Article

Not peer-reviewed version

Comparative Study of Pesticide-Induced Liver Damage in Common Carp (*Cyprinus carpio* Linnaeus, 1758): An Integrated Biomarker Approach

Vesela Yancheva , Stela Stoyanova , Elenka Georgieva , Eleonora Kovacheva , Bartosz Bojarski , Laszlo Antal , Ifeanyi Emmanuel Uzochukwu , Krisztian Nyeste *

Posted Date: 4 December 2025

doi: 10.20944/preprints202512.0512.v1

Keywords: common carp; liver; biomarkers; pesticides; pollution

 doi:10.20944/preprints202512.0512.v1

Article

Comparative Study of Pesticide-Induced Liver Damage in Common Carp (*Cyprinus carpio* Linnaeus, 1758): An Integrated Biomarker Approach

Vesela Yancheva [1], Stela Stoyanova [2], Elenka Georgieva [2], Eleonora Kovacheva [3,4,*], Bartosz Bojarski [5], László Antal [6,7], Ifeanyi Emmanuel Uzochukwu [6,8] and Krisztián Nyeste [6,7,*]

[1] Department of Ecology and Environmental Conservation, Paisii Hilendarski University of Plovdiv, 4000 Plovdiv, Bulgaria
[2] Department of Developmental Biology, Paisii Hilendarski University of Plovdiv, 4000 Plovdiv, Bulgaria
[3] Department of Medical Biology, Medical University-Plovdiv, 4000 Plovdiv, Bulgaria
[4] Research Institute, Medical University-Plovdiv, 4000 Plovdiv, Bulgaria
[5] Department of Animal Biology and Environment, Faculty of Animal Breeding and Biology, Bydgoszcz University of Science and Technology, Mazowiecka 28, 85-084 Bydgoszcz, Poland
[6] Department of Hydrobiology, University of Debrecen, 4032 Debrecen, Hungary
[7] National Laboratory for Water Science and Water Safety, University of Debrecen, 4032 Debrecen, Hungary
[8] Pál Juhász-Nagy Doctoral School of Biology and Environmental Sciences, University of Debrecen, 4032 Debrecen, Hungary
* Correspondence: nyeste.krisztian@science.unideb.hu

Abstract

The intensive use of pesticides in agriculture poses serious risks to aquatic ecosystems and non-target organisms, yet toxicological data remain limited. This study evaluated the acute effects of three widely used pesticides—pirimiphos-methyl (10 and 60 μg/L), propamocarb hydrochloride (40 and 80 μg/L), and 2,4-D (50 and 100 μg/L)—on the liver of common carp (*Cyprinus carpio* Linnaeus, 1758), a sentinel species in aquaculture, but also a species equally important in risk assessment and environmental monitoring. Fish were exposed for 96 hours under controlled conditions, and histopathological, histochemical, and biochemical biomarkers were analyzed. All tested pesticides induced significant histopathological alterations, predominantly circulatory and degenerative changes, with severity increasing at higher concentrations. Propamocarb hydrochloride and 2,4-D caused more pronounced and partly irreversible hepatotoxicity compared to pirimiphos-methyl. Histochemical assessment revealed altered glycogen metabolism, while biochemical assays showed inhibition of key liver enzymes, such as ALAT and ASAT, but also ChE and LDH, indicating disrupted metabolic processes. These findings highlight the vulnerability of aquatic organisms to pesticide exposure and support the use of fish liver biomarkers as effective tools in ecotoxicology research. The study also emphasizes the need for stricter regulation and environmental monitoring of pesticide contamination in aquatic systems.

Keywords: common carp; liver; biomarkers; pesticides; pollution

1. Introduction

The global pesticide use, associated with an increasing risk of exposure to all organisms and the environment, has increased manifold in recent decades. Modern agriculture is heavily dependent on the use of pesticides, primarily to protect crops and increase yields [1, 2]. The benefit of their application is undeniable, since crop productivity would decrease drastically without their use. On the other hand, there is clear evidence that the widespread use of pesticide substances causes irreversible damage to ecosystems and their inhabitants, including humans. Their excessive and

 doi:10.20944/preprints202512.0512.v1

incorrect application is one of the reasons for the presence of pesticide residues in various food products, which eventually threatens human health. This is especially exacerbated by frequent or prolonged consumption of contaminated foods. In this regard, in recent decades, a worldwide increase in the incidence of several types of allergies, an increase in the number of various genetic defects, and last but not least, a sharp increase in cancer diseases has been observed. This worrying trend is largely due to the highly polluted environment, primarily as a result of human activity. One of the major pollutants is pesticides [3]. Other factors that pose a potential risk to humans are treatment with unauthorized preparations, the use of unregulated imported products, and the presence of numerous warehouses with outdated pesticides, which are released into the environment under the influence of atmospheric conditions [2]. In addition, the spread of pesticides outside agricultural lands can also affect crops grown near the application sites where they are not permitted or desired [4] and affect protected natural areas [5, 6], a high number of plants, as well as aquatic organisms [7]. Moreover, the irrational use of pesticides causes biodiversity loss and damage to ecosystem functions, including their significant impact on aquatic ecosystems [8, 9]. Pesticides also cause negative impacts due to their accumulation in the environment, including the aquatic one, causing long-term negative effects on ecosystems and people. Once in the biosphere, they are included in the cycle of substances in nature, with the main circulation routes being water, air, and soil. The intensity of these processes is determined by the conditions of the external environment and the chemical nature of the pesticides, with their solubility and volatility being of the most significant importance [10]. Water pollution can be defined as the alteration of its characteristics due to the addition of anthropogenic pollutants to an extent that it cannot serve human drinking purposes or support the survival of biotic communities. This occurs when pollutants are discharged directly or indirectly without adequate treatment to remove harmful components [11].

Fish occupy high levels in the trophic chain; therefore, they represent an integrated picture of the entire ecosystem and can be used as bioindicators for the state of the environment [12].

In ecotoxicological studies, biomarkers are useful tools used in the monitoring of aquatic ecosystems. The inclusion of complex biomarkers at different levels of biological organization is a suitable approach to detect biological responses induced by pollutants. Therefore, it is necessary to apply them in monitoring studies of polluted ecosystems, providing an ecotoxicological assessment necessary for proper environmental management [13].

The liver is one of the organs frequently used for investigating complex biomarkers in aquatic organisms, including fish. According to Aniladevi et al. [14], assessing the changes in this organ may indicate disturbances in the detoxification mechanism of waste products, drugs, heavy metals, and organic pollutants, such as pesticides. Exposure to pesticides can cause histopathological, histochemical, and biochemical changes in the liver, which are important biomarkers for the determination of the pollution degree and effects in aquatic ecosystems [15]. Furthermore, conducting experimental studies related to the identification of morphological and biochemical changes at the tissue or cellular level can show the state of the studied organ as a result of the action of a certain environmental pollutant, and this can also affect the health of the whole organism [16-19].

According to Slaninova et al. [20], monitoring a single biomarker is not sufficient to determine the impact of pesticides on the fish organism. According to the authors, it is necessary to prepare a comprehensive multi-biomarker assessment, including tissue changes and changes in the enzymatic activity. Therefore, with the present study, we aimed to investigate the pesticide-induced liver damage after exposure to three different pesticides following histopathological, histochemical, and biochemical assessment.

2. Materials and Methods

The animal study protocol was approved by the Research Ethics Committee at Paisii Hilendarski University of Plovdiv, Faculty of Biology (protocol code No. 13/14.05.2025; date of approval: May 14, 2025).

2.1. Experimental Fish

 doi:10.20944/preprints202512.0512.v1

The test species of this study was common carp (*C. carpio*), which is one of the most preferred species for industrial breeding, compared to other freshwater fish. Common carp, a prominent freshwater species domesticated for more than 8000 years, is one of the most widely farmed fish globally due to its adaptability, rapid growth, and high protein content, making it integral to both fisheries and aquaculture [21, 22]. Furthermore, common carp is a species that not only has a high commercial value worldwide, but is widely used as a bioindicator of toxicity in the freshwater environment [23].

2.2. Experimental Pesticides

Pirimiphos-methyl

Pirimiphos-methyl is an organophosphorus insecticide that is commonly used for the prevention and control of insects during the storage of agricultural produce [24]. Residues in crops resulting from its excessive application pose a health risk to humans and animals [25]. Pirimiphos-methyl is also widely used against the malaria mosquito *Anopheles* sp. , especially in Africa [26, 27]. The mechanisms of resistance to pirimiphos-methyl are poorly understood, but organophosphates, such as carbamates, block the action of the AChE enzyme by competitive binding to its active site [28]. Pirimiphos-methyl is also responsible for the phosphorylation of AChE, which regulates the hydrolysis of acetylcholine in the synaptic cleft of the insect nervous system [29]. Like AChE, the circulating enzyme BchE, known as pseudocholinesterase, is also a target for phosphorylation and inhibition by the insecticide [30].

Propamocarb hydrochloride

Propamocarb hydrochloride was first introduced into the European markets for the control of oomycete pathogens in ornamental crops and some vegetables in 1978 [31]. Propamocarb hydrochloride is relatively unstable, stable to photodegradation in water, photodegradable in soil with a half-life of 35 days, degraded fairly rapidly by microbially-mediated metabolism, resistant to anaerobic metabolism, rapidly dissipates under field conditions, and has limited hydrolytic potential and bioconcentration in fish [32]. Propamocarb hydrochloride has a systemic activity following absorption through leaves, stems, and roots and through transport through the vascular system in treated plants [33]. The fungicide has good protective and curative activity against downy mildew without phytotoxic effects in fruits and vegetables, such as cucumbers, tomatoes, and potatoes [34-36]. It causes mild cytotoxicity to cortical neurons and has moderate effects on intracellular membrane potential, glucose consumption, ATP levels, and cytoskeleton [37].

2,4-D

2,4-D is a synthetic auxin herbicide commonly used to control dicotyledonous weeds in cereal crops. The herbicide was developed during World War II and introduced to the market in the 1940s [38] for the control of broadleaf weeds [39]. Due to its efficacy, selectivity, low cost, and broad spectrum of pest control, it has become a widely used herbicide in agricultural and urban areas around the world [40]. In 2025, 2,4-D was the most common herbicide ingredient used residentially in the U.S. [41]. Moreover, 2,4-D is registered as an ingredient in approximately 1500 agricultural and domestic pesticides, either as the sole active ingredient or in combination with others. The compound mimics natural plant auxins and promotes division, differentiation, and elongation of plant cells, and therefore acts as a growth regulator [38, 42]. However, even at low concentrations, 2,4-D has herbicidal effects on dicotyledons due to its ability to induce uncoordinated cell growth, damage to vascular tissues and roots, and malformation of leaves and stems [43].

2.3. Experimental Exposure

Juvenile fish used in the present experiment were supplied by the "Institute of Fisheries and Aquaculture", Plovdiv City, Bulgaria, where they are bred and raised under strictly controlled conditions. The experimental individuals were of the same size and age group (length: 10 cm ± 2.5 cm; weight: 22 g ± 1.5 g), without any external pathological changes. Twenty individuals were used for each experimental concentration (in 100-L glass tanks), as well as in the control and solvent control groups (total n = 160). They were left for 14 days to acclimatize before the experimental exposure began in two 100-L tanks, where they were fed on a diet based on their weight. During the

 doi:10.20944/preprints202512.0512.v1

experiment, the fish were not fed, and the water was renewed on the second day (48 hours). The experiment lasted 96 hours.

Dechlorinated in advance tap water with different concentrations of pesticides dissolved in it was used. Two concentrations were prepared for each pesticide (pirimiphos-methyl – 10 µg/L and 60 µg/L; propamocarb hydrochloride – 40 µg/L and 80 µg/L; 2,4-D – 50 µg/L and 100 µg/L). As previously described, the experimental concentrations were selected based on LC_{50} values provided by the manufacturers—600000 µg/L for pirimiphos-methyl (96 hours, aquatic invertebrates), 80000 µg/L for propamocarb hydrochloride, and 100000 µg/L for 2,4-D—corresponding to 1/60000 and 1/10000 (pirimiphos-methyl), and 1/2000 and 1/1000 (propamocarb and 2,4-D) of their respective LC_{50} values [18]. A control group and a solvent control group of fish were also used for comparison, in which no toxicant was added, but only dechlorinated tap water or solvent (acetone) at low concentrations not expected to cause detectable histological or biochemical alterations in the liver. All aquariums were equipped with aerator pumps to supply the necessary oxygen. The water parameters, such as temperature, pH, dissolved oxygen, and electrical conductivity, were recorded every day until the experiment lasted [44]. The fish were then dissected according to the procedure described by Rosseland et al. [45], which complied with the requirements for the humane treatment of experimental animals as outlined in Directive 2010/63/EU [46].

2.41. Histopathological Assessment

The histopathological processing of the materials was carried out according to the standard methodology of Romeis [47], which includes fixation of the sample in 10% neutral buffered formalin, tissue processing (alcohol dehydration, xylene clearing, paraffin wax infiltration), embedding, microtome sectioning (Leica RM 2125 RTS), and hematoxylin and eosin (H&E) staining. The prepared slides were evaluated using a Leica DM 2000 microscope and imaging software (LAS V4.13). The histopathological changes in the liver were further assessed using the scale described by Bernet et al. [48]. The alterations were classified into four groups: circulatory, proliferative, degenerative, and inflammation-related changes, with each group encompassing specific changes that affected functional units of the organ or the entire organ. In addition, a 5-point scale was used to determine the severity of each change according to Saraiva et al. [49], as follows: (0)—no changes in the hepatic structure (up to 10% of the organ structure); (1)—very mild changes in the hepatic structure (from 10% to 20% of the organ structure); (2)—mild degree of changes in the hepatic structure (from 20% to 30% of the organ structure); (3)—moderate degree of changes in the hepatic structure (from 30% to 50% of the organ structure); (4)—severe degree of changes in the hepatic structure (from 50% to 80% of organ structure); (5)—very severe degree of changes in the hepatic structure (over 80% of the organ structure). The pathological degree of each change was also determined using the significance factor (W) according to Bernet et al. [48] —W is a constant value and is categorized according to the author as follows: (1)—minimal pathological significance, reversible change after cessation of the toxicant; (2)—low pathological significance, the lesion is reversible in most cases if the stressor is neutralized; (3)—high pathological significance, irreversible changes, leading to partial or complete loss of organ function. The indices of histopathological changes are the result of the product of the degree of each specific change in the group multiplied by W. The summation of these final values for the organ determines the index (I) for the respective group of changes (circulatory I_C, degenerative I_R, proliferative I_P, and inflammatory I_I). The sum of all indices also determines the organ index (I_O) for pathological change—I_O refers to a class, according to Zimmerli et al. [50], as follows: class I (Index ≤ 10)—normal histological structure with mild pathological changes (reversible); class II (Index 11–20)—normal histological structure with moderate pathological changes (reversible); class III (Index 21–30)—moderate degree of change in the histological structure (reversible); class IV (Index 31–40)—severe degree of change in the histological structure (irreversible); class V (Index > 40)—very severe degree of change in the histological structure (irreversible).

2.5. Histochemical Assessment

The liver samples from the individuals were processed using a Leica CM 1520 freezing microtome (Leica Microsystems, Wetzlar, Germany). 5 µm-thick cryosections were prepared from

 doi:10.20944/preprints202512.0512.v1

the material. PAS-reaction analysis (Schiff-Iodic acid) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) according to McManus [51] for polysaccharides (liver glycogen) was performed. The histochemical changes in the liver of experimental individuals were presented semi-quantitatively according to our modified scale based on Mishra & Mohanty [52], as follows: (0) – negative reaction of histochemical staining; (1) – very weak positive histochemical reaction; (2) – weak positive histochemical reaction; (3) – moderate positive reaction to histochemical staining; (4) – strong positive histochemical reaction in the fish hepatocytes.

2.6. Biochemical Assessment

A biochemical evaluation of hepatic ASAT, ALAT, ChE, and LDH activities was conducted. Liver samples were rapidly thawed on ice and homogenized in chilled phosphate buffer (50 mM, 300 mM NaCl, pH 7.4) using a Pyrex Potter–Elvehjem tissue grinder fitted with a PTFE pestle (Thermo Fisher Scientific, Waltham, MA, USA). The resulting homogenates were centrifuged at 9000 rpm for 15 min at 4 °C in a refrigerated centrifuge (MPW, Poland). Determination of the activity of the enzyme LDH (LDH FL DGKC, Chema Diagnostica, Via Campania 2/4, 60030 Monsano (AN) - ITALY – UE, assay kit) was carried out according to the method of Vassault [53]. Determination of the activity of the enzymes ASAT (GOT/AST FL IFCC, Chema Diagnostica, Via Campania 2/4, 60030 Monsano (AN) - ITALY – UE, assay kit) and ALAT (GPT/ALT FL IFCC, Chema Diagnostica, Via Campania 2/4, 60030 Monsano (AN) - ITALY – UE, assay kit) was carried out according to a method developed in parallel by Henley & Pollard [54] and Wroblewski & Ladue [55], which was modified and improved by Reitman & Frankel [56]. Determination of ChE activity (ChE DGKC FL, Chema Diagnostica, Via Campania 2/4, 60030 Monsano (AN) - ITALY – UE, assay kit) was performed according to Burtis & Ashwood [57].

All enzymatic activities were measured spectrophotometrically, using a Beckman Coulter Spectrophotometer DU 800 (Beckman Coulter, Inc., Brea, CA, USA) at 25 °C. The total protein content of the supernatant for each test was measured according to Bradford [51] with Coomassie Brilliant Blue G-250, using bovine serum albumin at an absorbance of 595 nm, and presented as milligrams protein per milliliter homogenate. The specific enzyme activity is the number of enzyme units per ml divided by the protein concentration in mg/ml. Thus, the activity of the enzymes tested was expressed as specific enzyme activity (U/mg protein).

2.7. Statistical Analysis

All statistical analyses were performed using treatment groups as the independent factor. Before hypothesis testing, each dataset was evaluated for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. When both assumptions of normality and homoscedasticity were met, the data were analyzed using a one-way analysis of variance (ANOVA). The significant ANOVA results were followed by Tukey's Honestly Significant Difference (HSD) post hoc test to determine pairwise differences among treatments. If either assumption was violated, a non-parametric Kruskal–Wallis test was applied. When the Kruskal–Wallis test indicated significant differences, Mann–Whitney U tests were used for post hoc pairwise comparisons. A significance level of $p < 0.05$ was used in all analyses. This decision-making framework (parametric vs. non-parametric) was consistently applied across all biochemical datasets, including LDH, ASAT, ALAT, and ChE activities. All graphs were generated using Python 3.11 with the following scientific libraries: NumPy (v1.26) for numerical computation; Pandas (v2.1) for data handling and restructuring; Matplotlib (v3.8) for graph production; Statsmodels (v0.14) for ANOVA, Tukey HSD, and other statistical tests. All visualizations (bar charts, SD error bars, and compact letter displays) were produced programmatically using these packages.

3. Results

3.1. Histopathological Assessment

The study of the changes that occurred in the hepatic cells and liver tissue under the influence of the studied pesticides showed that even very low concentrations of pirimiphos-methyl, propamocarb hydrochloride, and 2,4-D caused a highly toxic effect.

 doi:10.20944/preprints202512.0512.v1

Control group

The results of the histopathological analysis showed normal morphology of the histological structure of the liver in the control group. According to the five-point (0–5) scale for the severity of changes, the control liver sections were assigned a value of 0 (Table 1 and Figure 1). The same was found for the solvent control, which will not be further discussed, as we did not observe any harm to fish liver histology.

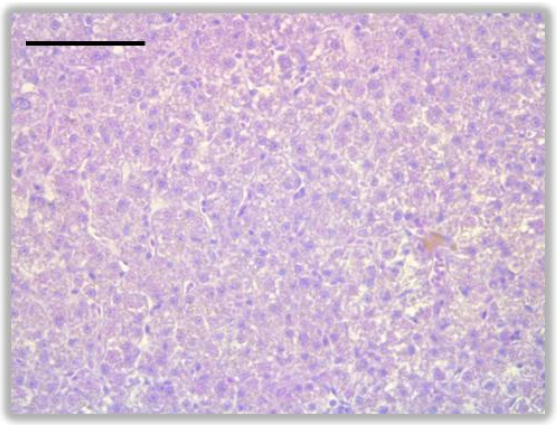

Figure 1. Normal morphology of the liver histological structure of common carp from the control group, x400, H&E. ***Histopathological alterations in the liver of common carp after acute exposure to pirimiphos-methyl.***

Upon exposure to pirimiphos-methyl in the liver of the tested fish, changes in the circulatory system were detected. They were mainly expressed as hyperemia of the blood vessels of the organ. The observed manifestation was similar at both experimental concentrations and was of a medium degree (Table 1, Figure 2 F). Intracellular edema was not detected at either experimental concentrations. The indices of histopathological changes in the peripheral circulatory system (I_{LC}) for both concentrations were 3.

Degenerative changes in the liver showed an increasing degree of manifestation proportional to increasing pesticide concentration (Figure 2 A, B, C, D, E). Granular degeneration was found to be very severe at a concentration of 10 µg/L and severe at a concentration of 60 µg/L of pirimiphos-methyl. The higher degree of degenerative changes in the liver cells was vacuolar degeneration, which was observed to be severe at both experimental concentrations. When analyzing the histopathological sections, fatty degeneration was also found, which was described as mild at a concentration of 10 µg/L and moderate at a concentration of 60 µg/L pirimiphos-methyl (Table 1).

Changes in hepatocytes associated with necrobiotic processes were also found, and they were very mild in both applied pirimiphos-methyl concentrations. The presence of necrosis with typical areas of necrotic masses was found only in single areas, which encompassed about 1-4 cells. Therefore, the degree of necrosis in hepatocytes was determined to be very mild at the lower concentration and mild at the higher concentration of insecticide applied. When comparing the indices of degenerative changes (I_{LR}), for the lower concentration of pirimiphos-methyl of 10 µg/L, the value was I_{LR} – 20, while at a concentration of 60 µg/L pirimiphos-methyl, the value was 23. This clearly shows that the influence of the higher concentration of the pesticide was reflected in a more severe degree of degenerative changes in the liver histological structure and therefore had a more serious negative effect, which undeniably affects the functioning of the organ.

Table 1. Histopathological alterations in the liver of common carp after acute (96 hours) exposure to pirimiphos-methyl.

Reaction pattern	Organ	Alteration	Importance factor	Score value		
				Control	Pirimiphos-methyl	
					10 µg/L	60 µg/L
	Liver	Hyperaemia	$W_{LC1} = 1$	0^{A}	3^{B}	3^{B}

 doi:10.20944/preprints202512.0512.v1

Changes in the circulatory system		Intracellular edema	$W_{LC2} = 1$	0^A	0^A	0^A
Index for the circulatory system				$I_{LC}=0$	$I_{LC}=3$	$I_{LC}=3$
Degenerative changes	*Liver*	Granular degeneration	$W_{LR1} = 1$	0^A	5^B	4^C
		Vacuolar degeneration	$W_{LR2} = 2$	0^A	4^B	4^B
		Necrobiosis	$W_{LR3} = 2$	0^A	1^B	1^B
		Necrosis	$W_{LR4} = 3$	0^A	1^B	2^C
		Fatty degeneration	$W_{LR5} = 1$	0^A	2^B	3^C
Index for the degenerative changes				$I_{LR} = 0$	$I_{LR}=20$	$I_{LR}=23$
Proliferative changes	*Liver*	Hypertrophy	$W_{LP1} = 1$	0^A	0^A	0^A
Index for the proliferative changes				$I_{LP} = 0$	$I_{LP} = 0$	$I_{LP} = 0$
Inflammation	*Liver*	Activation of RES	$W_{LI1} = 1$	0^A	0^A	0^A
		Lymphocyte infiltration	$W_{LI2} = 2$	0^A	0^A	0^A
Index for the inflammatory processes				$I_{LI} = 0$	$I_{LI} = 0$	$I_{LI} = 0$
Index for the organ				$I_L = 0$	$I_L = 23$	$I_L = 26$

0 – no changes in the histological structure of the organ; (1) – very mild changes in the histological structure of the organ; (2) – mild changes in the histological structure of the organ; (3) – moderate changes in the histological structure of the organ; (4) – severe changes in the histological structure of the organ; (5) – very severe changes in the histological structure of the organ. Different uppercase letters within the same row indicate statistically significant differences between groups based on the Mann–Whitney U test ($p < 0.05$).

Proliferative changes were not detected, nor well as disorders associated with lymphocytic infiltration and activation of the reticuloendothelial system (indicators of an inflammatory process). The index of these changes was respectively with a value $I_{LP}=0$ (Table 1).

From the determined histopathological lesions, the liver index (I_L) of the treated fish was also calculated, as at a concentration of 10 µg/L pirimiphos-methyl, the value was 23, and at a concentration of 60 µg/L, it was 26.

Based on the results obtained and the scale proposed by Zimmerli et al. [50], it was calculated that the liver index (I_L) falls into class III (index 21-30) for both applied concentrations of pirimiphos-methyl. This is an indicator that the observed alterations in the liver were of a moderate degree of change in the histological structure, and the processes could be reversible.

Acute exposure to pirimiphos-methyl caused pronounced histopathological alterations in the liver of common carp compared to the control and solvent groups. The Kruskal–Wallis test revealed significant treatment effects for the majority of degenerative lesions ($p < 0.05$). Mann–Whitney U tests demonstrated that both pesticide concentrations differed significantly from the control, as indicated by the distinct letter groupings (A, B, C). Hyperaemia showed a clear elevation in both treatment groups, while granular degeneration displayed a dose-dependent response, with the highest score observed at 60 µg/L. Similarly, necrosis and fatty degeneration increased significantly with concentration, reaching their highest levels in the 60 µg/L group. In contrast, intracellular edema and proliferative changes remained absent in all treatments, indicating that these lesion types were not triggered by acute pirimiphos-methyl exposure.

Degenerative changes formed the dominant reaction pattern, with the index for degenerative lesions increasing from 0 in the control to 20 and 23 in the treated groups. The overall liver index (I_L) showed a strong, concentration-dependent increase (0 → 23 → 26), confirming that pirimiphos-methyl induces substantial and escalating hepatic damage.

 doi:10.20944/preprints202512.0512.v1

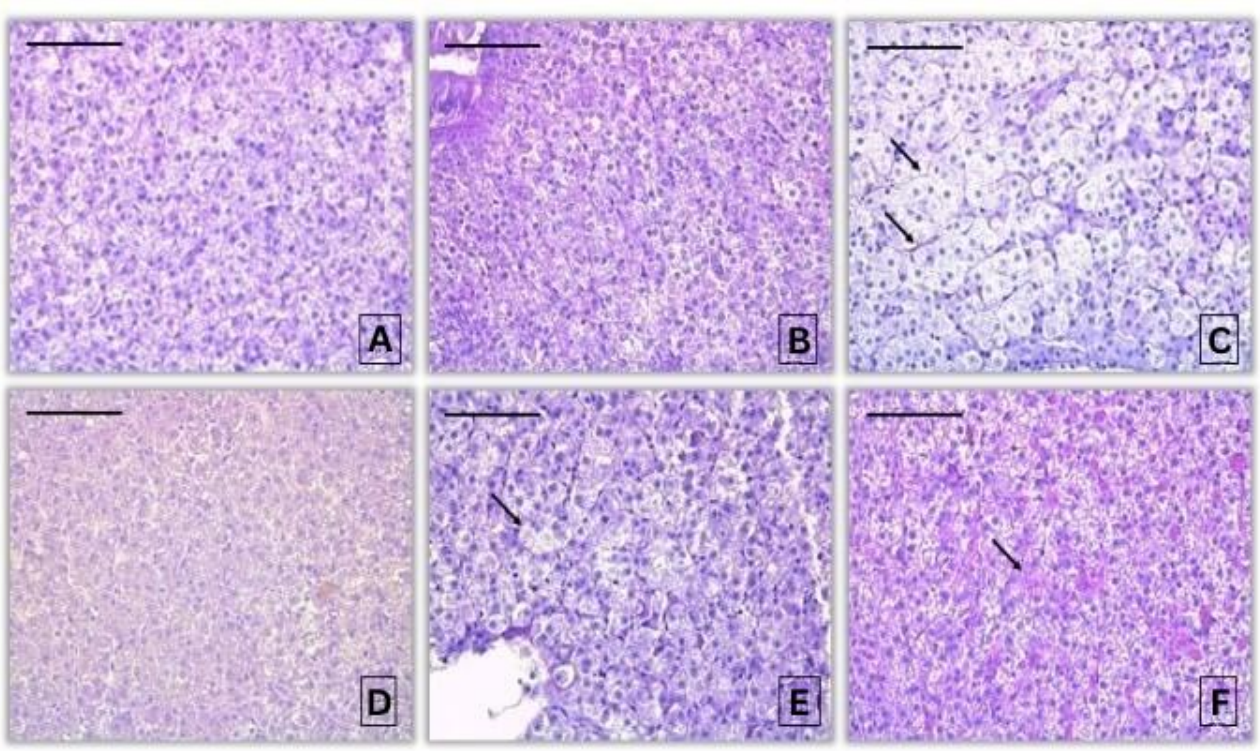

Figure 1. Histopathological alterations in the liver of common carp after acute (96 hours) exposure to pirimiphos-methyl (H&E), x400: A, B – granular degeneration (10 μg/L); C – vacuolar degeneration (10 μg/L); D – granular degeneration (60 μg/L); E – karyolysis (60 μg/L); F – hyperaemia (60 μg/L).

Histopathological alterations in the liver of common carp after acute exposure to propamocarb hydrochloride

After exposure to propamocarb hydrochloride, the prepared histopathological sections from each treated individual were analyzed similarly to the acute exposure to pirimiphos-methyl. Changes in the blood vessels of the liver were observed. Hyperemia was noted in many areas of the organ parenchyma at both applied concentrations of the pesticide. According to the histopathological scale, this disorder was defined as severe at both propamocarb hydrochloride concentrations, 40 μg/L and 80 μg/L (Table 2, Figure 3 F). When analyzing the samples, intracellular edema was not detected at either experimental concentrations. The calculated index of changes in the circulatory system (I_{LC}) was scored as 4 for both pesticide concentrations.

Table 2. Histopathological alterations in the liver of common carp after acute (96 hours) exposure to propamocarb hydrochloride.

Reaction pattern	Organ	Alteration	Importance factor	Score value		
				Control	Propamocarb hydrochloride	
					40 μg/L	80 μg/L
Changes in the circulatory system	*Liver*	Hyperaemia	$W_{LC1} = 1$	0^A	4^B	4^B
		Intracellular edema	$W_{LC2} = 1$	0^A	0^A	0^A
Index for the circulatory system				$I_{LC}=0$	$I_{LC}=4$	$I_{LC}=4$
Degenerative changes	*Liver*	Granular degeneration	$W_{LR1} = 1$	0^A	5^B	5^B
		Vacuolar degeneration	$W_{LR2} = 2$	0^A	4^B	5^C
		Necrobiosis	$W_{LR3} = 2$	0^A	1^B	1^B
		Necrosis	$W_{LR4} = 3$	0^A	2^B	2^B

 doi:10.20944/preprints202512.0512.v1

		Fatty degeneration	$W_{LR5} = 1$	0^A	2^B	4^C
Index for the degenerative changes				$I_{LR} = 0$	I_{LR}=23	I_{LR}=27
Proliferative changes	*Liver*	Hypertrophy	$W_{LP1} = 1$	0^A	0^A	0^A
Index for the proliferative changes				$I_{LP} = 0$	$I_{LP} = 0$	$I_{LP} = 0$
Inflammation	*Liver*	Activation of RES	$W_{LI1} = 1$	0^A	0^A	0^A
		Lymphocyte infiltration	$W_{LI2} = 2$	0^A	1^B	1^B
Index for the inflammatory processes				$I_{LI} = 0$	$I_{LI} = 2$	$I_{LI} = 2$
Index for the organ				$I_L = 0$	$I_L = 29$	$I_L = 33$

0 – no changes in the histological structure of the organ; (1) – very mild changes in the histological structure of the organ; (2) – mild changes in the histological structure of the organ; (3) – moderate changes in the histological structure of the organ; (4) – severe changes in the histological structure of the organ; (5) – very severe changes in the histological structure of the organ. Different uppercase letters within the same row indicate statistically significant differences between groups based on the Mann–Whitney U test ($p < 0.05$).

Along with the circulatory system lesions, degenerative changes in the hepatocytes were also observed (Figure 3 A, B, C, D, E). Granular degeneration was found to be very severe at both propamocarb hydrochloride concentrations. Vacuole degeneration was severe at the lower concentration and very severe at the higher concentration of the pesticide. Necrobiosis was detected and reported to be very mild, while necrosis was observed to be mild at both tested concentrations. Fatty degeneration was found, but it was mild at a concentration of 40 μg/L and severe at a concentration of 80 μg/L of propamocarb hydrochloride. The calculated index of degenerative changes in the liver (I_{LR}) was 23 for the concentration of 40 μg/L and 27 for the concentration of 80 μg/L propamocarb hydrochloride.

No proliferative changes were detected, and accordingly, the index for these changes (I_{LP}) was 0.

From the group of inflammatory processes, lymphocyte infiltration was found to a very mild extent at both concentrations, while activation of the reticuloendothelial system was not detected. The inflammatory change index (I_{LI}) was the same for both concentrations – 2.

Exposure to propamocarb hydrochloride also resulted in statistically significant hepatic alterations. The Kruskal–Wallis test indicated significant differences among groups for most degenerative lesions ($p < 0.05$). Mann–Whitney U post hoc comparisons showed that both concentrations differed significantly from the control and solvent control groups, but in several cases the two exposed groups did not differ from each other (shared letter "B"). Hyperaemia increased sharply in both treatment groups, and granular degeneration reached the maximum score at both experimental concentrations. Vacuolar degeneration and fatty degeneration demonstrated clear dose-dependent patterns, with the highest doses showing significantly greater alterations. Necrobiosis and necrosis were elevated, but reached similar values at both propamocarb hydrochloride concentrations.

The degenerative index (I_{LR}) increased dramatically from 0 in the controls to 23 and 27 in the treated fish. Mild inflammatory responses also appeared at both concentrations ($I_{LI} = 2$), unlike in Table 1. The overall liver index increased from 0 to 29 and 33, confirming that propamocarb hydrochloride causes extensive hepatic injury, particularly affecting degenerative pathways.

 doi:10.20944/preprints202512.0512.v1

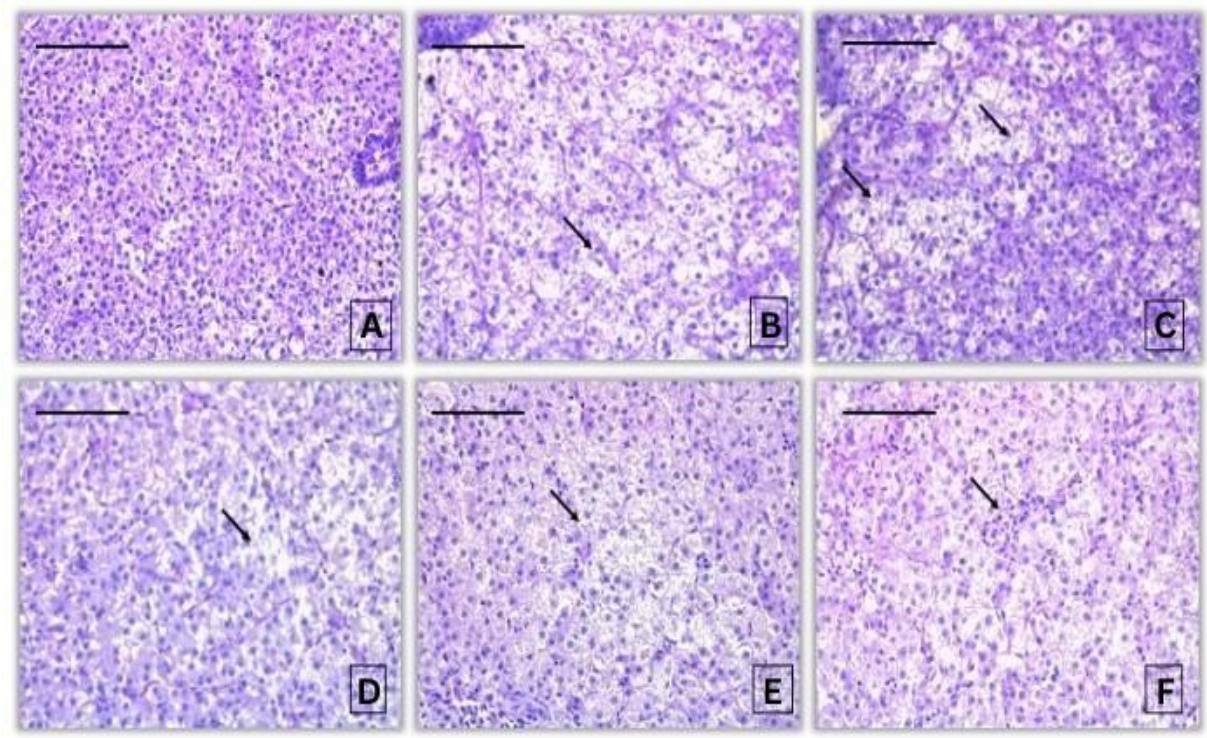

Figure 3. Histopathological alterations in the liver of common carp after acute (96 hours) exposure with propamocarb hydrochloride (H&E), x400: A – granular degeneration (40 μg/L); B, C – vacuolar degeneration (40 μg/L); D – karyolysis (80 μg/L); E – fatty degeneration (80 μg/L); F – hyperaemia (80 μg/L).

The organ index (I_L) of fish treated with propamocarb hydrochloride was 29 at a concentration of 40 μg/L, and in those treated with a concentration of 80 μg/L, the value was 33 (Table 2).

From the obtained results and according to the scale proposed by Zimmerli et al. [50], it was calculated that the liver index (I_L) at a concentration of 40 μg/L falls into class III (index 21-30). Based on this, the observed changes in the organ were of a moderate degree of change in the histological structure, and the process was reversible. I_L at a concentration of 80 μg/L falls into class IV (index 31-40). Therefore, the changes in the organ were of a severe degree in the histological structure, and the processes were already irreversible. The obtained results clearly indicate that the higher concentration of propamocarb hydrochloride has a higher degree of hepatotoxicity in the exposed fish.

Histopathological alterations in the liver of common carp after acute exposure to 2,4-D

Similar to the treatment with pirimiphos-methyl and propamocarb hydrochloride, lesions in the circulatory system of the organ were detected during the last herbicide exposure, which were expressed in a severe degree of hyperemia at both experimental concentrations, 50 μg/L and 100 μg/L (Table 3, Figure 4 A, C, E). Intracellular edema was not detected at either of the experimental concentrations. The index of circulatory changes was calculated at both concentrations with a value of $I_{LC} = 4$.

Degenerative changes in the liver parenchyma were registered (Figure 4 B, D, F). The observed granular degeneration in the hepatocytes was reported to be severe at a concentration of 50 μg/L and very severe at a concentration of 100 μg/L of 2-4 D. Hepatocyte vacuolar degeneration was also found, which was determined to be severe and very severe, respectively, at concentrations of 50 μg/L and 100 μg/L. Furthermore, fatty degeneration was observed, and at the lower concentration, it was determined to be mild. At the higher concentration of the herbicide, this change was detected to be very severe. In addition, cells with morphological alterations were also found in the hepatocytes, which unquestionably indicated the development of necrobiotic processes associated with karyopyknosis, karyorrhexis, and karyolysis. These changes, however, were found in single cells from separate areas of the organ parenchyma. Therefore, the degree of manifestation of necrobiosis was described as very mild at both applied concentrations. In the histopathological sections,

 doi:10.20944/preprints202512.0512.v1

hepatocytes with necrosis development were also observed, with the presence of destroyed cell membranes and necrotic masses. Taking into account the involvement of this disorder in the organ parenchyma, the degree of manifestation (necrosis) was determined as mild. Based on the degenerative changes found, the index (I_{LR}) was calculated for the concentration of 50 µg/L was 22, while for the concentration of 100 µg/L, it was 28.

Table 3. Histopathological alterations in the liver of common carp after acute (96 hours) exposure to 2, 4-D.

Reaction pattern	Organ	Alteration	Importance factor	Score value		
				Control	2, 4-D	
					50 µg/L	100 µg/L
Changes in the circulatory system	*Liver*	Hyperaemia	$W_{LC1} = 1$	0^A	4^B	4^B
		Intracellular edema	$W_{LC2} = 1$	0^A	0^A	0^A
Index for the circulatory system				$I_{LC}=0$	$I_{LC}=4$	$I_{LC}=4$
Degenerative changes	*Liver*	Granular degeneration	$W_{LR1} = 1$	0^A	4^B	5^C
		Vacuolar degeneration	$W_{LR2} = 2$	0^A	4^B	5^C
		Necrobiosis	$W_{LR3} = 2$	0^A	1^B	1^B
		Necrosis	$W_{LR4} = 3$	0^A	2^B	2^B
		Fatty degeneration	$W_{LR5} = 1$	0^A	2^B	5^C
Index for the degenerative changes				$I_{LR} = 0$	$I_{LR}=22$	$I_{LR}=28$
Proliferative changes	*Liver*	Hypertrophy	$W_{LP1} = 1$	0^A	0^A	0^A
Index for the proliferative changes				$I_{LP} = 0$	$I_{LP} = 0$	$I_{LP} = 0$
Inflammation	*Liver*	Activation of RES	$W_{LI1} = 1$	0^A	0^A	0^A
		Lymphocyte infiltration	$W_{LI2} = 2$	0^A	1^B	1^B
Index for the inflammatory processes				$I_{LI} = 0$	$I_{LI} = 2$	$I_{LI} = 2$
Index for the organ				$I_L = 0$	$I_L = 28$	$I_L = 34$

0 – no changes in the histological structure of the organ; (1) – very mild changes in the histological structure of the organ; (2) – mild changes in the histological structure of the organ; (3) – moderate changes in the histological structure of the organ; (4) – severe changes in the histological structure of the organ; (5) – very severe changes in the histological structure of the organ. Different uppercase letters within the same row indicate statistically significant differences between groups based on the Mann–Whitney U test ($p < 0.05$).

No proliferative changes (I_{LP}) were detected, and accordingly, the index for these changes was 0.

From the group of inflammatory processes, lymphocytic infiltration was found to a very mild extent at both 2,4-D concentrations, while activation of the reticuloendothelial system was not reported. The inflammatory change index (I_{LI}) at both concentrations was 2.

Based on the results obtained, the index of the analyzed organ (I_L) of the fish treated with 2,4-D at a concentration of 50 µg/L was calculated to be 28, and those treated with a concentration of 100 µg/L had a value of 34 (Table 3).

The herbicide 2,4-D induced significant dose-dependent liver damage in common carp. The Kruskal–Wallis test detected significant differences among treatments for nearly all degenerative lesions ($p < 0.05$). Mann–Whitney U tests showed clear separation between the controls and both exposed groups (A vs. B/C), with the high concentration consistently showing the most severe alterations (C). Hyperaemia was markedly elevated in both treatments. Granular and vacuolar degeneration reached high values in the 100 µg/L group (5C), indicating substantial hepatocellular

 doi:10.20944/preprints202512.0512.v1

impairment. Fatty degeneration showed a particularly strong dose-dependent pattern, increasing from 2B to 5C across concentrations. Necrobiosis and necrosis also increased significantly relative to controls, though differences between the two exposed groups were not evident for these lesions.

The degenerative index rose sharply from 0 in the control to 22 and 28 in the treated fish. Inflammatory changes were present in both treatments (I_{LI} = 2). The overall liver index increased progressively (0 → 28 → 34), representing the strongest hepatic impact among the three experimental pesticides. These results indicate that 2,4-D exerted potent and concentration-dependent hepatotoxic effects.

From the obtained results and according to the scale proposed by Zimmerli et al. [50], it was calculated that the liver index (I_L) at a concentration of 50 μg/L falls into class III (index 21-30), which indicates that the observed changes in the organ were of a moderate degree and the processes were still considered as reversible. I_L at a concentration of 100 μg/L falls into class IV (index 31-40), which means that the organ had a severe degree of alterations in the histopathological structure, and the processes were irreversible. The results clearly showed that the higher concentration of 2,4-D triggers a higher degree of hepatotoxicity in common carp.

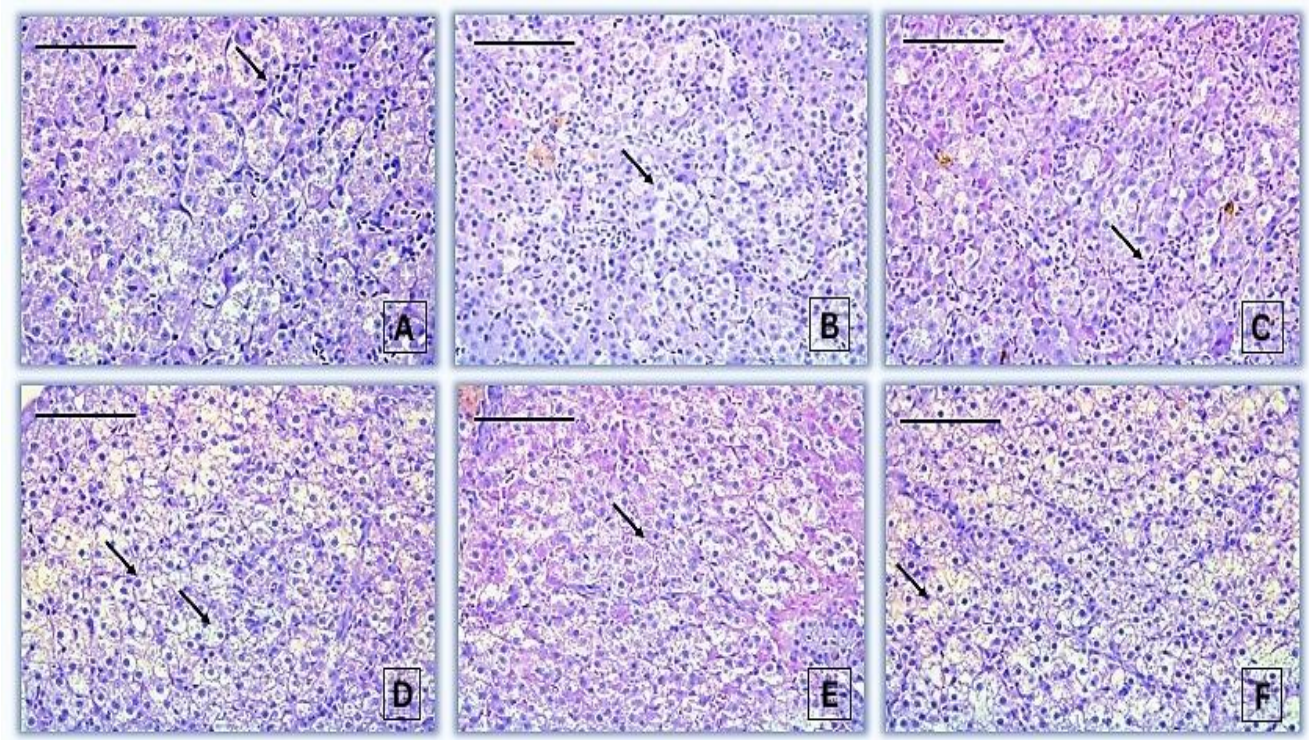

Figure 4. Histopathological alterations in liver of common carp after acute (96 hours) exposure to 2,4-D (H&E), x400: A – hyperaemia (50 μg/L); B – vacuolar degeneration (50 μg/L); C – hyperaemia (50 μg/L); D – fatty degeneration (100 μg/L); E – hyperaemia (100 μg/L); F – vacuolar degeneration (100 μg/L).

After summarizing the data based on the observed hepatic alterations according to the four categories, the degree of toxicity of the tested pesticides on fish can be summarized in the following descending order: for degenerative changes: propamocarb hydrochloride = 2,4-D > pirimiphos-methyl; for changes in the circulatory system: propamocarb hydrochloride = 2,4-D > pirimiphos-methyl; for inflammation: propamocarb hydrochloride = 2,4-D > pirimiphos-methyl. Since proliferative changes in the liver were not found, a comparison between the three pesticides according to this criterion could not be made. However, a general tendency of equalization of the indices of changes of propamocarb hydrochloride and 2,4-D was observed, while in pirimiphos-methyl, they were less pronounced.

3.2. Histochemical Assessment

 doi:10.20944/preprints202512.0512.v1

The histochemical analysis showed a negative impact of the pesticides applied on the structure of the liver of experimental fish. The intensity of the PAS-reaction in the liver of common carp differed significantly among treatments (Kruskal–Wallis test, $H = 32.25$, $p < 0.001$; Table 4). The control groups showed the weakest histochemical reaction, with PAS scores significantly lower than those of all pesticide-exposed groups (Mann–Whitney U test, $p < 0.05$). Moderate increases in PAS-positivity were observed in fish exposed to pirimiphos-methyl (10 and 60 μg/L), propamocarb hydrochloride (40 and 80 μg/L), and 2,4-D (50 μg/L). The strongest PAS-reaction was recorded in fish exposed to 100 μg/L 2,4-D (3.80 ± 1.10), which differed significantly not only from the controls, but also from the 60 μg/L pirimiphos-methyl treatment ($p < 0.05$). Overall, the results indicated a concentration-dependent enhancement of PAS-reactivity in response to all three pesticides.

Table 4. Mean ± SD of PAS-reaction intensity in the liver of common carp after acute (96 hours) exposure to pirimiphos-methyl, propamocarb hydrochloride, and 2,4-D.

Experimental pesticide	Controls	Pirimiphos-methyl		Propamocarb hydrochloride		2, 4-D	
Concentration μg/L		10 μg/L	60 μg/L	40 μg/L	80 μg/L	50 μg/L	100 μg/L
Intensity of PAS-reaction in Common carp liver	0.79± 0.43^{A}	2.60± 0.55$^{B, C}$	1.80± 0.84^{B}	2.40± 0.89$^{B, C}$	3.00± 0.71$^{B, C}$	3.00± 0.71$^{B, C}$	3.80± 1.10$^{B, C}$

Scores represent histochemical PAS-reaction intensity (0 = negative; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong). $^{A, B,C}$ Different superscript letters indicate statistically significant differences among treatments (Mann–Whitney U test, $p < 0.05$).

Histochemical alterations in the liver of common carp after acute exposure to pirimiphos-methyl

At a concentration of 10 μg/L pirimiphos-methyl, a moderate increase in glycogen levels was observed compared to the controls, indicating reduced glycogenolysis or increased glycogen synthesis. At a concentration of 60 μg/L pirimiphos-methyl, a slight increase in the glycogen levels compared to the controls was observed, but a decrease compared to the previous, lower concentration of the applied pesticide (Table 4, Figure 5).

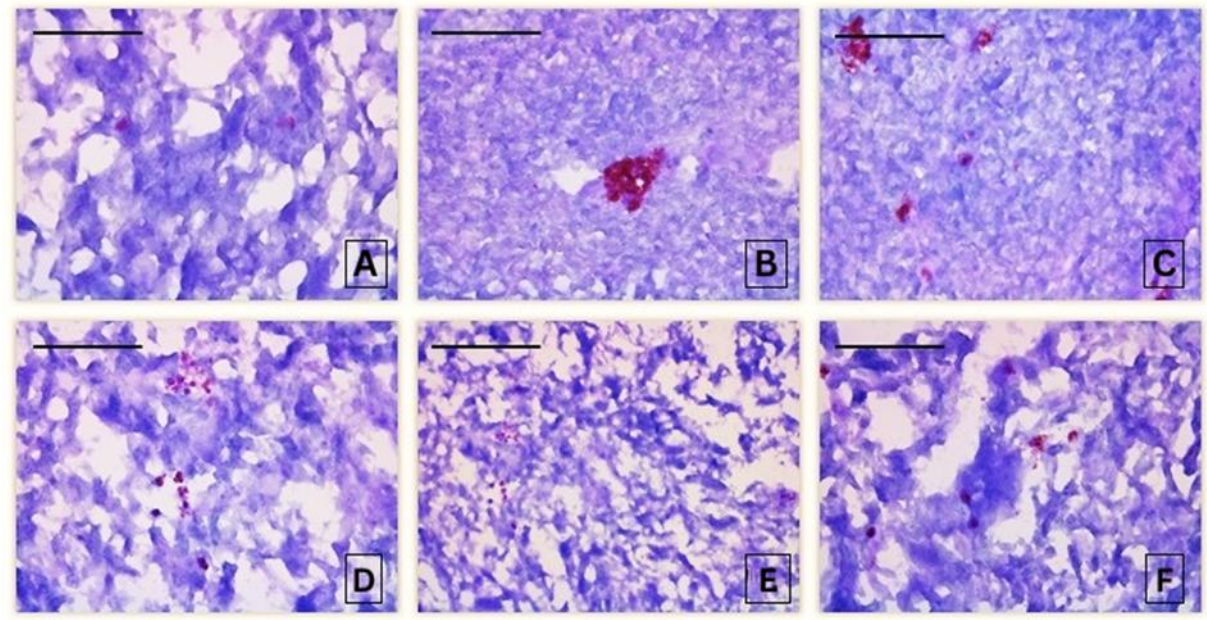

Figure 5. Intensity of the PAS-reaction in the liver of common carp after acute (96 hours) exposure to pirimiphos-methyl: A – control, x400; B, C – 10 μg/L, x400; D, E, F – 60 μg/L, x400.

 doi:10.20944/preprints202512.0512.v1

Histochemical alterations in the liver of common carp after acute exposure to propamocarb hydrochloride

The fungicide propamocarb hydrochloride showed a moderate increase in the amount of glycogen compared to the controls, in a similar manner at both experimental concentrations (Table 4, Figure 6) to propamocarb hydrochloride: A – control, x400; B, C –40 μg/L, x400; D, E, F –80 μg/L, x400.

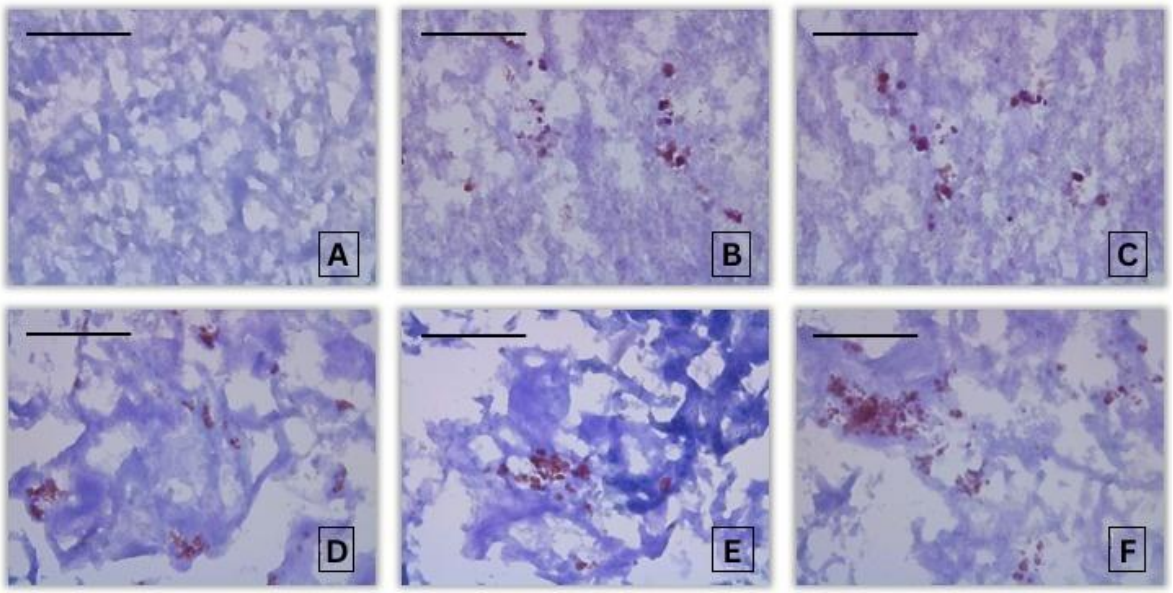

Figure 6. Intensity of the PAS-reaction in the liver of common carp after acute (96 hours) exposure.

Histochemical alterations in the liver of common carp after exposure to 2,4-D

The herbicide 2,4-D also showed a moderate accumulation of glycogen compared to the control group, at both experimental exposures (Table 4, Figure 7).

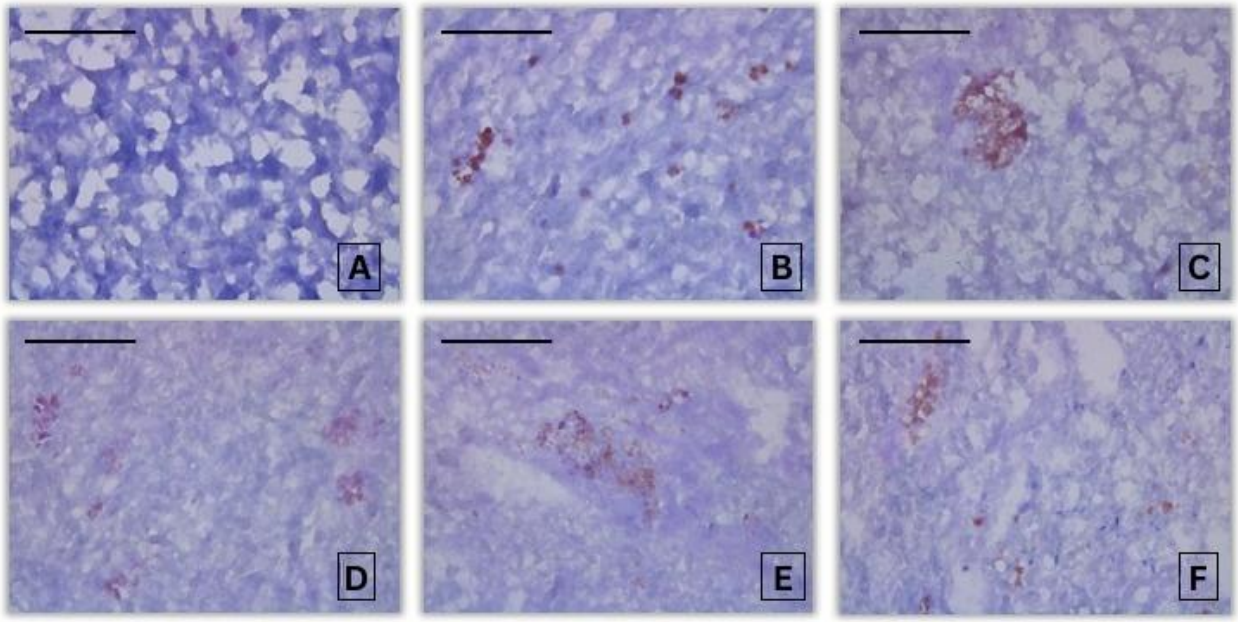

Figure 7. Intensity of the PAS-reaction in the liver of common carp after acute (96 hours) exposure to 2,4-D: A – control, x400; B, C –50 μg/L, x400; D, E, F –100 μg/L, x400.

The toxicity of the experimental pesticides can be summarized in the following descending order: propamocarb hydrochloride = 2,4-D > pirimiphos-methyl. Overall, the results on glycogen

 doi:10.20944/preprints202512.0512.v1

accumulation in the liver showed a similar degree of manifestation after exposure to propamocarb hydrochloride and 2,4-D, while after treatment with pirimiphos-methyl, the changes were expressed to a lesser extent.

3.3. Biochemical Assessment

In a similar manner to the histopathological and histochemical lesions in the liver, we also determined changes at the cellular level. The biochemical alterations in the liver of common carp after acute exposure to the tested pesticides are presented below.

Lactate dehydrogenase (LDH)

The LDH activity showed a significant overall treatment effect (one-way ANOVA: $F_{6,14} = 28.98$, $p < 0.001$). The control group exhibited the highest LDH activity, which was significantly greater than all pesticide-exposed groups (Tukey HSD, $p < 0.05$). Among the treatments, the exposure to 50 µg/L 2,4-D resulted in moderately elevated LDH activity compared to 60 µg/L pirimiphos-methyl, 40 µg/L propamocarb hydrochloride, and 80 µg/L propamocarb hydrochloride ($p < 0.05$), but it did not differ significantly from 10 µg/L pirimiphos-methyl or 100 µg/L 2,4-D. All pesticide treatments showed markedly reduced LDH activity relative to the controls, indicating a general suppression of LDH in response to pirimiphos-methyl, propamocarb hydrochloride, and 2,4-D exposures, with a partial increase at the 50 µg/L 2,4-D concentration. (Figure 8).

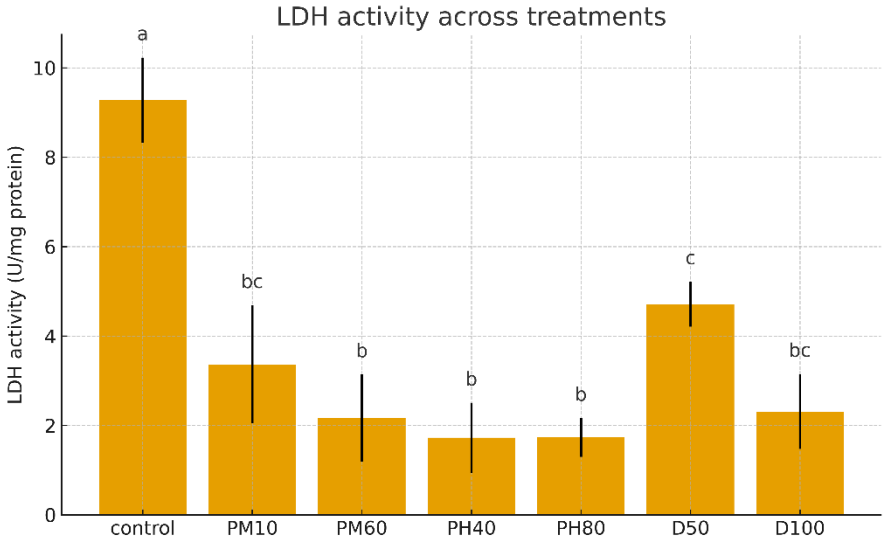

Figure 8. LDH activity (U/mg protein) in the liver of common carp after acute (96 hours) pesticide exposure. Treatments: control (no pesticide), PM10 = 10 µg/L pirimiphos-methyl, PM60 = 60 µg/L pirimiphos-methyl, PH40 = 40 µg/L propamocarb hydrochloride, PH80 = 80 µg/L propamocarb hydrochloride, D50 = 50 µg/L 2,4-D, D100 = 100 µg/L 2,4-D.
Values are mean ± SD. Different letters above the bars indicate significant differences among treatments (Tukey HSD, $p < 0.05$).

Aspartate aminotransferase (ASAT) and Alanine aminotransferase (ALAT)

Aspartate aminotransferase (ASAT) activity showed a strong overall treatment effect (one-way ANOVA: $F_{6,14} = 66.85$, $p < 0.001$). The control groups exhibited markedly higher ASAT activity compared with all pesticide-exposed treatments (Tukey HSD, $p < 0.05$). In contrast, none of the pesticide concentrations—10 µg/L pirimiphos-methyl, 60 µg/L pirimiphos-methyl, 40 µg/L propamocarb hydrochloride, 80 µg/L propamocarb hydrochloride, 50 µg/L 2,4-D, or 100 µg/L 2,4-D—differed significantly from each other. All exposed groups displayed uniformly reduced ASAT activity relative to the control, indicating a consistent suppressive effect of pirimiphos-methyl, propamocarb hydrochloride, and 2,4-D on aminotransferase activity in the common carp hepatic tissue (Figure 9).

 doi:10.20944/preprints202512.0512.v1

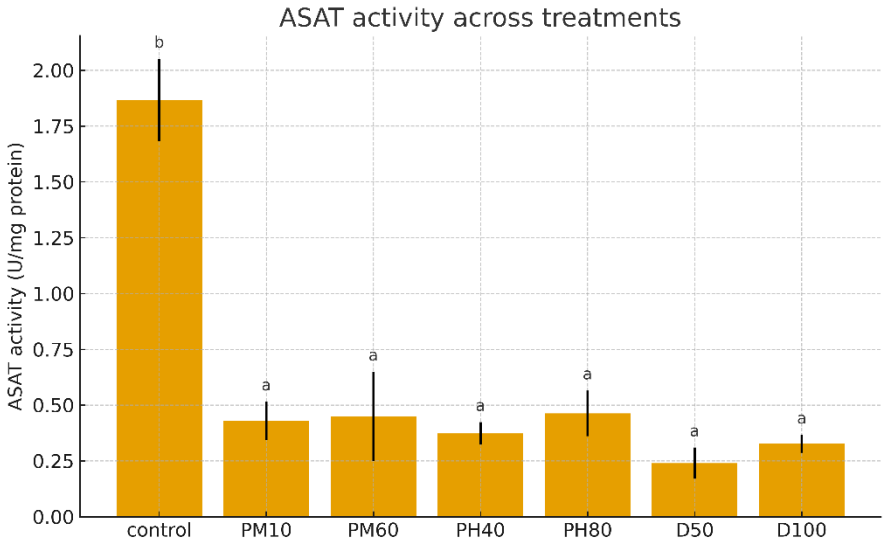

Figure 9. ASAT activity (U/mg protein) in the liver of common carp after acute (96 hours) pesticide exposure. Treatments: control (no pesticide), PM10 = 10 µg/L pirimiphos-methyl, PM60 = 60 µg/L pirimiphos-methyl, PH40 = 40 µg/L propamocarb hydrochloride, PH80 = 80 µg/L propamocarb hydrochloride, D50 = 50 µg/L 2,4-D, D100 = 100 µg/L 2,4-D. Values are mean ± SD. Different letters above the bars indicate significant differences among treatments (Tukey HSD, $p < 0.05$).

Alanine aminotransferase (ALAT) activity differed significantly among treatments (one-way ANOVA: $F_{6,14} = 17.77$, $p < 0.001$). The control groups showed the highest ALAT activity, which was significantly greater than the activity measured in fish exposed to 10 µg/L pirimiphos-methyl, 60 µg/L pirimiphos-methyl, 40 µg/L propamocarb hydrochloride, 50 µg/L 2,4-D, 100 µg/L 2,4-D, and 80 µg/L propamocarb hydrochloride (Tukey HSD, $p < 0.05$). Among the treatments, the lowest activity was detected in the 50 µg/L 2,4-D group, which differed significantly from 40 µg/L propamocarb hydrochloride, 60 µg/L pirimiphos-methyl, 80 µg/L propamocarb hydrochloride, and 100 µg/L 2,4-D. Several treatments—including 60 µg/L pirimiphos-methyl, 40 µg/L propamocarb hydrochloride, and 100 µg/L 2,4-D—formed a homogeneous subset, indicating comparable ALAT suppression. Overall, pesticide exposure resulted in a marked reduction of ALAT activity relative to the control, with the strongest inhibition occurring under 50 µg/L 2,4-D (Figure 10).

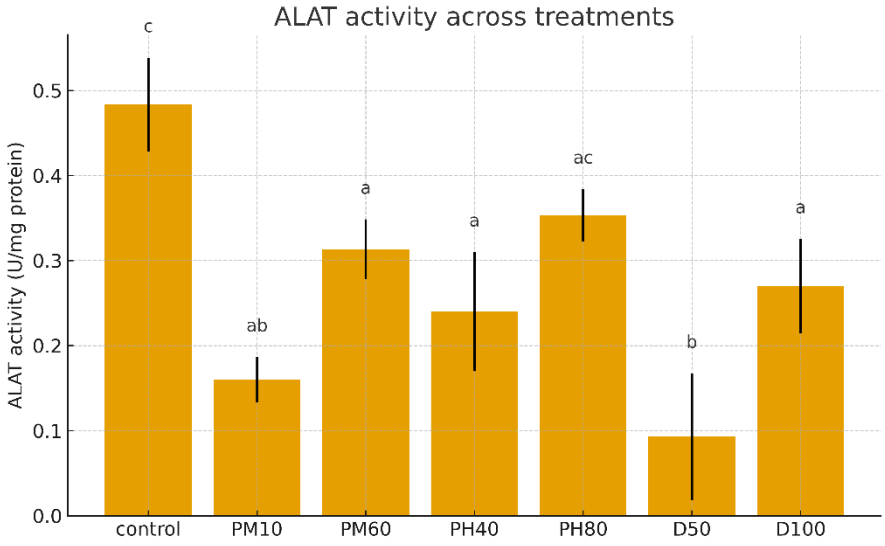

Figure 10. ALAT activity (U/mg protein) in the liver of common carp after acute (96 hours) pesticide exposure. Treatments: control (no pesticide), PM10 = 10 µg/L pirimiphos-methyl, PM60 = 60 µg/L pirimiphos-methyl, PH40 = 40 µg/L propamocarb hydrochloride, PH80 = 80 µg/L propamocarb hydrochloride, D50 = 50 µg/L 2,4-D, D100

 doi:10.20944/preprints202512.0512.v1

= 100 µg/L 2,4-D. Values are mean ± SD (n = 3). Different letters above bars indicate significant differences among treatments (Tukey HSD, $p < 0.05$).

Cholinesterase (ChE)

Cholinesterase (ChE) activity also differed markedly among treatments (one-way ANOVA: $F_{6,14} = 198.26$, $p < 0.001$). The highest activity was observed in the control groups, which was significantly greater than in all pesticide-exposed fish (Tukey HSD, $p < 0.05$). Among the exposed groups, тхе fish treated with 10 µg/L pirimiphos-methyl exhibited the second-highest ChE activity, followed by 40 µg/L propamocarb hydrochloride and 50 µg/L 2,4-D. In contrast, the lowest ChE activities were recorded in the 100 µg/L 2,4-D, 80 µg/L propamocarb hydrochloride, and 60 µg/L pirimiphos-methyl groups, which did not differ significantly from each other but were significantly lower than all other treatments. These results demonstrated a strong dose-dependent suppression of ChE activity across all three pesticides, with the greatest inhibition occurring at the higher concentrations of 2,4-D, propamocarb hydrochloride, and pirimiphos-methyl (Figure 11).

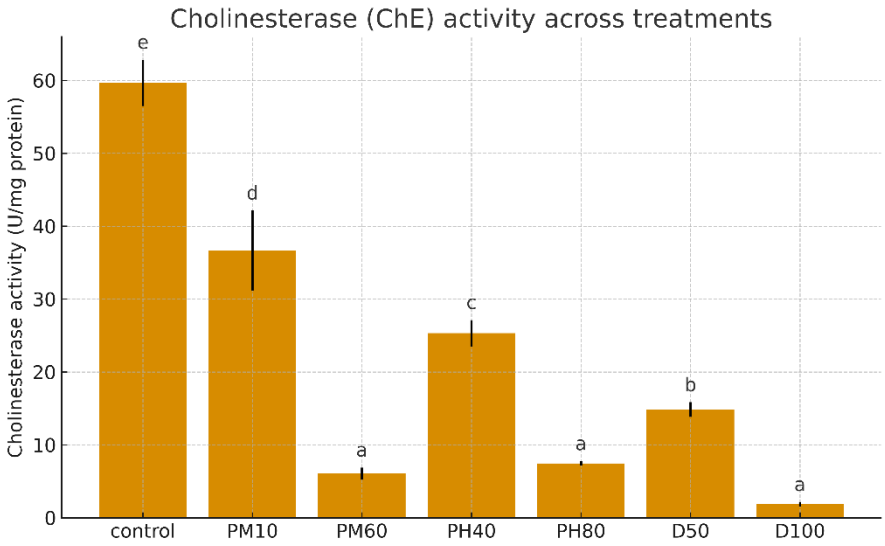

Figure 11. Cholinesterase (ChE) activity (U/mg protein) in the liver of common carp after acute (96 hours) pesticide exposure. Treatments: control (no pesticide), PM10 = 10 µg/L pirimiphos-methyl, PM60 = 60 µg/L pirimiphos-methyl, PH40 = 40 µg/L propamocarb hydrochloride, PH80 = 80 µg/L propamocarb hydrochloride, D50 = 50 µg/L 2,4-D, D100 = 100 µg/L 2,4-D. Values are mean ± SD. Different letters indicate significant differences among treatments (Tukey HSD, $p < 0.05$).

4. Discussion

4.1. Histopathological Alterations in the Liver of Common Carp After Acute Exposure to the Experimental Pesticides

The liver of fish and other vertebrates is the main detoxifying organ, and the effects of its constant exposure to pollutants, including pesticides, can be evident at the cellular and tissue level [58].

The results obtained from the present study did not show proliferative changes in the liver tissue after exposure to the tested concentrations of the three pesticides. This can be explained by the fact that proliferative changes are a compensatory-restorative response of the organism to the experimental toxicants. In general, they develop due to increased functional activity and can also contribute to organ damage. The high toxicity of pesticides, however, most likely did not provide the fish liver with the opportunity for such a response in our case. Moreover, the pesticide toxicity was mainly expressed in a high degree of degenerative changes.

When comparing the indices of degenerative changes in the liver, the highest degree of manifestation of the changes was found upon exposure to the test fungicide propamocarb hydrochloride. The lowest degree of degenerative disorders in the liver was observed upon exposure

 doi:10.20944/preprints202512.0512.v1

to the insecticide pirimiphos-methyl. Unlike the changes in the gills and kidneys in our previous study with the same experimental concentrations and pesticides [18, 19, 59], in which proliferative changes were significantly more pronounced than degenerative changes, the changes in the liver tissue were absolutely opposite – a lack of proliferative changes and relatively high degrees of manifestation of degenerative changes. This can be explained by the fact that the toxic compounds that enter the fish's body are directed to processes of neutralization of their action in the liver, where significant regressive changes in the tissue are induced. It should be emphasized that the observed changes in hepatocytes are the result of the action of pesticides in a short-term exposure of 96 hours. With chronic exposure, we could assume that the degenerative changes would be even higher, more serious, and even lethal for the fish.

Compared with the findings of Mohammod Mostakim et al. [60], who reported severe vascular rupture, hemorrhage, hypertrophy, and extensive necrosis in fish exposed to quinalphos, the pesticides in the present study primarily induced degenerative and circulatory changes without vessel rupture, hemorrhage, or hypertrophic responses, and necrosis remained mild and localized. Overall, the hepatic damage observed here was less structurally destructive and largely reversible compared to the more advanced and progressive lesions described for quinalphos.

The observed circulatory changes in the liver with the three applied pesticides were of similar degrees of manifestation, mostly moderate and severe. Lesions related to inflammatory processes, which are expressed in lymphocyte infiltration, were observed with exposures to propamocarb hydrochloride and 2,4-D, while with pirimiphos-methyl, they were absent. We presume that the tested pesticide exposure causes serious disorders in the vascular system of the organ, an effusion of lymphocytes, which is in response to the deviations in ion exchange that probably have occurred. Oxidative stress has probably also developed. In a similar manner, serious disorders in the circulatory system were found in the gills under the action of the tested pesticides [18, 19, 59]. This is an indicator of the pronounced toxic effect of pesticides on the condition of the vessels, not only in the gills, but also in the liver, in the current study.

Similar to our results, Nagaraju & Rathnamma [61] found cytoplasmic degeneration, atrophy, necrosis, vacuolation, blood vessel rupture, and hepatocyte wall loss in hepatocytes exposed to novaluron. We support the idea of Babatunde et al. [62], who reported that increased vacuolization could be attributed to abnormal lipid accumulation in the liver, while liver cell necrosis could be due to the inability of fish to regenerate new liver cells as a result of continuous pesticide exposure.

In comparison with the hepatic alterations described for oxadiargyl and pendimethalin [63]—where fish exhibited marked necrosis, fibrosis, hemorrhage, bile duct hyperplasia, melanomacrophage proliferation, and pronounced inflammatory and fibrotic encapsulation—the lesions observed in the present study were limited mainly to degenerative and circulatory disturbances, with no evidence of fibrosis, hemorrhage, or strong inflammatory activation. Thus, the liver damage caused by the experimental pesticides appeared less advanced and did not progress towards the structural remodeling reported for oxadiargyl and pendimethalin.

In contrast to the findings in *Clarias gariepinus* exposed to 2,4-D-amine, which reported advanced hepatitis accompanied by marked macrophage and lymphocyte infiltration, the present results revealed only very mild inflammatory involvement, with degenerative lesions predominating. Thus, the inflammatory response in our case was far less pronounced and did not progress to the hepatitic condition described for 2,4-D-amine exposure [64].

Relative to the strong inflammatory reactions documented in fish exposed to PCBs, PAHs, polluted effluents, and cadmium, where liver tissue often shows clear infiltration by granulocytes and other immune cells [65-70], our present findings indicate only a subtle inflammatory presence. The hepatic alterations, which we observed here, were mainly degenerative, suggesting that the pesticides can elicit a far milder immune response than those reported for some other contaminants [71].

Similar to our results, after exposure to glyphosate, the liver of common carp had slightly vacuolated cells, showing evidence of fatty degeneration. Necrosis was observed in some parts of the liver tissues, probably as a result of the excessive work required by the fish to eliminate the toxic substance from their bodies during the detoxification process. The inability of the organ to regenerate

 doi:10.20944/preprints202512.0512.v1

new liver cells may also have led to necrosis [72]. Alterations, such as hepatocyte hypertrophy, necrosis, hemorrhage, and vacuolization, were found in the liver of *C. gariepinus* treated with diazinon. We agree with [73] that the presence of fatty vacuoles in the liver of the exposed fish is evidence of fatty degeneration. In our case, the necrosis that occurred in the fish liver may be related to the excessive work of detoxifying the pesticides from the body, similarly to the findings reported by [74]. In addition, liver histomorphology in common carp, but after exposure to two sublethal concentrations of glyphosate, showed hepatocytes with mild vacuolization, leukocyte infiltration, fatty degeneration, nuclear degeneration, and vasodilation. In a similar manner, the histopathological observations revealed that the changes in liver structure were time and concentration-dependent, i.e., the severity of the aberrations increased with increasing concentrations as well as with exposure time [75].

In comparison to the study on fipronil-treated common carp, which reported extensive bile duct disruption, hepatocyte necrosis, karyorrhexis, karyolysis, vascular abnormalities, and cytoplasmic shrinkage [76], the present study observed primarily degenerative and circulatory changes without bile duct damage or widespread necrosis. This indicates that the hepatic lesions caused by the pesticides that we tested were less severe and structurally disruptive than those induced by fipronil.

4.2. Histochemical Alterations in the liver of Common Carp After Acute Exposure to the Experimental Pesticides

Glycogen plays a central role as the main energy reserve for animals, and its levels in the liver can serve as an important indicator of fish health. Our opinion is in line with previous studies showing that the rate of glycogen synthesis is regulated by the relative activities of glycogen synthase (GSase) and glycogen phosphorylase (GPase), with GPase catalyzing the rate-limiting step of glycogenolysis and exerting control over glycogenesis [77]. Similar to earlier observations, we also agree that carbohydrates are typically the first energy source mobilized to meet the demands of various metabolic processes, particularly under toxic stress or when tissue glycogen is depleted. On the other hand, stress triggers the release of adrenal hormones via activation of the sympathetic nervous system, which leads to increased secretion of catecholamines, glucagon, and growth hormone, further stimulating gluconeogenesis and glycogenolysis [78-84]. We believe that in the initial phases of toxicity, carbohydrates are preferentially used to cope with stress, whereas in later stages, lipids and proteins become the primary energy sources. In our opinion, pesticide-induced stress results from alterations in the hormones of the Hypothalamic-Pituitary-Adrenal (HPA) Axis, autonomic nervous system activation, cytokine signaling, and immune-neuroendocrine interactions, all of which favor hyperglycemia.

Furthermore, oxidative stress caused by pollutants, such as pesticides, can damage organs, including the liver, leading to disturbances in carbohydrate, lipid, and protein metabolism. Mitochondrial damage may further impair the activity of respiratory chains and Krebs cycle enzymes, deplete adenosine triphosphate (ATP), and promote anaerobic metabolism over aerobic pyruvate oxidation [85]. Similar to the findings in other studies, we also agree that hyperglycemia is one of the most common metabolic signs of pesticide toxicity [86-89]. Chronic exposure to low levels of organophosphorus and organochlorine pesticides has been reported to exert deleterious effects on carbohydrate metabolism [90]. That is why we also recommend that, in addition to metabolic liver enzymes, such experimental setups also include analyses of antioxidant enzyme activities, reactive oxygen species, and lipid peroxidation.

Our observations are consistent with the notion that organs involved in carbohydrate, lipid, and protein metabolism, particularly the liver, can be affected by pesticides through alterations in glycolysis, gluconeogenesis, and glycogenolysis [91]. On the other hand, we think that pesticides may influence systemic metabolism by modulating neuronal stimulation and hormonal secretion, thereby affecting the energy balance of the entire organism. Interestingly, we also note that reduction of glycogen or lipids may occur directly as a result of intoxication or secondarily due to compromised body condition from stress, exhaustion, or co-morbidity. Contrary to what might be expected, pesticide exposure can sometimes lead to paradoxical accumulation of fat or glycogen in the liver [92]. The significant increase in glycogen content in the liver may be due to the increased energy

 doi:10.20944/preprints202512.0512.v1

requirement, as suggested by Tendulkar and Kulkarni [93]. On the other hand, the decrease in glycogen in various tissues may be due to stress, leading to the breakdown of tissue glycogen [94] to meet the energy demand caused by toxic stress due to pesticide exposure. As explained by Tendulkar and Kulkarni [93], the decrease in glycogen content indicates its greater use for metabolic purposes and stress management. We assume that the higher toxicity is the reason for the lower glycogen accumulation at a concentration of 60 µg/L pirimiphos-methyl, compared to the other pesticide concentrations used.

Our opinion is also in line with Ayoola [95], who considers that the changes associated with changes in the amount of glycogen (and lipids) in the liver of experimental individuals can generally be attributed to changes in glycolysis processes, which in turn depend on the applied concentrations of the toxicant, the duration of action, or its chemical nature. Moreover, our findings on glycogen accumulation in the liver of common carp are in line with previous reports showing pesticide-induced alterations in carbohydrate metabolism. Similar to the study on *Marcia opima*, where acute and chronic exposure to cypermethrin led to a significant increase in glycogen content in the hepatopancreas [93], we also observed enhanced glycogen deposition following exposure to pirimiphos-methyl, propamocarb hydrochloride, and 2,4-D. On the other hand, contrary to studies with fenitrothion and carbofuran, where hepatic glycogen levels decreased through activation of glycogen phosphorylase [96], the pesticides in our study induced accumulation rather than depletion of glycogen. Our results also resonate with observations from acephate exposure, where increased glycogen content was accompanied by upregulation of gluconeogenic enzymes, including glucose-6-phosphatase and tyrosine aminotransferase [97], suggesting that enhanced glycogen deposition may reflect an adaptive metabolic response to detoxification stress.

4.3. Biochemical Alterations in the Liver of Common Carp After Exposure to the Experimental Pesticides

Enzymes play an important role due to their specificity and rapid response to environmental changes. Their use as biomarkers of environmental status is based on a change in their catalytic activity induced by toxicants [98]. Studies on biochemical changes in fish allow the determination of the dose-response relationship, the threshold limit value, and the reversible and irreversible nature of the effect of the pollutant in the aquatic ecosystem. In addition, biochemical indicators of toxicity obtained after a relatively short exposure time can be useful in predicting the appropriate threshold concentration for the development of chronic effects [99]. Most pesticides exert their harmful effects by disrupting the redox system, directly related to the production of energy for cells [100].

ASAT is found in high concentrations in the liver of fish, but ALAT is considered to be an enzyme that is more specific for this organ [101]. According to El-Shehawi et al. [102], aminotransferases are widely used for the diagnosis of the damage that toxins cause to the liver in fish, as well as to other organs, such as gills and muscles. According to El-Shehawi et al. [103] in ecotoxicological studies, the increase or decrease in the levels of ALAT and ASAT in the blood and other studied tissues of fish can be used as a good indicator of water pollution. A number of authors have monitored the activity of aminotransferases in different organs of Cyprinid fish under the influence of various toxic compounds. Our results are in accordance with those of Rao [104], who reported that the decrease in the activity of aminotransferases in the liver of *O. mossambicus* under the influence of organophosphorus pesticides may be due to liver damage, which is also supported by the high degrees of degeneration in our study. In addition, Malarvizhi et al. [105] found a decrease in the activity of ASAT and ALAT in the liver of common carp upon exposure to carbamazepine. According to Tripathi & Shasmal [106], a decrease in the specific activity of metabolic enzymes upon exposure to organophosphorus and pyrethroid pesticides indicates the direct impact of these toxicants on the enzyme activity. We also agree with Abhijith et al. [107] that the detoxification mechanism may not be effective enough to prevent the toxicity and effect of the pollutant on the organism.

Cholinesterases (ChE) are a family of enzymes that catalyze the hydrolysis of acetylcholine (Ach) to choline and acetic acid, a fundamental process that allows the regeneration of cholinergic neurons. Cholinesterases are divided into two groups: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). AChE is involved in cholinergic neurotransmission by hydrolyzing acetylcholine. It is

 doi:10.20944/preprints202512.0512.v1

expressed in nerve and blood cells. BuChE is known as plasma cholinesterase or pseudocholinesterase [108]. ChE is also present in the liver and participates in the detoxification process. According to Payne et al. [109], cholinesterase inhibition has been used as an ecological biomarker to demonstrate the contamination of aquatic ecosystems with organophosphorus compounds. ChE activity in fish is strongly influenced not only by organophosphorus and carbamate pesticides, but also by various other classes, as we confirmed in our study. Although organochlorine, pyrethroid, and neonicotinoid insecticides have different target molecules [110], ChE inhibition by these compounds in fish has also been described [111-114]. BuChE is a less selective enzyme than AChE, i.e., it has a broader substrate specificity, hydrolyzing the synthetic substrate butyrylcholine as well as acetylcholine at a relatively low rate [115, 116]. BuChE is thought to act on cell adhesion and as a xenobiotic scavenger, potentially protecting AChE from anticholinesterase agents [117]. Following exposure to chlorpyrifos, the increasing inhibition of ChE over time is due to the irreversible binding of organophosphorus pesticides to ChE [118]. Similar time-dependent actions have been observed previously in fish [119]. This is confirmed in the results of the present study, with specific enzyme activity decreasing in direct proportion to increasing the concentration of the applied pesticide.

LDH, an important glycolytic enzyme that is contained in the cytoplasm of cells and is involved in maintaining the balance in carbohydrate metabolism. Any change in the metabolism of organisms as a result of toxic stress harms the activity of LDH [120]. LDH is involved in carbohydrate metabolism, but also serves as a significant indicator of environmental chemical pollution. The study of LDH activity can provide useful information about cellular metabolism in organisms that are exposed to stress factors [121]. As Yousafzai & Shakoori [122] pointed out, changes in LDH activity signal changes in cellular energy metabolism as a result of water pollution. For example, the altered LDH activity can serve as an enzymatic biomarker for the diagnosis of various disorders in tissues of many vertebrate organisms, which is often a result of oxygen depletion. According to Tripathi & Shasmal [106], a decrease in LDH activity in the liver of *Heteropneustes fossilis* exposed to chlorpyrifos may be due to the binding of the pesticides or their metabolites to the enzyme molecule. In the present study, an overall decrease in LDH activity was found. We believe, like the authors, that this is a result of chemically induced stress and a most likely increased amount of free radicals. Furthermore, this leads to intensive processes of gluconeogenesis and glycogen accumulation, which is also proven by the histochemical analysis using the PAS reaction method. Along with the processes of gluconeogenesis, an enhanced process of glycolysis probably also occurred, which in turn led to the accumulation of pyruvate, from there Acetyl CoA and as a result to hyperlipidemia. The stronger inhibition of the enzyme may also be associated with the higher degree of fatty degeneration established by the histopathological and histochemical analysis, which again shows intensive processes of glycolysis and hyperlipidemia. Similar to us, Abhijith et al. [107] found a decrease in LDH activity in the liver, as they examined the activity of the enzyme in the gills of *Catla catla* under exposure to methylparathion. The results showed a higher degree of glycolysis under pesticide stress. According to the same authors, pesticides can inhibit aerobic and anaerobic metabolism of fish, which leads to a decrease in LDH activity. It is likely that the pesticides used by us have similar mechanisms of action.

5. Conclusions

In summary, we can conclude that all three applied pesticides, the insecticide pirimiphos-methyl, the fungicide propamocalb hydrochloride, and the herbicide 2,4-D, each tested in two experimental concentrations, which were times lower than the LC_{50}, negatively affected the hepatic structure of common carp. Along with this, compensatory-adaptive mechanisms were activated in the body, which affects the functionality of the carp organs and inevitably leads to a deterioration in the health of the whole organism. There was a tendency towards increased morphological lesions and the degree of their expression in direct proportion to the increasing concentrations of the applied pesticides. Moreover, the results of the histochemical study showed that all tested concentrations of different classes of pesticides caused a change in the carbohydrate profile of the exposed species. The results of the present study further showed that key metabolic hepatic enzymes exhibited reduced

 doi:10.20944/preprints202512.0512.v1

specific activity, providing further evidence of the toxic effects of the tested pesticides on the liver of common carp. The identification of histopathological, histochemical, and biochemical biomarkers in common carp could be used to determine maximum permissible concentrations of pesticides in biota, as well as for the Water Framework Directive and ecological biomonitoring, applying an assessment model based on correlation dependencies between the tested biomarkers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; methodology, X.X.; software, X.X.; validation, X.X., Y.Y. and Z.Z.; formal analysis, X.X.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

Funding: This work was supported by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project № BG-RRP-2.004-0001-C01, DUECOS.

Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

Acknowledgments: Project no. TKP2021-NKTA-32 was implemented with the support provided by the National Research, Development and Innovation Fund of Hungary, financed under the TKP2021-NKTA funding scheme. The research presented in the article was carried out within the framework of the Széchenyi Plan Plus program with the support of the RRF 2.3.1-21-2022-00008 project. Krisztián Nyeste and László Antal were supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Sjerps, R.M.A., et al., *Occurrence of pesticides in Dutch drinking water sources.* Chemosphere, 2019. **235**: p. 510-518.
2. Syafrudin, M., et al., *Pesticides in Drinking Water-A Review.* Int J Environ Res Public Health, 2021. **18**(2).
3. Ahmad, M.F., et al., Pesticides impacts on human health and the environment with their mechanisms of action and possible countermeasures. Heliyon, 2024. **10**(7): p. e29128.
4. Gomez-Ramos, M.D.M., et al., Pesticide residues evaluation of organic crops. A critical appraisal. Food Chem X, 2020. **5**: p. 100079.
5. Bruhl, C.A., et al., Direct pesticide exposure of insects in nature conservation areas in Germany. Sci Rep, 2021. **11**(1): p. 24144.
6. Zaller, J.G., et al., Pesticides in ambient air, influenced by surrounding land use and weather, pose a potential threat to biodiversity and humans. Sci Total Environ, 2022. **838**(Pt 2): p. 156012.
7. Stehle, S. and R. Schulz, *Agricultural insecticides threaten surface waters at the global scale.* Proc Natl Acad Sci U S A, 2015. **112**(18): p. 5750-5.
8. Pérez-Parada, A., et al., *Recent advances and open questions around pesticide dynamics and effects on freshwater fishes.* Current Opinion in Environmental Science & Health, 2018. **4**: p. 38-44.
9. Sharma, A., et al., Worldwide pesticide usage and its impacts on ecosystem. Sn Applied Sciences, 2019. **1**(11).

 doi:10.20944/preprints202512.0512.v1

10. Rajan, S., M. Parween, and N.J. Raju, Pesticides in the hydrogeo-environment: a review of contaminant prevalence, source and mobilisation in India. Environmental Geochemistry and Health, 2023. **45**(8): p. 5481-5513.
11. Babuji, P., et al., Human Health Risks due to Exposure to Water Pollution: A Review. Water, 2023. **15**(14).
12. Abdel-Moneim, A.M., M.A. Al-Kahtani, and O.M. Elmenshawy, Histopathological biomarkers in gills and liver of Oreochromis niloticus from polluted wetland environments, Saudi Arabia. Chemosphere, 2012. **88**(8): p. 1028-35.
13. Souza-Bastos, L.R., et al., Evaluation of the water quality of the upper reaches of the main Southern Brazil river (Iguacu river) through in situ exposure of the native siluriform Rhamdia quelen in cages. Environ Pollut, 2017. **231**(Pt 2): p. 1245-1255.
14. Hossain, M.A., et al., Toxic effects of chlorpyrifos on the growth, hematology, and different organs histopathology of Nile tilapia, Oreochromis niloticus. Saudi J Biol Sci, 2022. **29**(7): p. 103316.
15. Rana, S., et al., Enhancement of body coloration of sword-tail fish (Xiphophorus helleri): Plant-derived bio-resources could be converted into a potential dietary carotenoid supplement. Heliyon, 2023. **9**(4): p. e15208.
16. Elenka Georgieva, E.K., Vesela Yancheva, Iliana Velcheva, Petar Hrischev, Pepa Atanassova, Stoil Tomov, Stela Stoyanova, *Pesticides induce fatty degeneration in liver of Cyprinus carpio (Linnaeus 1758) after acute exposure.* Ecologia Balkanika, 2023. **15**: p. 77-82.
17. Sachi, I.T.C., et al., Biochemical and morphological biomarker responses in the gills of a Neotropical fish exposed to a new flavonoid metal-insecticide. Ecotoxicol Environ Saf, 2021. **208**: p. 111459.
18. Stoyanova, S., et al., Kidneys Under Siege: Pesticides Impact Renal Health in the Freshwater Fish Common Carp (Cyprinus carpio Linnaeus, 1758). Toxics, 2025. **13**(7).
19. Georgieva, E., et al., Which Is More Toxic? Evaluation of the Short-Term Toxic Effects of Chlorpyrifos and Cypermethrin on Selected Biomarkers in Common Carp (Cyprinus carpio, Linnaeus 1758). Toxics, 2021. **9**(6).
20. Slaninova, A., et al., *A review: oxidative stress in fish induced by pesticides.* Neuro Endocrinol Lett, 2009. **30 Suppl 1**: p. 2-12.
21. Nakajima, T., et al., Common carp aquaculture in Neolithic China dates back 8,000 years. Nat Ecol Evol, 2019. **3**(10): p. 1415-1418.
22. Lingran Wang, C.L., Di Feng, Jintai Huang, Zhan Jin, Fangran Ma, Jiaxin Xu, Yuyue Xu, Meng Zhang, Miao Yu, Hongxia Jiang, Zhigang Qiao, Lei Wang, *Effect of exercise intensity on growth performance, serum biochemistry parameters, liver antioxidant capacity, and intestinal health of common carp (Cyprinus carpio) in recirculating aquaculture system.* Aquaculture, 2025. **595**: p. 741631.
23. Ahmad, H., et al., Pollution Problem in River Kabul: Accumulation Estimates of Heavy Metals in Native Fish Species. Biomed Res Int, 2015. **2015**: p. 537368.
24. Mhadhbi, L. and R. Beiras, Acute Toxicity of Seven Selected Pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the Marine Fish (Turbot,
25. *) (Retraction of vol 223, pg 5917, 2012).* Water Air and Soil Pollution, 2016. **227**(8).
26. Stepic, S., et al., Effects of individual and binary-combined commercial insecticides endosulfan, temephos, malathion and pirimiphos-methyl on biomarker responses in earthworm Eisenia andrei. Environ Toxicol Pharmacol, 2013. **36**(2): p. 715-723.
27. Oxborough, R.M., Trends in US President's Malaria Initiative-funded indoor residual spray coverage and insecticide choice in sub-Saharan Africa (2008-2015): urgent need for affordable, long-lasting insecticides. Malar J, 2016. **15**: p. 146.
28. Dengela, D., et al., Multi-country assessment of residual bio-efficacy of insecticides used for indoor residual spraying in malaria control on different surface types: results from program monitoring in 17 PMI/USAID-supported IRS countries. Parasit Vectors, 2018. **11**(1): p. 71.
29. Oakeshott, J.G., et al., Comparing the organophosphorus and carbamate insecticide resistance mutations in cholin- and carboxyl-esterases. Chem Biol Interact, 2005. **157-158**: p. 269-75.
30. Donarski, W.J., et al., Structure-activity relationships in the hydrolysis of substrates by the phosphotriesterase from Pseudomonas diminuta. Biochemistry, 1989. **28**(11): p. 4650-5.

 doi:10.20944/preprints202512.0512.v1

31. John, H., et al., *Toxicokinetic Aspects of Nerve Agents and Vesicants.* Handbook of Toxicology of Chemical Warfare Agents, 2nd Edition, 2015: p. 817-856.
32. E.A. Pieroh, W.K., C. Hemmen, *Propamocarb, einneues Fungizid zur Abwehr von Oomyceten im Zierpflanzen- und Gemüsebau.* Meded. Fac. Landbouw. Rijksuniv. Gent, 43, 933-942, 1978.
33. USEPA. 1995. U.S. Environmental Protection Agency. Propamocarb Hydrochloride R.E.D. FACTS - Prevention, Pesticides and Toxic Substances (7508W.
34. Elliott, M., S.F. Shamoun, and G. Sumampong, Effects of systemic and contact fungicides on life stages and symptom expression of
35. *and.* Crop Protection, 2015. **67**: p. 136-144.
36. Taylor, J.C., et al., Determination of residues of propamocarb in wine by liquid chromatography-electrospray mass spectrometry with direct injection. Food Addit Contam, 2004. **21**(6): p. 572-7.
37. Hiemstra, M. and A. de Kok, Determination of propamocarb in vegetables using polymer-based high-performance liquid chromatography coupled with electrospray mass spectrometry. J Chromatogr A, 2002. **972**(2): p. 231-9.
38. Abd-Alrahman, S.H. and M.M. Almaz, Degradation of propamocarb-hydrochloride in tomatoes, potatoes and cucumber using HPLC-DAD and QuEChERS methodology. Bull Environ Contam Toxicol, 2012. **89**(2): p. 302-5.
39. Schmuck, G. and F. Mihail, Effects of the carbamates fenoxycarb, propamocarb and propoxur on energy supply, glucose utilization and SH-groups in neurons. Arch Toxicol, 2004. **78**(6): p. 330-7.
40. Islam, F., et al., Potential impact of the herbicide 2,4-dichlorophenoxyacetic acid on human and ecosystems. Environ Int, 2018. **111**: p. 332-351.
41. Aquino, A.J.A., et al., Interaction of the 2.4-dichlorophenoxyacetic acid herbicide with soil organic matter moieties: a theoretical study. European Journal of Soil Science, 2007. **58**(4): p. 889-899.
42. Dehnert, G.K., et al., Effects of low, subchronic exposure of 2,4-Dichlorophenoxyacetic acid (2,4-D) and commercial 2,4-D formulations on early life stages of fathead minnows (Pimephales promelas). Environ Toxicol Chem, 2018. **37**(10): p. 2550-2559.
43. Jazan, E., T. Griffin, and M. Woodin, Mapping temporal trends of pesticide use and identifying potential non-occupation population exposure using a geospatial approach. Sci Rep, 2025. **15**(1): p. 23441.
44. Song, Y., Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. J Integr Plant Biol, 2014. **56**(2): p. 106-13.
45. von Stackelberg, K., A Systematic Review of Carcinogenic Outcomes and Potential Mechanisms from Exposure to 2,4-D and MCPA in the Environment. J Toxicol, 2013. **2013**: p. 371610.
46. APHA. 2005. Standard methods for examination of water and wastewater, 21st Ed. Washington, American Public Health Association.
47. Rosseland, B.O., et al., Brown Trout in Lochnagar: Population and Contamination by Metals and Organic Micropollutants, in Lochnagar: The Natural History of a Mountain Lake, N.L. Rose, Editor. 2007, Springer Netherlands: Dordrecht. p. 253-285.
48. The European Parliament and the Council of the European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Off. J. Eur. Union 2010, 276, 33–79. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063 (accessed on 18 June 2025).
49. Romeis, B., *Mikroskopische Technik; Walter de Gruyter: Berlin, Germany.* https://books.google.co.uk/books?id=NDOnDwAAQBAJ, 2015.
50. Bernet, D., et al., Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of Fish Diseases, 1999. **22**(1): p. 25-34.
51. Saraiva, A., et al., A histology-based fish health assessment of farmed seabass (
52. *L.).* Aquaculture, 2015. **448**: p. 375-381.
53. Zimmerli, S., et al., Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches. Aquatic Sciences, 2007. **69**(1): p. 11-25.
54. Mc, M.J., Histological and histochemical uses of periodic acid. Stain Technol, 1948. **23**(3): p. 99-108.

 doi:10.20944/preprints202512.0512.v1

55. Mishra, A.K. and B. Mohanty, Acute toxicity impacts of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the freshwater fish, Channa punctatus (Bloch). Environ Toxicol Pharmacol, 2008. **26**(2): p. 136-41.
56. Vassault, A., Lactate dehydrogenase, In: Methods of Enzymatic Analysis, Enzymes: Oxireductases, Transferases, Bergmeyer M.O. (Ed.). Academic Press, New York., 1983.
57. Henley, K.S. and H.M. Pollard, A new method for the determination of glutamic oxalacetic and glutamic pyruvic transaminase in plasma. J Lab Clin Med, 1955. **46**(5): p. 785-9.
58. Wroblewski, F. and J.S. Ladue, *Serum glutamic pyruvic transaminase in cardiac with hepatic disease.* Proc Soc Exp Biol Med, 1956. **91**(4): p. 569-71.
59. Reitman, S. and S. Frankel, A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol, 1957. **28**(1): p. 56-63.
60. C.A. Burtis, E.R.A., *Tietz Text-book of Clinical Chemistry. 2th Ed. W.B. .* Sunders Company, Philadelphia., 1994.
61. Thomas A. Minarik, J.A.V., Melissa M. Schultz, Stephen E. Bartell, Dalma Martinovic-Weigelt, Daniel C. Rearick, Heiko L. Schoenfuss, *On-Site Exposure to Treated Wastewater Effluent Has Subtle Effects on Male Fathead Minnows and Pronounced Effects on Carp.* JAWRA Journal of the American Water Resources Association, 2014.
62. Kovacheva, E., Georgieva, E., Velcheva, I., Nikolova, M., Atanassova, P., Todorova, B., Todorova-Bambaldokova, D., Yancheva, V., Stoyanova, S., & Tomov, S. , *Acute Histopathological Changes in Common carp (Cyprinus carpio Linnaeus, 1785) Gills: Pirimiphos-methyl, 2, 4 - Dichlorophenoxyacetic Acid and Propamocarb Hydrochloride Effects. .* Ecologia Balkanica, 2022.
63. Mohammod Mostakim, G., et al., Alteration of Blood Parameters and Histoarchitecture of Liver and Kidney of Silver Barb after Chronic Exposure to Quinalphos. J Toxicol, 2015. **2015**: p. 415984.
64. Nagaraju, B. and V.R. Vakita, HISTOPATHOLOGICAL CHANGES IN THE GILL AND LIVER OF FRESHWATER FISH LABEO ROHITA (HAMILTON) EXPOSED TO NOVALURON. 2014.
65. Babatunde, M., O. A.A, and A. Rafindadi, Histopathological Changes in the Gills, Livers and Brains of O. niloticus (TREWAVAS) Exposed to Paraquat in Chronic Bioassay. International Journal of Science and Technology, 2014. **4**: p. 101-110.
66. Emadi, H., et al., Evaluation of toxicity and biochemical effects of the Oxadiargyl in Common carp (Cyprinus carpio L.). International Journal of Aquatic Biology, 2018. **6**.
67. Ladipo, M., F. Doherty, and S. Oyebadejo, Acute Toxicity, Behavioural Changes and Histopathological Effect of Paraquat Dichloride on Tissues of Catfish (Clarias Gariepinus). International Journal of Biology, 2011. **3**.
68. Bucher, F. and R. Hofer, The effects of treated domestic sewage on three organs (gills, kidney, liver) of brown trout (Salmo trutta). Water Research, 1993. **27**(2): p. 255-261.
69. Adams, S.M., et al., *Downstream gradients in bioindicator responses: Point source contaminant effects on fish health.* Canadian Journal of Fisheries and Aquatic Sciences, 2011. **53**: p. 2177-2187.
70. Schwaiger, J., et al., *The use of histopathological indicators to evaluate contaminant-related stress in fish.* Journal of Aquatic Ecosystem Stress and Recovery, 1997. **6**: p. 75-86.
71. Bernet, D., et al., Evaluation of Two Monitoring Approaches to Assess Effects of Waste Water Disposal on Histological Alterations in Fish. Hydrobiologia, 2004. **524**: p. 53-66.
72. Agamy, E., Histopathological liver alterations in juvenile rabbit fish (Siganus canaliculatus) exposed to light Arabian crude oil, dispersed oil and dispersant. Ecotoxicol Environ Saf, 2012. **75**(1): p. 171-9.
73. Fu, D., et al., Using a multi-biomarker approach to assess the effects of pollution on sand flathead (Platycephalus bassensis) from Port Phillip Bay, Victoria, Australia. Mar Pollut Bull, 2017. **119**(1): p. 211-219.
74. Guardiola, F.A., et al., Accumulation, histopathology and immunotoxicological effects of waterborne cadmium on gilthead seabream (Sparus aurata). Fish Shellfish Immunol, 2013. **35**(3): p. 792-800.
75. Sigamani, D., Effect of herbicides on fish and histological evaluation. 2015: p. 437-440.
76. Lawrence Ikechukwu, E. and E. Ogbomida, *Histopathological Effects of Gammalin 20 on African Catfish (Clarias gariepinus).* Applied and Environmental Soil Science, 2010. **2010**.

 doi:10.20944/preprints202512.0512.v1

77. Al-Otaibi, A.M., et al., Toxicity bioassay and sub-lethal effects of diazinon on blood profile and histology of liver, gills and kidney of catfish, Clarias gariepinus. Braz J Biol, 2019. **79**(2): p. 326-336.
78. Bawa, V., et al., Biochemical and Histological Effects of Glyphosate on the Liver of Cyprinus carpio (Linn.). American Journal of Life Sciences, 2017. **5**: p. 71-80.
79. Ghaffar, A., et al., Fipronil (Phenylpyrazole) induces hemato-biochemical, histological and genetic damage at low doses in common carp, Cyprinus carpio (Linnaeus, 1758). Ecotoxicology, 2018. **27**(9): p. 1261-1271.
80. Connett, R. and K. Sahlin, Control of Glycolysis and Glycogen Metabolism. 2011.
81. Hart, B.B., et al., *Catecholamines: study of interspecies variation.* Crit Care Med, 1989. **17**(11): p. 1203-22.
82. Siegel, J.H., et al., Physiological and metabolic correlations in human sepsis. Invited commentary. Surgery, 1979. **86**(2): p. 163-93.
83. Chiasson, J.L., et al., Hormonal regulation of glycogen synthase phosphorylation in perfused rat skeletal muscle. FEBS Lett, 1981. **127**(1): p. 97-100.
84. Frayn, K.N., et al., The relationship of plasma catecholamines to acute metabolic and hormonal responses to injury in man. Circ Shock, 1985. **16**(3): p. 229-40.
85. Little, R.A., et al., Plasma catecholamine concentrations in acute states of stress and trauma. Arch Emerg Med, 1985. **2**(1): p. 46-7.
86. Hunt, D.G. and J.L. Ivy, *Epinephrine inhibits insulin-stimulated muscle glucose transport.* J Appl Physiol (1985), 2002. **93**(5): p. 1638-43.
87. Gustavson, S.M., et al., Glucagon's actions are modified by the combination of epinephrine and gluconeogenic precursor infusion. Am J Physiol Endocrinol Metab, 2003. **285**(3): p. E534-44.
88. Karami-Mohajeri, S. and M. Abdollahi, Toxic influence of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: a systematic review. Hum Exp Toxicol, 2011. **30**(9): p. 1119-40.
89. Matschinsky, F.M., et al., Quantitative histochemistry of glucose metabolism in the islets of Langerhans. Curr Probl Clin Biochem, 1971. **3**: p. 143-82.
90. Koundinya, P.R. and R. Ramamurthi, Effect of organophosphate pesticide Sumithion (Fenitrothion) on some aspects of carbohydrate metabolism in a freshwater fish, Sarotherodon (Tilapia) mossambicus (Peters). Experientia, 1979. **35**(12): p. 1632-3.
91. Clement, J.G. and M.J. Lee, Soman-induced convulsions: significance of changes in levels of blood electrolytes, gases, glucose, and insulin. Toxicol Appl Pharmacol, 1980. **55**(1): p. 203-4.
92. Deotare, S.T. and C.H. Chakrabarti, Effect of acephate (orthene) on tissue levels of thiamine, pyruvic acid, lactic acid, glycogen and blood sugar. Indian J Physiol Pharmacol, 1981. **25**(3): p. 259-64.
93. Garg, U.K., et al., Pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides on bone health of broiler chicks. Toxicol Pathol, 2004. **32**(3): p. 364-9.
94. Rahimi, R. and M. Abdollahi, *A review on mechanisms involved in hyperglycemia induced by organophosphorus insecticides.* Pesticide Biochemistry and Physiology - PESTIC BIOCHEM PHYSIOL, 2007. **88**: p. 115-121.
95. Wolf, J.C. and M.J. Wolfe, *A brief overview of nonneoplastic hepatic toxicity in fish.* Toxicol Pathol, 2005. **33**(1): p. 75-85.
96. Tendulkar, M. and A. Kulkarni, Cypermethrin-induced toxic effect on glycogen metabolism in estuarine clam, marcia opima (gmelin, 1791) of ratnagiri coast, maharashtra. J Toxicol, 2012. **2012**: p. 576804.
97. McLeay, D.J. and D.A. Brown, Effects of Acute Exposure to Bleached Kraft Pulpmill Effluent on Carbohydrate Metabolism of Juvenile Coho Salmon (Oncorhynchus kisutch) During Rest and Exercise. Journal of the Fisheries Research Board of Canada, 1975. **32**(6): p. 753-760.
98. Ayoola, S.O., Histopathological Effects of Glyphosate on Juvenile African Catfish (Clarias gariepinus). American-Eurasian J Agric Environ Sci, 2007. **4**.
99. Bretaud, S., et al., Biochemical and behavioral effects of carbofuran in goldfish (Carassius auratus). Environ Toxicol Chem, 2002. **21**(1): p. 175-81.
100. Joshi, A.K. and P.S. Rajini, Reversible hyperglycemia in rats following acute exposure to acephate, an organophosphorus insecticide: role of gluconeogenesis. Toxicology, 2009. **257**(1-2): p. 40-5.
101. Stoyanova, S., et al., Multi-Biomarker Assessment in Common Carp (Cyprinus carpio, Linnaeus 1758) Liver after Acute Chlorpyrifos Exposure. Water, 2020. **12**.

 doi:10.20944/preprints202512.0512.v1

102. Gill, T.S. and J.C. Pant, Effect of organomercurial poisoning on the peripheral blood and metabolite levels of a freshwater fish. Ecotoxicol Environ Saf, 1985. **10**(2): p. 150-8.
103. Rajak, P., et al., Protective Potential of Vitamin C and E against Organophosphate Toxicity: Current Status and Perspective. Journal of Ecophysiology and Occupational Health, 2022. **22**: p. 141-154.
104. Samsonova, M.V., T.I. Lapteva, and B. Filippovich Iu, *[Aminotransferases in early development of salmonid fish].* Ontogenez, 2005. **36**(2): p. 96-101.
105. Ahmed M. El-Shehawi, F.K.A.a.M.A.S., Estimation of water pollution by genetic biomarkers in tilapia and catfish species shows species-site interaction. African Journal of Biotechnology 2007. **6**(7): p. 840-846.
106. El-Shehawi, A., F. Abdel-Gawad, and M. Seehy, *Estimation of water pollution by genetic biomarkers in tilapia and catfish species shows species-site interaction.* African Journal of Biotechnology (ISSN: 1684-5315) Vol 6 Num 7, 2007. **6**.
107. Venkateswara Rao, J., Sublethal effects of an organophosphorus insecticide (RPR-II) on biochemical parameters of tilapia, Oreochromis mossambicus. Comp Biochem Physiol C Toxicol Pharmacol, 2006. **143**(4): p. 492-8.
108. Malarvizhi, A., et al., Carbamazepine (CBZ) induced enzymatic stress in gill, liver and muscle of a common carp, Cyprinus carpio. Journal of King Saud University - Science, 2012. **24**(2): p. 179-186.
109. Tripathi, G. and J. Shasmal, Concentration related responses of chlorpyriphos in antioxidant, anaerobic and protein synthesizing machinery of the freshwater fish, Heteropneustes fossilis. Pesticide Biochemistry and Physiology - PESTIC BIOCHEM PHYSIOL, 2011. **99**: p. 215-220.
110. Abhijith, B., M. Ramesh, and P. Rk, Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of Catla catla during methyl parathion exposure. The Journal of Basic & Applied Zoology, 2016. **77**: p. 31-40.
111. Pohanka, M., *Cholinesterases, a target of pharmacology and toxicology.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2011. **155**(3): p. 219-29.
112. Payne, J.F., et al., Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Marine Pollution Bulletin, 1996. **32**(2): p. 225-231.
113. Casida, J.E. and K.A. Durkin, *Anticholinesterase insecticide retrospective.* Chem Biol Interact, 2013. **203**(1): p. 221-5.
114. Bonansea, R.I., D.A. Wunderlin, and M.V. Ame, Behavioral swimming effects and acetylcholinesterase activity changes in Jenynsia multidentata exposed to chlorpyrifos and cypermethrin individually and in mixtures. Ecotoxicol Environ Saf, 2016. **129**: p. 311-9.
115. Da Cuna, R.H., et al., Assessment of the acute toxicity of the organochlorine pesticide endosulfan in Cichlasoma dimerus (Teleostei, Perciformes). Ecotoxicol Environ Saf, 2011. **74**(4): p. 1065-73.
116. Tian, X., et al., Chronic brain toxicity response of juvenile Chinese rare minnows (Gobiocypris rarus) to the neonicotinoid insecticides imidacloprid and nitenpyram. Chemosphere, 2018. **210**: p. 1006-1012.
117. Vieira, C.E.D., et al., DNA damage and oxidative stress induced by imidacloprid exposure in different tissues of the Neotropical fish Prochilodus lineatus. Chemosphere, 2018. **195**: p. 125-134.
118. Massoulie, J., et al., *Molecular and cellular biology of cholinesterases.* Prog Neurobiol, 1993. **41**(1): p. 31-91.
119. Pope, C.N. and S. Brimijoin, *Cholinesterases and the fine line between poison and remedy.* Biochem Pharmacol, 2018. **153**: p. 205-216.
120. Albendin, G., et al., Characterization and in vitro sensitivity of cholinesterases of gilthead seabream (Sparus aurata) to organophosphate pesticides. Fish Physiol Biochem, 2017. **43**(2): p. 455-464.
121. Fulton, M.H. and P.B. Key, Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem, 2001. **20**(1): p. 37-45.
122. Boone, J.S. and J.E. Chambers, Time course of inhibition of cholinesterase and aliesterase activities, and nonprotein sulfhydryl levels following exposure to organophosphorus insecticides in mosquitofish (Gambusia affinis). Fundam Appl Toxicol, 1996. **29**(2): p. 202-7.
123. Mommsen, T.P., *Glucagon-like Peptide-1 in Fishes: The Liver and Beyond1.* American Zoologist, 2015. **40**(2): p. 259-268.

 doi:10.20944/preprints202512.0512.v1

124. Monteiro, D.A., et al., Oxidative stress biomarkers in the freshwater characid fish, Brycon cephalus, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). Comp Biochem Physiol C Toxicol Pharmacol, 2006. **143**(2): p. 141-9.
125. Yousafzai, A. and A. Shakoori, *Hepatic Responses of A Freshwater Fish Against Aquatic Pollution**. Pakistan journal of zoology, 2011. **43**: p. 209-221.