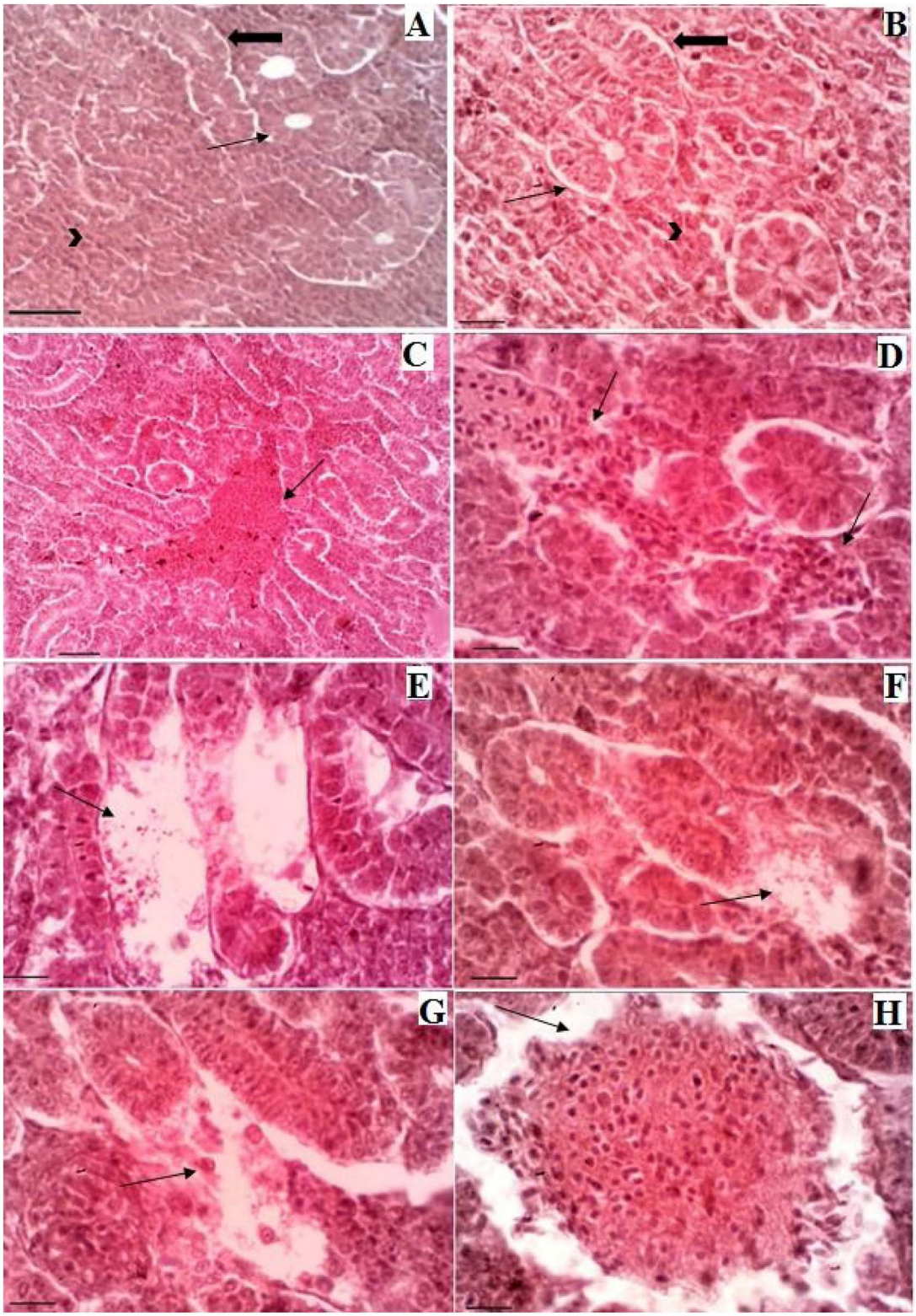

Fig. 5. Histological alterations in Caspian white fish kidneys with intraperitoneal exposure to fipronil (750 mg/kg). A (x 725) and B (x 2900): Normal histological structure, proximal tubule (narrow arrow), distal tubule (thick arrow), and hematopoietic tissue (arrowhead). C (x 725) and D (x 2900): Hemorrhaging (arrow). E, F and G (x 2900): Degeneration and necrosis of the tubule (arrow). H (x 2900): thrombosis (arrow). (H & E, scale bar: 0.05 mm (A and C) and 0.02 mm).

fipronil caused severe vacuolation in the molecular layer, necrosis of neurons in the granular layer, vacuolation in the gray matter and degeneration of purkinje cell layer with loss of nissl substance in the brain of mice after 28 d of fipronil exposure. Clasen et al. [44] showed increasing lipid peroxidation in the brain of common carp after 90 days exposure to a commercial formulation containing fipronil. In this study, H & E staining showed no histological alterations in the brain. However, nissl staining showed some alterations in distribution of purkinje cells in the ganglionic layer of the cerebellum. In contrast to mammalian brains, purkinje cells in the ganglionic layer of fish brains (the purkinje layer in mammalian brains) are arranged less regularly between the molecular and granular layers [45]. Microscopic observations showed

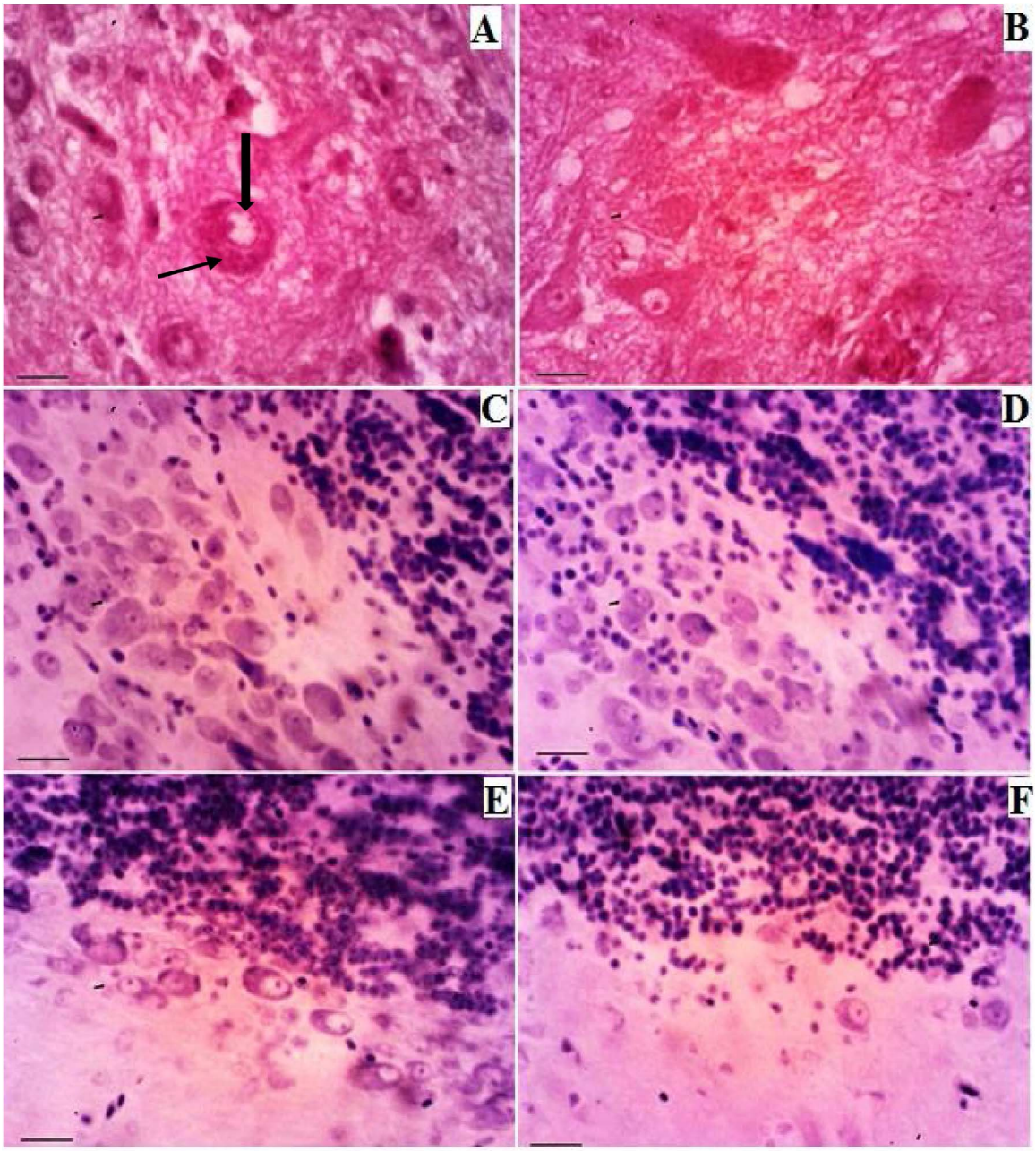

Fig. 6. Brain histopathology of Caspian white fish, exposed to fipronil via the waterborne route (700 μg/L). A (control) and B (exposed): Normal histological structure of the brain; nissl bodies (thin arrow) and perikaryon (thick arrow) are shown (H & E). C and D: Normal histological structure of the cerebellum; normal purkinge and granular cells are shown (nissl staining). E and F: Alteration in the distribution of purkinge cells resulting from waterborne exposure to fipronil (nissl staining). Scale bar: 0.02 mm, x 2900.

that purkinje cells (GABAergic neurons) in the cerebellum of control fish had a more concentrated distribution compared to that in the treatment groups with sparser distribution. Semi-circular swimming behavior and muscle shivering observed as a clinical sign in the exposed fish may be correlated with this alteration, which needs more study.

5. Conclusion

This study showed that the i.p. route of fipronil exposure was less toxic in Caspian white fish compared to w.b. exposure. DTC values measured for the important organs showed that the gills and kidneys had severe damage and were the most affected organs studied with fipronil exposure with w.b. and i.p. exposure, respectively.

Declaration of conflicts of interest

There are no potential or actual conflicts of interest.

Acknowledgments

The authors would like to thank Dr. Sara Rastgar at the Khorrmashahr University for helping and performing experimental work, and Professor Joe M. Regenstein at Cornell University for the critical reading, editing, and scientific review of the paper. This work was supported by Khorramshahr University of Marine Science and Technology, and done at the Babol University of Medical Sciences.

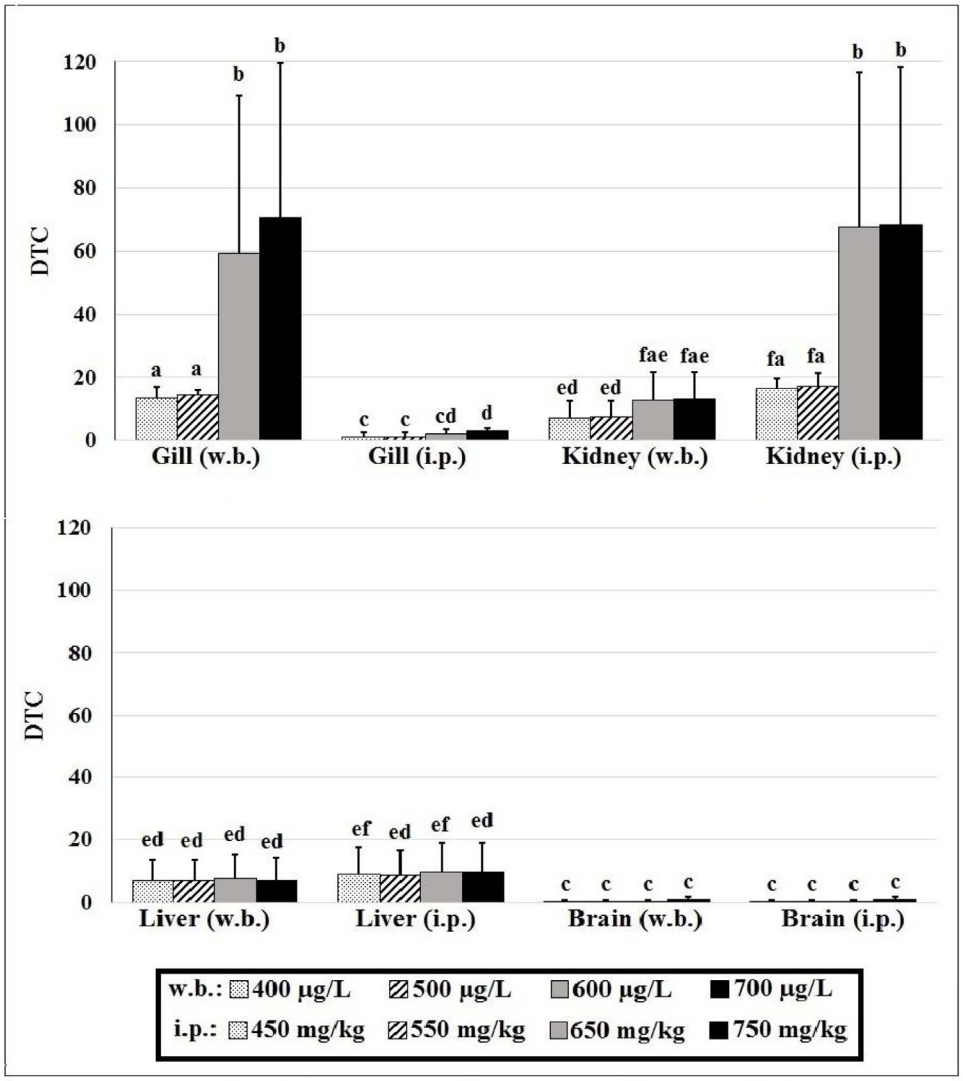

Fig. 7. Mean degree of tissue changes (DTC) for gills, livers, kidneys and brains of *Rutilus frisii kutum* exposed via intraperitoneal and waterborne routes to fipronil. This figure shows the DTC for the fish organs exposed to 450, 550, 650 and 750 mg fipronil/kg fish weight and 400, 500, 600 and 700 µg fipronil/L water. Graph shows the mean ± SD.

References

[1] L.M. Cole, R.A. Nicholson, J.E. Casida, Action of phenylpyrazole insecticides at the GABA-gated chloride channel, Pestic. Biochem. Physiol. 46 (1993) 47–54.
[2] C.C. Tingle, J.A. Rother, C.F. Dewhurst, S. Lauer, W.J. King, Fipronil: environmental fate, ecotoxicology, and human health concerns, Rev. Environ. Contam. Toxicol. 176 (2003) 1–66.
[3] P.B. Key, K.W. Chung, A.D. Opatkiewicz, E.F. Wirth, M.H. Fulton, Toxicity of the insecticides fipronil and endosulfan to selected life stages of the grass shrimp (*Palaemonetes pugio*), Bull. Environ. Contamin. Toxicol. 70 (2003) 533–540.
[4] S. Beggel, I. Werner, R.E. Connon, J.P. Geist, Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (*Pimephales promelas*), Sci. Total Environ. 408 (2010) 3169–3175.
[5] R. Peveling, S.A. Demba, Toxicity and pathogenicity of Metarhizium anisopliae var. acridum (Deuteromycotina, Hyphomycetes) and fipronil to the fringe-toed lizard Acanthodactylus dumerili (Squamata: lacertidae), Environ. Toxicol. Chem. 22 (2003) 1437–1447.
[6] M. Kitulagodage, W.A. Buttemer, L.B. Astheimer, Adverse effects of fipronil on avian reproduction and development: maternal transfer of fipronil to eggs in zebra finch *Taeniopygia guttata* and in ovo exposure in chickens *Gallus domesticus*, Ecotoxicol 20 (2011) 653–660.
[7] P.R. De Oliveira, G.H. Bechara, S.E. Denardi, R.J. Oliveira, M.I.C. Mathias, Genotoxic and mutagenic effects of fipronil on mice, Exp. Toxicol. Pathol. 64 (2012) 569–573.
[8] A. Planchart, C.J. Mattingly, D. Allen, P. Ceger, W. Casey, D. Hinton, J. Kanungo, S.W. Kullman, T. Tal, M. Bondesson, S.M. Burgess, Advancing toxicology research using in vivo high throughput toxicology with small fish models, ALTEX 33 (2016) 435–452.
[9] A. Aajoud, P. Ravanel, M. Tissut, Fipronil metabolism and dissipation in a simplified aquatic ecosystem, J. Agric. Food Chem. 51 (2003) 1347–1352.
[10] A. Bobe, P. Meallier, J.-F. Cooper, C.M. Coste, Kinetics and mechanisms of abiotic degradation of fipronil (hydrolysis and photolysis), J. Agric. Food Chem. 46 (1998) 2834–2839.
[11] A.S. Gunasekara, T. Truong, K.S. Goh, F. Spurlock, R.S. Tjeerdema, Environmental fate and toxicology of fipronil, J. Pestic. Sci. 32 (2007) 189–199.
[12] L.L. Johnson, C.M. Stehr, O.P. Olson, M.S. Myers, S.M. Pierce, C.A. Wigren, B.B. McCain, U. Varanasi, Chemical contaminants and hepatic lesions in winter flounder (*Pleuronectes americanus*) from the northeast coast of the United States, Environ. Sci. Technol. 27 (1993) 2759–2771.
[13] I. Altinok, E. Capkin, Histopathology of rainbow trout exposed to sublethal concentrations of methiocarb or endosulfan, Toxicol. Pathol. 35 (2007) 405–410.
[14] E. Capkin, S. Birincioglu, I. Altinok, Histopathological changes in rainbow trout (*Oncorhynchus mykiss*) after exposure to sublethal composite nitrogen fertilizers, Ecotoxicol. Environ. Saf. 72 (1999) 1999–2004.
[15] J.M. Parent, V.V. Valentin, D.H. Lowenstein, Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone?olfactory bulb pathway, J. Neurosci. 22 (2002) 3174–3188.
[16] V. Poleksic, V. Mitrovic-Tutundzic, Fish gills as a monitor of sublethal and chronic effects of pollution, in: R. Müller, R. Lloyd (Eds.), Sublethal and Chronic Effects of Pollutants on Freshwater Fish, Oxford, Fishing News Books, 1994(Pp. 339–352).
[17] P.V. Hodson, D.G. Dixon, K.L. Kaiser, Estimating the acute toxicity of waterborne chemicals in trout from measurements of median lethal dose and the octanol-water partition coefficient, Environ. Toxicol. Chem. 7 (1988) 443–454.
[18] Environmental Protection Agency (EPA), United States Environmental Protection Agency, Ecological Risk Assessment, USA, 2012.
[19] C.J. Driver, D.B. Drown, M.W. Ligotke, P. Van Voris, B.D. McVeety, B.J. Greenspan, Routes of uptake and their relative contribution to the toxicologic response of northern bobwhite (*Colinus virginianus*) to an organophosphate pesticide, Environ. Toxicol. Chem. 10 (1991) 21–33.
[20] K.A. Pickford, R.E. Thomas-Jones, B. Wheals, C.R. Tyler, J.P. Sumpter, Route of exposure affects the oestrogenic response of fish to 4-tert-nonylphenol, Aquat. Toxicol. 65 (2003) 267–279.
[21] B.J. Konwick, A.W. Garrison, M.C. Black, J.K. Avants, A.T. Fisk, Bioaccumulation, biotransformation, and metabolite formation of fipronil and chiral legacy pesticides in rainbow trout, Environ. Sci. Technol. 40 (2006) 2930–2936.

[22] S. Reynaud, I.A. Worms, S. Veyrenc, J. Portier, A. Maitre, C. Miaud, M. Raveton, Toxicokinetic of benzo [a] pyrene and fipronil in female green frogs (*Pelophylax kl. esculentus*), Environ. Poll. 161 (2012) 206–214.
[23] C.R. Carbis, G.T. Rawlin, G.F. Mitchell, J.W. Anderson, I. McCauley, The histopathology of carp, Cyprinus carpio L., exposed to microcystins by gavage, immersion and intraperitoneal administration, J. Fish Dis. 19 (1996) 199–207.
[24] J.M. McKim, Physiological and biochemical mechanisms that regulate the accumulation and toxicity of environmental chemicals in fish, Bioavailability: Physical, Chemical and Biological Interactions, Lewis Publishers, London, 1994.
[25] T.S. Gill, J.C. Pant, J. Pant, Gill, liver, and kidney lesions associated with experimental exposures to carbaryl and dimethoate in the fish (*Puntius conchonius Ham.*), Bull. Environ. Contam. Toxicol. 41 (1988) 71–78.
[26] E. Fanta, F.S.A. Rios, S. Romão, A.C.C. Vianna, S. Freiberger, Histopathology of the fish *Corydoras paleatus* contaminated with sublethal levels of organophosphorus in water and food, Ecotoxicol. Environ. Saf. 54 (2003) 119–130.
[27] S.L. Levine, J.T. Oris, T.E. Wissing, Comparison of P-4501A1 monooxygenase induction in gizzard shad (Dorosoma cepedianum) following intraperitoneal injection or continuous waterborne-exposure with benzo [a] pyrene: temporal and dose-dependent studies, Aquat. Toxicol. 30 (1994) 61–75.
[28] S. Bhagwant, K.B. Elahee, Pathologic gill lesions in two edible lagoon fish species, *Mulloidichtys flavolineatus* and *Mugil cephalus*, from the Bay of Poudre d, Or, Mauritis, Westem Indian Ocean, J. Mar. Sci. 1 (2002) 35–42.
[29] B. Nowak, Histological changes in gills induced by residues of endosulfan, Aquat. Toxicol. 23 (1992) 65–84.
[30] M. Rosety-Rodriguez, F.J. Ordoez, M. Rosety, J.M. Rosety, A. Ribelles, C. Carrasco, Morpho-histochemical changes in the gills of turbot, Scophthalmus maximus L., induced by sodium dodecyl sulfate, Ecotoxicol. Environ. Saf. 51 (2002) 223–228.
[31] I.Z. Qureshi, A. Bibi, S. Shahid, M. Ghazanfar, Exposure to sub-acute doses of fipronil and buprofezin in combination or alone induces biochemical, hematological, histopathological and genotoxic damage in common carp (*Cyprinus carpio* L.), Aquat. Toxicol. 179 (2016) 103–114.
[32] N.D.C. Ghisi, W.A. Ramsdorf, M.V.M. Ferraro, M.I.M. de Almeida, C.A.D.O. Ribeiro, M.M. Cestari, Evaluation of genotoxicity in *Rhamdia quelen* (Pisces, Siluriformes) after sub-chronic contamination with Fipronil, Environ. Monit. Assess. 180 (2011) 589–599.
[33] P. Neelima, L. Cyril, A.J. Kumar, C.S. Rao, N.G. Rao, Histopathological alterations in gill, liver and kidney of cyprinus carpio (Linn.) exposed to cypermethrin (25% EC), Int. J. Adv. Res. Biol. Sci. 2 (2015) 34–40.
[34] E. Neiboer, D.H.S. Richardson, The replacement of nondescript term heavy metals by a biologically and chemically significant classification of metals ions, Environ. Pollut. 1 (1980) 3–26.
[35] S.K. Gupta, A.K. Pal, N.P. Sahu, N. Saharan, S.C. Mandal, C. Prakash, M.S. Akhtar, A.K. Prusty, Dietary microbial levan ameliorates stress and augments immunity in *Cyprinus carpio fry* (Linnaeus, 1758) exposed to sublethal toxicity of fipronil, Aquac. Res. 45 (2014) 893–906.
[36] R.C. Dalela, M.C. Bhatnagar, A.K. Tyagi, S.R. Verma, Adenosine triphosphatase activity in a few tissues of a freshwater teleost, *Channa gaucha* following *in vivo* exposure to endosulfan, Toxicol 11 (1978) 361–368.
[37] A.G. Heath, Water Pollution and Fish Physiology, Second ed., CRC press, Boca Raton, FL, USA, 1995.
[38] B.D. Banerjee, V. Seth, R.S. Ahmed, Pesticide-induced oxidative stress: perspective and trends, Rev. Environ. Health 16 (2001) 1–40.
[39] P.C. Badgujar, N.N. Pawar, G.A. Chandratre, A.G. Telang, A.K. Sharma, Fipronil induced oxidative stress in kidney and brain of mice: protective effect of vitamin E and vitamin C, Pestic. Biochem. Physiol. 118 (2015) 10–18.
[40] R. Van der Oost, J. Beyer, N.P. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharmacol. 13 (2003) 57–149.
[41] A.T.H. Mossa, E.S. Swelam, S.M.M. Mohafrash, Sub-chronic exposure to fipronil induced oxidative stress, biochemical and histotopathological changes in the liver and kidney of male albino rats, Toxicol. Rep. 2 (2015) 775–784.
[42] S.A. Ali, A.A.R. Mohamed, H. Ali, K.M. Elbohi, Sublethal effect of fipronil exposure on liver and kidney tissues with evaluation of the recovery ability of Japanese quail (*Coturnix japonica*), Japan J. Veter. Res. 64 (2016) 131–138.
[43] S. Thophon, M. Kruatrachue, E.S. Upathan, P. Pokethitiyook, S. Sahaphong, S. Jarikhuan, Histopathological alterations of white seabass, *Lates calcarifer* in acute and subchronic cadmium exposure, Environ. Poll. 121 (2003) 307–320.
[44] B. Clasen, V.L. Loro, R. Cattaneo, B. Moraes, T. Lópes, L.A. de Avila, R. Zanella, G. Boschmann, B. Baldisserotto, Effects of the commercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: Implications for rice- fish cultivation, Ecotoxicol. Environ. Saf. 77 (2012) 45–51.
[45] F. Genten, E. Terwinghe, A. Danguy, Atlas of Fish Histology, Science Publishers, NH, USA, 2009.