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

Rashid Alijani Ardeshira, Hossein Zolgharninea, Abdolali Movahedinia, Negin Salamatb, Ebrahim Zabihib⁎

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

ARTICLE INFO

Chemical compounds studied in this article:
- Fipronil (PubMed CID: 15278226)
- Phenoxethanol (PubMed CID: 17848643)
- Haematoxylin (PubMed CID: 442514)
- Eosin (PubMed CID: 11048)
- Picric acid (PubMed CID: 6954)
- Acetic acid (PubMed CID: 712)
- Formaldehyde (PubMed CID: 716)
- Ethanol (PubMed CID: 702)
- M-xylene (PubMed CID: 7929)

Keywords:
- Fipronil
- Caspian white fish
- Acute toxicity
- Administration route

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 (LC50) and dose (LD50) 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, LD50 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 LD50 and LC50 estimate expressed toxicity, LD50 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 LD50, 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 LD50 of fipronil in fish is related to its low/moderate water solubility [9] which makes it...
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 LC50 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 LD50 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 LD50 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 air pump aeration and static system were used for determining the LC50. 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.

2.2.2. Preparation and injection of fipronil solution

Fipronil (98% purity, 50:50 racemic mixture) was purchased from the Moshkmam 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 phenoxethanol, 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.

2.2.3. Experimental design for LD50

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 LC50 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 LC50. 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 the degree of tissue alteration, stage I (without alteration), stage II (some to severe damage) and stage III (very severe and irreparable damage), respectively.

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 gills, livers, kidneys and brains were normal (Fig. 3A). In

Table 2

| Dose (mg/kg) | Day 1 | Day 2 | Day 3 | Day 4 | Decapitation on day 5 |
|-------------|------|------|------|------|-----------------------|
| 300         | 0    | 0    | 0    | 0    | 21                    |
| 450         | 0    | 0    | 0    | 0    | 16                    |
| 550         | 0    | 0    | 0    | 0    | 14                    |
| 650         | 0    | 0    | 0    | 0    | 10                    |
| 750         | 0    | 0    | 0    | 0    | 9                     |
| 850         | 0    | 0    | 0    | 0    | 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                     |
| LD$_{20}$   | 485         | 403.6                   |
| LD$_{50}$   | 632.4       | 585.4                   |
| LD$_{80}$   | 779.9       | 724.6                   |
| LD$_{90}$   | 857         | 788.6                   |
| LD$_{99}$   | 1040        | 934.1                   |

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.

nucleus and sinusoid dilation (Fig. 4C and D). There was no significant difference (P ≥ 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
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. LD50 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 LD50 for chemicals in fish is a simpler, faster and less expensive alternative than LC50 [17].

According to the US Environmental Protection Agency [18], LD50 measured in this study for fipronil (632 mg/kg) would have a slight toxicity (501–2000 mg/kg) while the measured LC50 (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 LC50 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/L) [2]. Comparison of the LC50 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 aneurysms 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. Gill

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. Ghi et al. [32] observed aneurysms, 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 ≤ DTC ≤ 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.
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 ≤ DTC ≤ 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 Japanese 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]. Badgajar 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).
Slight toxicity of fi pronil 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).

### 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)  |         |       |
| Gill                    | Epithelial lifting                          | 4          | 4          | 4       | 1     |
|                         | Hypertrophy                                 | 4          | 4          | 4       | 1     |
|                         | Hyperplasia                                 | 3          | 3          | 4       | 1     |
|                         | Deletion                                    | 3          | 3          | 3       | 0     |
|                         | Aneurysm                                    | 3          | 3          | 4       | 3     | II    |
|                         | Necrosis                                    | 0          | 0          | 1       | 2     | III   |
|                         | Lamellar fusion                             | 4          | 4          | 4       | 0     | I     |
| Liver                   | Pyknosis                                    | 3          | 3          | 4       | 3     | II    |
|                         | Structural alteration                        | 1          | 1          | 1       | 2     | I     |
|                         | Congestion of blood vessels                 | 2          | 2          | 2       | 2     | I     |
|                         | Sinusoid dilation                           | 2          | 2          | 3       | 3     | I     |
| Kidney                  | Thrombosis                                  | 3          | 3          | 3       | 3     | I     |
|                         | Hemorrhage                                  | 2          | 3          | 3       | 3     | II    |
|                         | Congestion, hemolysis, and edema of blood vessels | 2          | 1          | 2       | 2     | 1     | I     |
|                         | Degeneration of hematopoietic tissue        | 1          | 1          | 1       | 1     | II    |
|                         | Thrombosis                                  | 1          | 1          | 1       | 1     | I     |
|                         | Degeneration of the tubule                  | 4          | 4          | 4       | 3     | II    |
|                         | Necrosis of the tubule                      | 0          | 0          | 2       | 2     | III   |
| Brain                   | Alteration in distributions of purkinje cells | 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).](image)

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
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. 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).
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 Khorramshahr 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 μg fipronil/L water. Graph shows the mean ± SD.
