Hindawi
Aquaculture Research
Volume 2023, Article ID 3396066, 9 pages
https://doi.org/10.1155/2023/3396066

Research Article

The Effect of Malathion Concentration and Exposure Time on Histopathological Changes in the Liver and Gill of Rainbow Trout

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Received 15 March 2023; Revised 4 June 2023; Accepted 11 August 2023; Published 31 August 2023

Academic Editor: Mohamed Abdelsalam

Exposure of aquatic organisms to organophosphorus pollutants is a subject of keen interest to biologists and environmental scientists. Examining histopathological changes in the tissues of exposed animals can provide great insights to understand the health condition of the organisms. This study examined the effects of malathion concentration and exposure time on the liver and gill tissues of the rainbow trout (*Oncorhynchus mykiss*) in a laboratory condition and tried to provide a quantitative assessment for the analysis of these effects. The experiment was conducted in three treatments including 0.025, 0.05, and 0.075 mg/L of malathion for 1, 5, and 9 days with a nonexposed group as control, in three replicates. The liver and gill samples were fixed in buffered formalin. About 5 μ tissue sections were prepared using the conventional histological methods and stained using the hematoxylin–eosin method. Histopathological changes in the liver and gill tissues were quantified by grading and the resulting data were analyzed by rank-based estimation. The results showed that histopathological changes in the liver and gill tissues are more affected by the malathion concentration than by the duration of the exposure. However, longer exposure had an intensifying effect on the tissue damage caused by the malathion at higher concentrations. The presence of melanomacrophages as an indicator of malathion toxicity was determined. The fish exposed to 0.075 mg/L malathion for 9 days showed atrophy in the liver and gill tissues, indicating cell death and functional inactivation. Histopathological changes in the liver and gills confirmed the dose-dependent effect of malathion on the rainbow trout.

1. Introduction

In the absence of widely applicable biological methods of pest control, farmers have to inevitably use pesticides to protect their crops [1]. Many studies have reported the existence of these toxic substances in rivers, coastal, and estuarine waters, and even in the effluents of water treatment plants in the different parts of the world, including Iran [1, 2].

Excessive use of pesticides for pest control in farms, forests, and aquatic environments can cause extensive pollution in water, air, and soil [3–6]. While aquatic ecosystems are not target environment for the pesticides, the pollution caused by these substances tends to find its way into these ecosystems, causing genetic changes and biodiversity loss [7–10].

Malathion is an organophosphorus insecticide widely used in many countries including Iran. Malathion has low water solubility [9, 11] and much like other organophosphates inhibit the activity of a set of enzymes, including acetylcholinesterase [4, 12, 13]. Malathion has different effects on the different species of fish, which mainly depend on the fish's age, gender, body size, and environmental chemistry and climatic conditions [14, 15].

Various studies have shown that physiological changes in fish tend to manifest as histopathological changes in certain

Table 1: Analysis of the commercial feed for rainbow trout (Biomar VR (EFICO YS 887 F)).

Analyses	Composition (%)
Crude protein	39
Crude lipid	15
Crude fiber	2.5
Ash	9
Moisture	8
Digestible phosphorus	1.25

organs such as the liver and gills. Thus, these tissues can serve as a target to evaluate the effects of xenobiotics and other pollutants [16, 17]. However, for more scientifically assessment of these effects, such histopathological studies need to be based on the comparable quantitative data.

Previous studies on the effect of chemical pollutants such as pesticides on fish have shown that the liver and gills could be good indicators of water quality. However, histopathological studies in this field have mostly reported their findings as qualitative data, which makes them ill-suited for making the precise comparisons. Malathion is one of the first and most widely available organophosphorus pesticides, it is also used as an agricultural pesticide which has the least toxicity damage in the mammals. In this experimental–interventional study, the goal was to examine and grade the effects of malathion concentration and exposure time on the histopathological changes in rainbow trout's liver and gill to determine how important they (exposure time and concentration) are for the toxicity effect and also obtain a quantitative comparable measure of these effects for use in the future studies.

2. Materials and Methods

2.1. Experimental Fish and Design. Thirty-six rainbow trout weighing 70 ± 10 g were purchased from Karaj Aquaculture Center and transferred to the laboratory in aerated tanks. Before starting the experiment, the fish were kept in the laboratory for 1 week to adapt to new environment. During this period, the fish were fed with commercial feed (Biomar VR, EFICO YS 887F (Table 1)) at the rate of 2% of their body weight per day. In addition, the water quality parameters were checked during the trial. About 24 hr before the start of the experiments, the fish were transferred to 70 L tanks containing chlorine-free aerated water, and the feeding was stopped. After reviewing the literatures regarding the sublethal concentration of the malathion for rainbow trout, the 96 hr LC_{50} was determined to be 130 μg/L [18, 19]. Accordingly, the experiment was designed with four treatments with the different malathion concentrations (control, 0.025, 0.05, and 0.075 mg/L) in three replicates [5, 20]. The fish were placed in malathion-containing water for 1, 5, and 9 days. Given the degradability of malathion in water, it was decided not to prolong the experiment beyond Day 9. Daily, temperature, O_2, pH, and total hardness were checked and adjusted to 18 ± 2°C, 7–7.5 ppm, 7.5–8, and 185 mg/L ($CaCO_3$), respectively. pH and O_2 were measured with a portable pH meter (model TS) and a digital oxygen meter (model DO-5510), respectively.

Table 2: Grading of histopathological changes observed in liver tissues.

Grade of tissue damage	Histopathological changes
1	Normal liver
2	Hepatocyte necrosis; hepatocyte malformation; melanomacrophage centers
3	Hyperemia; sinusoid necrosis; degeneration; hepatocyte malformation
4	Bile duct necrosis; aneurysm; cholestasis; inflammatory cell infiltration
5	Hyperemia; hepatocyte necrosis; vacuolation; cell atrophy

Table 3: Grading of histopathological changes observed in gills tissues.

Grade of tissue damage	Histopathological changes
1	Normal gill
2	Distal cell hyperplasia (distal clubbing); secondary lamellae shortening; aneurysm
3	Aneurysm; basal cell hyperplasia; secondary lamellae epithelium protrusion; lamellae fusion; secondary lamellae curling
4	Secondary lamellae curling; secondary lamellae atrophy; aneurysm; distal clubbing
5	Secondary lamellae rupture; secondary lamellae atrophy; secondary lamellae shortening; aneurysm; extensive gill tissue damage

During the 9 days of the experiment, the physicochemical conditions were kept constant as much as possible so that the pollution would be the only variable affecting the fish [21].

2.2. Histopathological Assay. At Days 1, 5, and 9 after the start of the experiment, for histological studies, three fish from each treatment were randomly caught and anesthetized with clove powder solution (200 ppm) [5]. Before taking liver and gill sample, fish were killed by a sharp blow to the head and the middle part of the liver and second left gill arch samples were collected and fixed in the 10% buffered formalin [22, 23]. The typical tissue preparation processes including dewatering, clearing with xylol, and soaking in paraffin were performed in a tissue processor machine. After preparing the tissue sections, the samples were stained using the hematoxylin–eosin staining method the resulting sections were examined under an Olympus BX60 light microscope with 100x magnification, and, when needed, photomicrographs were taken with a digital camera (COOLPIX 950, Nikon, China) [24].

The results of the histopathological examination of liver and gill tissues were graded as shown in Tables 2 and 3.

2.3. Data Analysis. After rating the observed liver and gill tissue changes into five grades, the data were imported into R software and analyzed with the R-fit package (rank-based estimation for linear models) at the 0.05 significance level to determine the effect of malathion concentration and exposure time on tissue damage. The images showing the severity of histopathological changes with descriptive captions were also prepared for each treatment [25].

3. Results

There was no mortality in any of the groups during the experiment. Histopathological examinations of the fish exposed to malathion showed structural abnormalities in the liver tissue (Figure 1). Liver damage was found to be significantly affected by the concentration and concentration–exposure time, but not by the exposure time alone. Histopathological changes observed in the experimental groups were much different from those in the control group and were more intense in the fish exposed to the higher malathion concentrations.

The results of R-fit for liver tissue showed the statistical significance of the effect of concentration and the interactive effect of the concentration and exposure time on the liver tissue (Table 4).

As the grading diagram for the liver tissue shows, the highest damage was observed at concentrations of 0.05 and 0.075, which were not significantly different in this respect. In terms of exposure time, the greatest damage was observed after 9 days exposure (Figure 2).

As mentioned, none of the fish in any of the groups died during the experiment. Histopathological examinations showed structural abnormalities in the gill tissue of the exposed fish (Figure 3). As with liver tissue, changes in gill tissues were found to be significantly affected by the concentration and concentration–exposure time, but not by the exposure time alone. Likewise, histopathological changes of gill tissues in the experimental groups were much different from those in the control group and were more intense in the fish exposed to the higher malathion concentrations.

The results of R-fit for gill tissue also showed the statistical significance of the effect of concentration and the interactive effect of concentration and exposure time on the gill tissue (Table 5).

As with the liver, the ranking diagram for the gill tissue shows the highest damage at concentrations of 0.05 and 0.075, which again are not significantly different in this respect. In terms of exposure time, the greatest tissue damage was again observed after 9 days of exposure (Figure 4).

4. Discussion

Based on our results, the tested doses, exposure time, and the poison concentrations had a greater effect on the histopathological lesions of the fish liver and gills.

The greatest tissue damage in both liver and gills was observed at Day 9 of the exposure and at the highest malathion concentration. The most notable pathological changes observed in the liver tissue were hepatocyte necrosis and malformation, melanomacrophage centers, sinusoid hyperemia, degeneration, cholestasis, vacuolation, inflammatory cell infiltration, and cell atrophy.

The liver is the primary organ for metabolism, detoxification of xenobiotics, and elimination of harmful substances [26, 27]. Exposure to high concentrations of toxic substances for a prolonged period can disrupt the liver's detoxification mechanisms, eventually causing more severe damage to the liver tissue [28, 29]. Therefore, examining the histopathological changes of the liver could be a highly accurate method for determining the impacts of different toxic compounds on fish in the field and laboratory studies [10, 30].

The fish exposed to the lowest dose (0.025 mg/L) showed melanomacrophage centers in the liver tissue, which can be used as an indicator of toxicity and a biological marker of exposure to the pesticide [31]. Melanomacrophages in fish are aggregates of pigmented phagocyte cells that are responsible for cleaning catabolites, natural cell wastes, and foreign substances [32]. A change observed in the liver tissue of all treatment groups was hepatocyte malformation. According to Braunbeck and Völkl [33], a change in the shape and size of nuclei is often a sign of increased metabolic activity, which may have a pathological origin and be caused by a pollutant's effect on the organism; a statement that is consistent with our results. Since hepatocyte degeneration and malformation took place from the first day of the experiment and even under the lowest malathion concentration, it can be inferred that these changes are the most common effects of malathion exposure on the liver of the fish. It can also be concluded that even the presence of low amounts of this toxic substance in the environment may be created some changes in the liver. In a study on the effect of malathion on the liver of catfish (*Heteropneustes fossilis*), the fish developed symptoms such as hepatocyte swelling, hepatocyte disintegration, hepatocyte nucleus necrosis, and pyknosis [34]. In a study that investigated the effect of two sublethal concentrations of diazinon on the liver tissue of rainbow trout, the most visible effects were the hypertrophy of liver cells, vacuolation of the cell cytoplasm, and cloudy swelling [35]. We also observed all of these effects, except cloudy swelling in the rainbow trout exposed to malathion.

Reduced bile inside the liver cells is a indicator of a change in metabolism [36, 37]. If continued, cholestasis can disrupt major physiological mechanisms of the liver, leading to liver damage. In this study, cholestasis was observed at Day 5 of exposure in the fish exposed to 0.075 mg/L of malathion.

Another change observed in the present study was necrosis, which has also been reported in many similar histopathological studies of liver tissue [38, 39]. In our study, necrosis appeared on the Ist day of the experiment and peaked on the 9th day. The necrosis and destruction of liver cells in the tested fish are indicators of the damaging effect of malathion on the cell wall, which triggers necrosis in the cells. This necrosis can be attributed to various reasons, including the inability of the fish to regenerate new liver cells or its effort to clean the toxic substance from the body through the detoxification process [40]. In a study conducted by Banaee et al. [41] on rainbow trout exposed to diazinon, hepatocyte necrosis, and vacuolation took place under the lowest dose

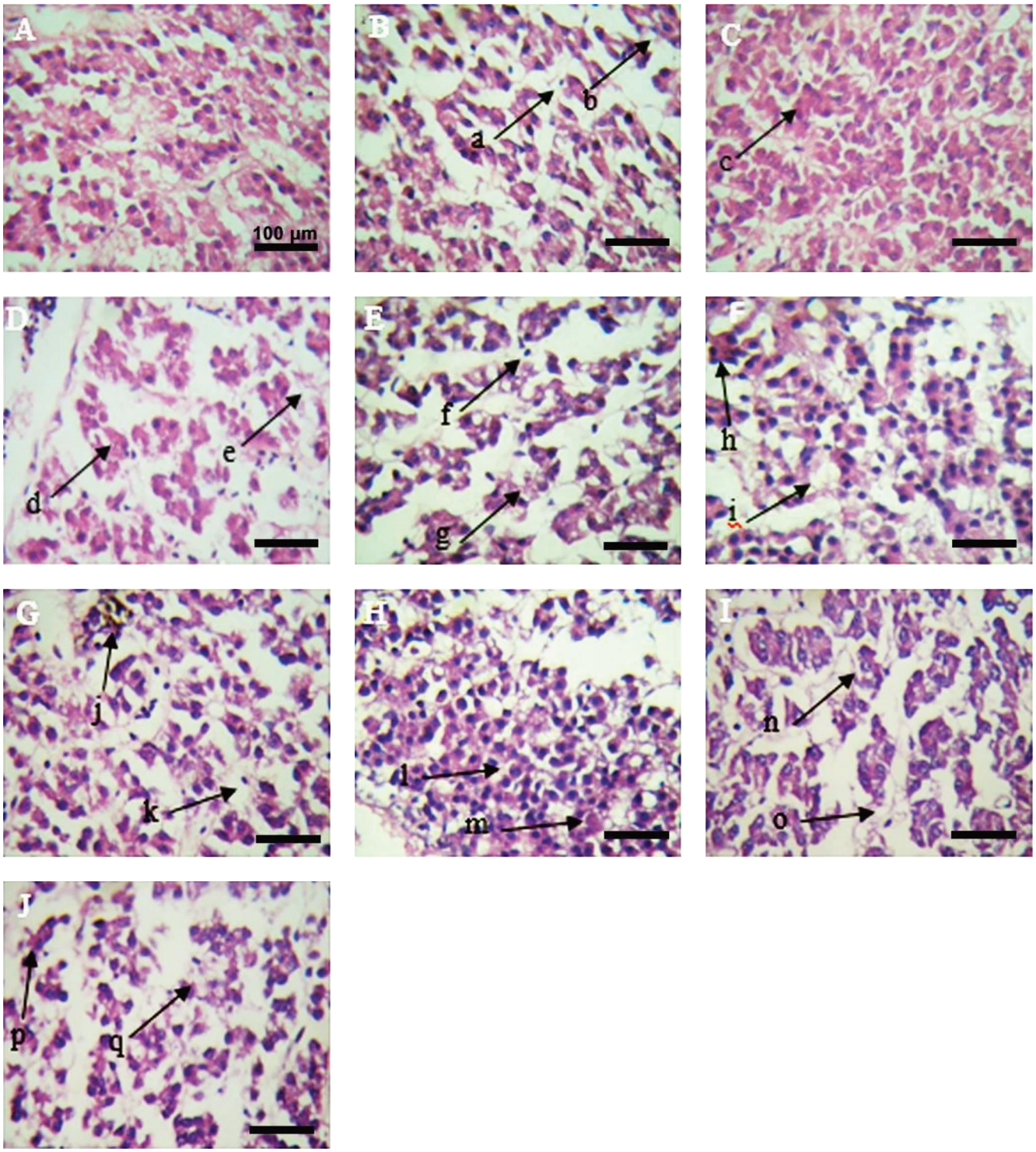

Figure 1: Histopathological changes in the liver tissue of rainbow trout exposed to malathion (hematoxylin–eosin staining, 400x magnification). In the control group, there was no abnormal change in the histological structure of the liver (A). At the end of Day 1, the second group (0.025 mg/L), (B) showed hepatocyte malformation (a) and melanomacrophage centers (b), the third group (0.05 mg/L) (C) showed sinusoid necrosis (c), and the fourth group (0.075 mg/L) (D) showed hyperemia (d) and degeneration (e). At the end of Day 5, the second group (0.025 mg/L) (E) showed hepatocyte malformation (f) and necrosis (g), the third group (0.05 mg/L) (F) showed hyperemia (h) and sinusoid necrosis (i), and the fourth group (0.075 mg/L) (G) showed cholestasis (j) and hepatocyte malformation (k). At the end of Day 9, the second group (0.025 mg/L) (H) showed inflammatory cell infiltration (l) and hyperemia (m), the third group (0.05 mg/L) (I) showed vacuolation (n) and necrosis (o), and the fourth group (0.075 mg/L) (J) showed hyperemia (p) and cell atrophy (q).

Table 4: Results of rank-based estimation for linear models (R-fit) for the changes observed in the liver tissue of different groups.

p Value	t Value	Standard error	Estimate	Intercept
0.00791	2.8333	8.235377	23.333336	Concentration
0.32877	0.9917	0.064495	0.063962	Time
0.02151	2.4173	1.378962	3.333332	Concentration × time

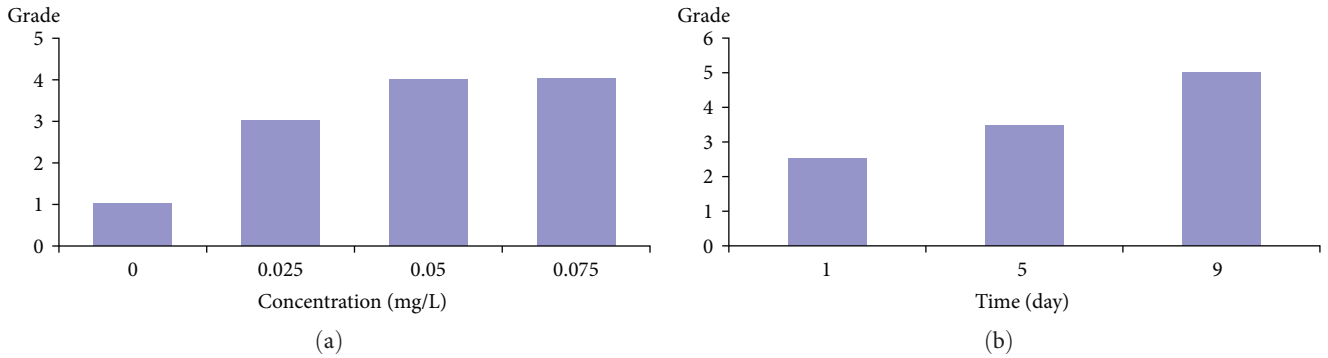

Figure 2: Median grade of liver damage in rainbow trout exposed to malathion: (a) for different concentrations and (b) for different exposure times.

and increased with the increasing concentration, which is fully consistent with our observations. The results of a study where *Esomus danricus* was exposed to malathion also showed hepatocyte necrosis, vacuolation, and swelling in the fish [35]. According to Sanad et al. [42], liver cell necrosis can be caused by the inhibition of DNA synthesis required for the liver growth and maturation.

In this study, we observed hepatocyte vacuolation in the fish exposed to 0.050 mg/L of malathion on the 9th day of the experiment. In the study of Sastry and Sharma [43], cytoplasm necrosis and vacuolation were observed in the *Channa punctatus* exposed to sublethal concentrations of diazinon, which was consistent with our results. Vacuolation of hepatocytes can signify a mismatch between the rate of synthesis of substances and the rate of their release in the hepatocyte [37]. Rahman et al. [44] reported observing cytoplasm necrosis and vacuolation in the fish exposed to the lowest concentration of diazinon and that the effects intensified with increasing diazinon concentration, which is not consistent with our observation.

In our study, atrophy in the liver tissue was observed on the 9th day of the experiment under the highest malathion concentration. Atrophy is an abnormal irreversible state in which the number and volume of cells decrease because of extensive cell death [45]. Similar to our study, the study of Fanta et al. [36] on *Corydoras paleatus* exposed to organophosphorus pesticide also reported observing atrophy in the liver tissue under the highest concentration. Banaee et al. [46] also observed hepatocyte cell atrophy in their rainbow trout, which is completely consistent with our results.

As an organ with a large surface area that is in constant contact with the external environment, gills tend to be the first place affected by pollutants [47]. In this study, the prominent tissue changes observed in the gill tissue of rainbow trout exposed to malathion were distal cell hyperplasia, aneurysm, necrosis, primary lamellae artery rupture, secondary lamellae epithelium protrusion, secondary lamellae curling, secondary lamellae shortening, secondary lamellae–epithelial separation, and secondary lamellae fusion, and atrophy. In a study on the histopathological effects of sublethal concentrations of malathion (0.01 and 0.02 mg/L) on the gills tissue of Gambozia after 10, 20, and 30 days of exposure, the observed changes included necrosis and peeling of the secondary lamellae epithelium, epithelium protrusion, intraepithelial edema, secondary lamellae adhesion, primary lamellae hemorrhage, secondary lamellae collapse, and rupture, and hypertrophy in the epithelial cells, and the intensity of these changes was dependent on the exposure dosage and time [47].

Epithelial hyperplasia, referring to an abnormal increase in the number of gill epithelium cells, can directly affect breathing, and in severe cases even prevent gas exchange [38]. In this study, this hyperplasia appeared in all treatment groups starting from the first day. Epithelium hyperplasia and distal clubbing of the secondary lamellae are a defensive reaction against toxic and harmful pollutants, as they decrease the gill surface [47]. In our study, distal clubbing of the secondary lamellae was observed from the very Ist day of the experiment even in the fish exposed to the lowest concentration of malathion. Similar changes have also been reported in other studies conducted on the gill tissue of fish exposed to the different pollutants [47].

Secondary lamellae epithelium protrusion disrupts gas exchange and oxygen absorption by increasing the distance between water and blood cells, although fish can increase their breathing rate to compensate the reduced oxygen absorption [48]. In our study, this change appeared in the fish exposed to 0.075 mg/L of malathion starting from the Ist day of the experiment and was most intense at the highest concentration.

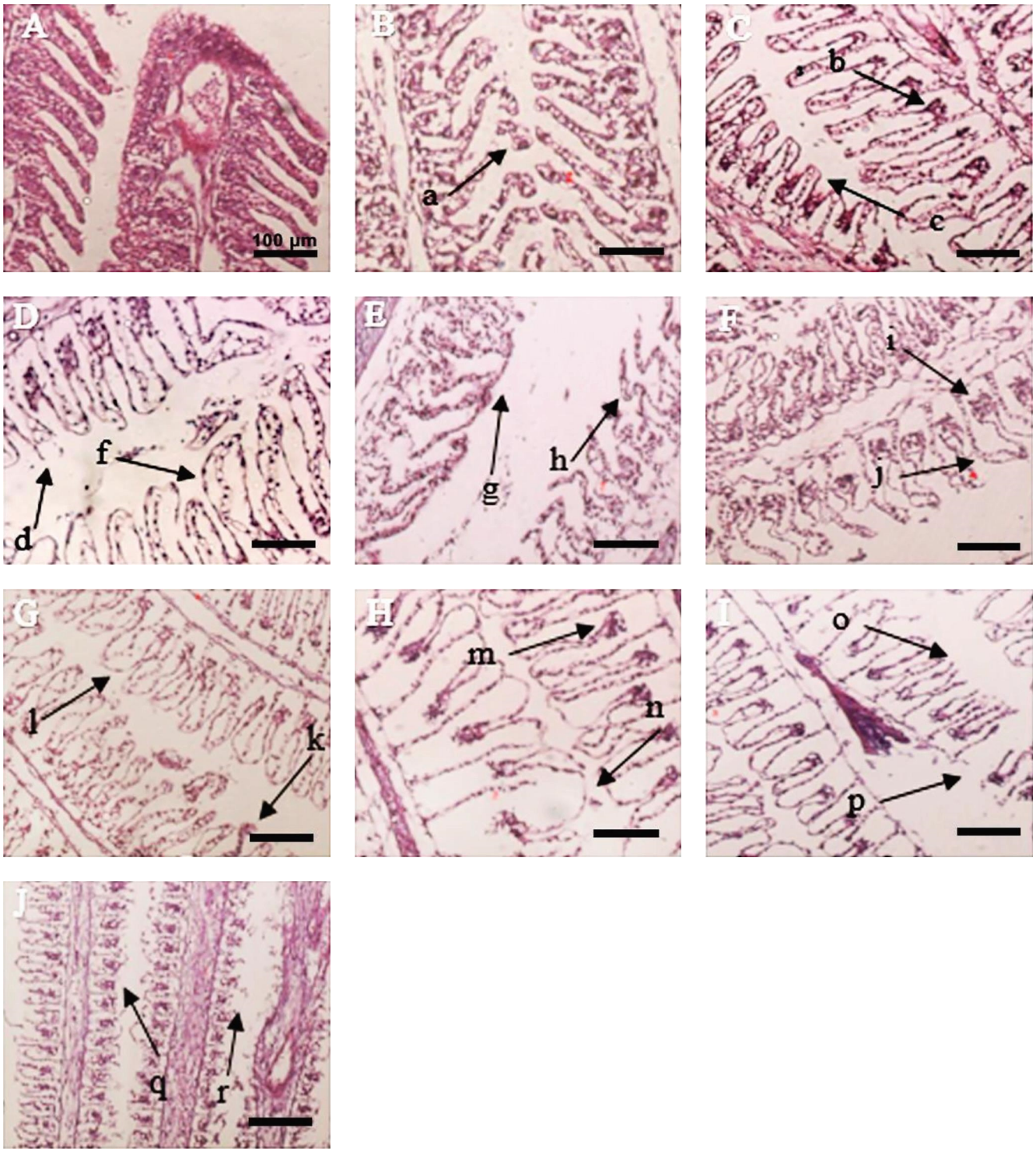

Figure 3: Histopathological changes in the gill tissue of rainbow trout exposed to malathion (hematoxylin–eosin staining, 400x magnification). In the control group, there was no abnormal change in the gill, including primary and secondary lamellae and chloride cells (A). At the end of Day 1, the second group (0.025 mg/L) (B) showed distal clubbing (a), the third group (0.05 mg/L) (C) showed aneurysm (b), and secondary lamellae shortening (c), and the fourth group (0.075 mg/L) (D) showed secondary lamellae rupture (d) and secondary lamellae epithelium protrusion (f). At the end of Day 5, the second group (0.025 mg/L) (E) showed lamellae fusion (g) and secondary lamellae curling (h), the third group (0.05 mg/L) (F) showed aneurysm (i) and secondary lamellae curling (j), and the fourth group (0.075 mg/L) (G) showed secondary lamellae epithelium protrusion (l) and distal clubbing (k). At the end of Day 9, the second group (0.025 mg/L) (H) showed aneurysm (m) and secondary lamellae epithelium protrusion (n), the third group (0.05 mg/L) (I) showed secondary lamellae rupture (o) and primary lamellae artery rupture (p), and the fourth group (0.075 mg/L) (J) showed hyperemia and secondary lamellae atrophy (q) and secondary lamellae shortening and secondary lamellae–epithelial separation (r).

Table 5: Results of rank-based estimation for linear models (R-fit) for the changes observed in the gill tissue of different groups.

p Value	t Value	Standard error	Estimate	Intercept
8.998e-09	7.6954	3.0321e + 00	23.3333e + 01	Concentration
1	0.0000	2.3746e02	4.8502e-07	Time
2.134e-07	6.5654	5.0771e-01	3.33333e + 00	Concentration × time

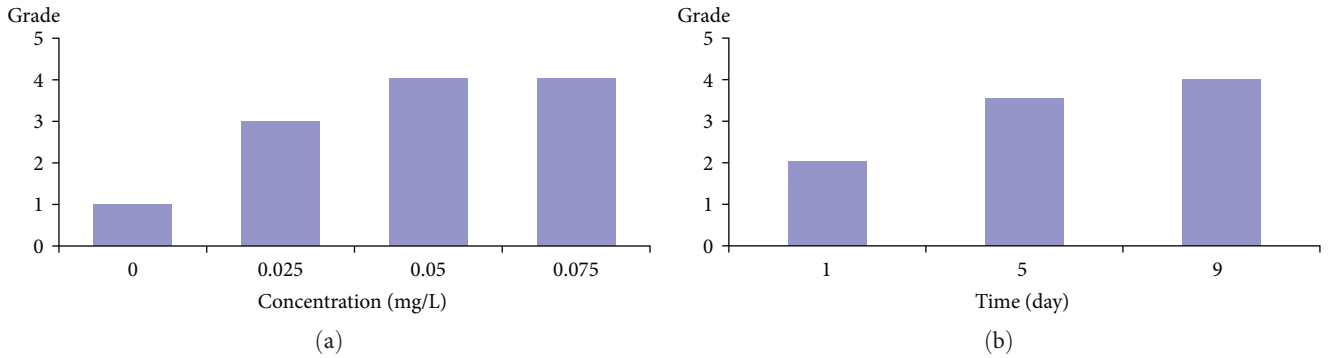

Figure 4: Median grade of gill damage in rainbow trout exposed to malathion: (a) for different concentrations and (b) for different exposure times.

Histopathological examination of the gills of the fish exposed to the highest concentration of malathion (0.075 mg/L) on the 9th day of the experiment showed atrophy or in other words the destruction of the gills tissue.

5. Conclusion

In this study, the intensity of structural changes and damage in the liver and gills tissues of rainbow trout exposed to malathion was found to increase with the increase in malathion concentration and exposure time. The results showed that malathion harms hematopoietic and melanomacrophage centers that are present in different organs of fish, especially the liver. Histopathological examinations of the effect of this insecticide also showed that it can cause tissue damage in the liver and gills even at the lowest concentration. Overall, the results confirmed the dose-dependent adverse effects of this toxic compound on the liver and gills. Therefore, histopathological changes in the liver and gills of fish can serve as a good biomarker for measuring the pollution of fish breeding ponds or natural environments such as rivers.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were performed following the protocol approved by the committee of ethics of the Baharavaran Nastaran Agricultural Applied Scientific Training Center, Applied Scientific University, Qom, Iran (1074; 2022).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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