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# „ASSESSMENT OF POLLUTION OF AQUATIC ECOSYSTEMS WITH PRIORITY ORGANIC POLLUTANTS USING MORPHOFUNCTIONAL BIOMARKERS IN *CYPRINUS CARPIO* (LINNAEUS 1785)"

# ABSTRACT

of a dissertation
for the acquisition of an educational and scientific degree "PhD"

Field: 4. **Natural sciences, mathematics and informatics**
Professional direction: **4.3. Biological Sciences**
Doctoral program: **Morphology**

**Supervisor:**
Prof. Elenka Stoilova Georgieva, PhD

**Plovdiv, 2024**

The dissertation contains 183 pages, 20 tables and 34 figures. 519 sources are cited, 10 of which are in Cyrillic.

The experimental work on the dissertation was conducted in the vivarium of the Faculty of Biology, the scientific laboratory of Embryology and Histology at the Department of Developmental Biology and the Biochemistry laboratory at the Department of Biochemistry and Microbiology of Plovdiv University “Paisiy Hilendarski”.

The dissertation was discussed and proposed for defense at a meeting of the Department of Developmental Biology, Faculty of Biology, Plovdiv University ”Paisiy Hilendarski”, held on February 12, 2024 (Protocol No. 388/February 12, 2024).

The defense of the dissertation will take place on 17.05.2024, 11 h. at the Faculty of Biology, Plovdiv University "Paisiy Hilendarski", Str. "Todor Samodumov" №2 at an open meeting of the scientific jury.

The defense materials are available in the Dean's Office of the Faculty of Biology, Plovdiv University "Paisiy Hilendarski", Str. “Todor Samodumov” №2.

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**Title:**
Assessment of pollution of aquatic ecosystems with priority organic pollutants using morphofunctional biomarkers in *Cyprinus carpio* (Linnaeus, 1785)

# INTRODUCTION

The global use of pesticides, associated with an increasing risk of exposure to all organisms and the environment, has increased manifold in recent decades (Tang et al., 2021; Zaller, 2020). Modern agriculture is highly dependent on the use of pesticides, primarily for crop protection and yield enhancement (Sharma et al., 2019; Sjerps et al., 2019; Rizzo et al., 2021; Syafrudin et al., 2021). The benefit of their application is undeniable, but on the other hand, there is clear evidence that the widespread importation of pesticide substances causes irreversible damage to ecosystems and their inhabitants, including humans (Bogoeva, 2014). Pesticide use causes biodiversity loss and has a significant impact on aquatic ecosystems (Pérez-Parada et al., 2018; Sharma et al., 2019; de Souza et al., 2020). Any pollution of water bodies also affects the organisms living in them (Agrawal et al., 2010). In ecotoxicological studies, biomarkers are useful tools used in monitoring aquatic ecosystems. Incorporating complex biomarkers at different levels of biological organization is a suitable approach to detect pollutant-induced responses (Souza-Bastos et al., 2017). In aquatic ecosystems, one of the commonly used organs for investigating complex biomarkers for ecotoxicological assessment are fish gills. They are sensitive to chemicals pollution of the aquatic environment due to their large surface area coming into contact with it. This makes them efficient tools for biomonitoring the potential effects of various toxicants. Another commonly used target organ is the liver of hydrobionts, as exposure to pesticides can cause histological changes in the liver, which are important biomarkers for pollution assessment of aquatic ecosystems (Yön et al., 2014). The fish kidney is also a commonly used organ in ecotoxicological studies. The function of the kidney and the established changes in its histostructure under the influence of toxicants found in the water are associated with a violation in the maintenance of homeostasis (Iqbal et al., 2004). On this basis, conducting research related to the establishment of morphological and biochemical changes at a cellular and tissue level can show the state of the studied organ as a result of the action of a certain environmental pollutant, and this can affect the health of the whole organism (Sachi et al., 2021). Current regulations governing the use of pesticides are still focused on ecological risk assessments for individual active ingredients. An important step is the construction of a conceptual model of the composition of the plant protection products (PPPs) used, their fate in the aquatic environment and the potential for toxicity to non-target organisms (Scholz et al., 2012). In the current dissertation, pesticides from different groups - insecticides, herbicides and fungicides - are used, which are part of widely applicable in agricultural practice PPP. Considering the topicality of the problem, research in this area is important, both for aquatic ecosystems and for improving legislation nationally and globally.

## I. AIM AND TASKS

The aim of the present study is to assess the contamination of aquatic ecosystems with priority organic pollutants by using morphofunctional biomarkers in *Cyprinus carpio* (Linnaeus 1785).

To achieve the aim of the present study, the **following tasks** were set:

**1. Conducting a short-term 96-hour toxicity test with the pesticides pirimiphos-methyl, propamocarb hydrochloride and 2,4-dichlorophenoxyacetic acid (2,4-D), including:**

**1.1.** Adaptation of test groups of carp (*Cyprinus carpio* L.).

**1.2.** Conducting an acute 96-hour exposure with applied different concentrations of the pesticides.

**1.3.** Collection and processing of samples from the gills, liver and kidney of the experimental groups of carp (*Cyprinus carpio* L.) for histological, histochemical and biochemical examination.

**1.4.** Histopathological analysis of gills, liver and kidney of common carp (*Cyprinus carpio* L.) under the influence of the applied pesticide concentrations.

**1.5.** Histochemical analysis of liver of common carp (*Cyprinus carpio* L.) under the influence of the applied pesticide concentrations.

**1.6.** Biochemical analysis of liver of common carp (*Cyprinus carpio* L.) under the influence of the applied pesticide concentrations.

**1.7.** Comparative analysis of the changes that occurred under the action of the tested pesticides.

**2. Evaluation of the histopathological, histochemical and biochemical changes in the target organs of common carp (*Cyprinus carpio* L.) under the influence of the applied pesticides, and their application as biomarkers of contamination of aquatic ecosystems with pesticides.**

## II. MATERIALS AND METHODS

### 1. Materials

### 1.1. Characteristics of common carp (*Cyprinus carpio* L.) and the applied pesticides

The object of the present study is common carp (*Cyprinus carpio* L.), which has a high commercial value worldwide and is widely used as a bioindicator of toxicity in aquatic environments (Ahmad et al., 2015). It inhabits freshwater environments, especially lakes and rivers, rarely salt water (Barus et al., 2001).

The PPPs used are Actelik 50 EK, Rival and Aminopielik, respectively with the active ingredients pirimiphos-methyl, propamocarb hydrochloride and 2,4-D. *Pirimiphos-methyl* is an organophosphorus insecticide that is responsible for the phosphorylation of acetylcholinesterase (AChE), regulating the hydrolysis of acetylcholine in the synaptic cleft of the insect nervous system (Donarski et al., 1989; Eleršek & Filipic, 2011; Khan, 2021). *Propamocarb hydrochloride* was first introduced to control oomycete pathogens in ornamental crops and some vegetables (Pieroh et al., 1978), used to control *Pythium spp*. and *Phytophthora spp*. (USEPA,

1995) and belongs to the carbamate family of pesticides (Wang et al., 2016; Li et al., 2020). 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin herbicide that, due to its effectiveness, selectivity, low cost and broad spectrum in pest control, has become one of the most frequently used herbicides in agricultural and urban areas worldwide (Dehnert et al., 2018).

## 2. Methods

### 2.1. Experimental set

The fish used in the present experiment were supplied by the Institute of Fisheries and Aquaculture, Plovdiv. The experimental individuals were of the same size-age group, without external pathological changes. For the experiment, 7 aquariums (100 L.) were used, with n=10 for each experimental concentration and for the control group, which was no added toxicant. Two concentrations were used for each pesticide: pirimiphos-methyl – 10 μg/L and 60 μg/L, propamocarb hydrochloride – 40 μg/L and 80 μg/L and 2,4-D – 50 μg/L and 100 μg/L L. The duration of each of the experiments is 96 hours. Water parameters ($t^oC$, pH, dissolved oxygen, electrical conductivity) were recorded every day of the experiment (APHA, 2005).

### 2.2. Histopathological analysis of gills, liver and kidney of *Cyprinus carpio* L.

Fish dissection was performed according to Rosseland et al. (2003), respecting the animal welfare requirements of Directive 2010/63/EU. The histological processing of the samples was carried out according to the standard methodology of Romeis (1989) in the scientific laboratory of "Histology and Embryology" at the Department of "Developmental Biology", Faculty of Biology. The assessment of histopathological changes in gills, liver and kidney was performed according to the scale of Bernet et al. (1999) for the groups of alterations and modified by Saraiva et al. (2015). According to Bernet et al. (1999) is also defined a Significance Factor (W), which has a constant value and is categorized into three grades. According to Saraiva et al. (2015), a 5-point scale was used to determine the severity of each change in the histological structure of the organ, as follows: (0) – no changes (up to 10%); (1) – very mild degree of change (from 10% to 20%); (2) – mild degree of change (from 20% to 30%); (3) – moderate degree of change (from 30% to 50%); (4) – severe degree of changes (from 50% to 80%); (5) – very severe degree of alteration (over 80%).

The indices of the established histopathological changes for each group of changes are the product of the degree of the specific disorder in the group and W. The sum of these final values determines the Index (I) for the corresponding group of changes. The sum of all indices determines the Organ Index ($I_O$) for pathological change. $I_O$ refers to a class, according to Zimmerli et al. (2007) 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 histological structure change (reversible); Class IV (Index 31–40)–severe degree of histological structure change (irreversible); Class V (Index > 40) – very severe degree of histological structure change (irreversible).

### 2.3. Histochemical analysis of liver of *Cyprinus carpio* L.

The histochemical examination was carried out in the scientific laboratory of "Histology and Embryology" at the Faculty of Biology of the PU "Paisii Hilendarski". Cryostat sections of excised livers were stained to demonstrate lipids with Sudan Black B (Culling, 1974; Sheehan & Hrapchak, 1980) and PAS-reaction (McManus, 1948) to demonstrate glycogen, as described by Pearse (1972). Histochemical changes in the liver of the experimental subjects were presented according to a semi-quantitative scale described by Mishra & Mohanty (2008).

### 2.4. Biochemical analysis of liver of *Cyprinus carpio* L.

The biochemical study of the liver enzymes lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), cholinesterase (ChE) and catalase (CAT) was carried out at the Technological Center of Plovdiv University. Measurement of LDH activity was performed according to the method of Vassault (1983). ALAT and ASAT activity was measured according to a method developed in parallel by Henley & Pollard (1955) and Wroblewski & Ladue (1956), modified by Reitman & Frankel (1957). ChE activity was measured according to Burtis-Ashwood (1994) and CAT according to Beers & Sizer (1952). The amount of total protein was determined according to the method of Bradford (1976) by measuring absorbance at 595 nm. The activity of the studied enzymes is presented as specific enzyme activity (U/mg protein).

### 2.5. Statistical data processing

It was performed using GraphPad Prism v.9.2.0. Differences between concentrations and control were tested for significance by Student's t-test for independent samples, at significance level ($p<0.05$), with normally distributed data.

## III. RESULTS AND DISCUSSION

### 1. Histopathological alterations in carp gills after acute exposure with the tested pesticides

### 1.1. Control group

The results show normal morphology of the gill histological structure in the control group (**Table 1**, **Figure 1)**.

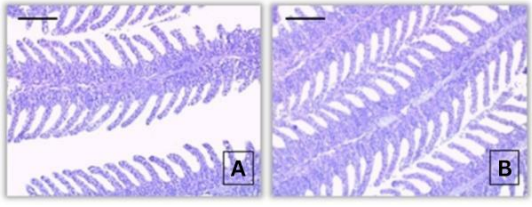


**Figure 1. Normal morphology of the gill histological structure in the control group, x200, H&E**

## 1.2. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute exposure with pirimiphos-methyl

**Table 1. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with pirimiphos-methyl**

| Reaction pattern | Functional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | Pirimiphos-methyl | |
| | | | | | 10 µg/L | 60 µg/L |
| **Changes in the circulatory system** | *Filament* | Vasodilation | $W_{GC1} = 1$ | 0 | 2* | 2* |
| | *Seconary lamellae* | Vasodilation | $W_{GC2} = 2$ | 0 | 3* | 4* |
| | *Secondary lamellae* | Aneurysm | $W_{GC3} = 2$ | 0 | 0 | 2* |
| ***Index for changes in the circulatory system*** | | | | **$I_{GC}$ =0** | **$I_{GC}$=8** | **$I_{GC}$=14** |
| **Degenerative changes** | *Gill epithelium (filament)* | Necrosis | $W_{GR1} = 3$ | 0 | 0 | 1 |
| | *Gill epithelium (secondary lamellae)* | Necrosis | $W_{GR2} = 3$ | 0 | 1 | 1 |
| ***Index for degenerative changes*** | | | | **$I_{GR}$ = 0** | **$I_{GR}$= 3** | **$I_{GR}$=6** |
| **Proliferative changes** | *Gill epithelium (filament)* | Edema | $W_{GP1} = 1$ | 0 | 1 | 1 |
| | | Proliferation of stratified epithelium | $W_{GP2} = 2$ | 0 | 3* | 2* |
| | | Proliferation of glandular cells | $W_{GP3} = 1$ | 0 | 1 | 0 |
| | | Fusion | $W_{GP4} = 3$ | 0 | 3* | 3* |
| | *Gill epithelium (secondary lamellae)* | Lamellar lifting | $W_{GP5} = 1$ | 0 | 1 | 2* |
| | | Proliferation of stratified epithelium | $W_{GP6} = 2$ | 0 | 2* | 2* |
| ***Index for proliferative changes*** | | | | **$I_{GP}$ = 0** | **$I_{GP}$ =22** | **$I_{GP}$=20** |
| ***Index for organ $I_G$*** | | | | **$I_G$ = 0** | **$I_G$=33** | **$I_G$=40** |

* p<0.05

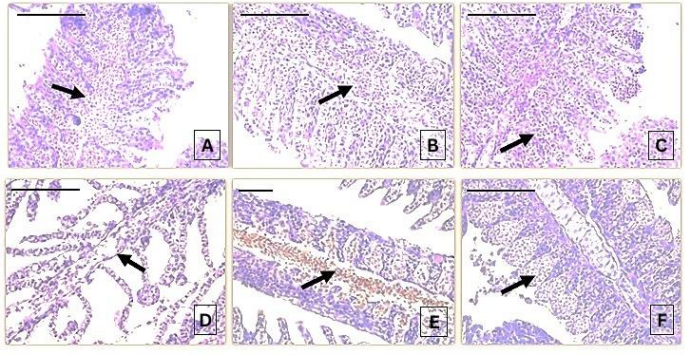


**Figure 2. Histopathological alterations in gills of *Cyprinus carpio* L. after acute (96 h) exposure with pirimiphos-methyl (H&E): A, B** – vasodilatation in the filament (10 µg/L), x 400; **C** – fusion of the secondary lamellae (10 µg/L), x 400; **D** – degenerative changes (necrosis) (60 µg/L), x 400; **E** – vasodilatation in the filament (60 µg/L), x 200; **F** – aneurysms of the secondary lamellae (60 µg/L), x 400.

## 1.3. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute exposure with propamocarb hydrochloride

**Table 2. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with propamocarb hydrochloride**

| Reaction pattern | Funtional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | Propamocarb hydrochloride | |
| | | | | | 40 µg/L | 80 µg/L |
| **Changes in the circulatory system** | *Filament* | Vasodilation | $W_{GC1} = 1$ | 0 | 2* | 2* |
| | *Secondary lamellae* | Vasodilation | $W_{GC2} = 2$ | 0 | 2* | 2* |
| | *Secondary lamellae* | Aneurysms | $W_{GC3} = 2$ | 0 | 1 | 0 |
| ***Index for changes in the circulatory changes*** | | | | **$I_{GC}$=0** | **$I_{GC}$=8** | **$I_{GC}$= 6** |
| **Degenerative changes** | *Gill epithelium (filament)* | Necrosis | $W_{GR1} = 3$ | 0 | 0 | 0 |
| | *Gill epithelium (secondary lamellae)* | Necrosis | $W_{GR2} = 3$ | 0 | 0 | 0 |
| ***Index for degenerative changes*** | | | | **$I_{GR}$=0** | **$I_{GR}$=0** | **$I_{GR}$=0** |
| **Proliferative changes** | *Gill epithelium (filament)* | Edema | $W_{GP1} = 1$ | 0 | 3* | 2* |
| | | Proliferation of stratified epithelium | $W_{GP2} = 2$ | 0 | 5* | 4* |
| | | Proliferation of glandular cells | $W_{GP3} = 1$ | 0 | 1 | 0 |
| | | Fusion | $W_{GP4} = 3$ | 0 | 4* | 4* |
| | *Gill epithelium (secondary lamellae)* | Lamellar lifting | $W_{GP5} = 1$ | 0 | 2* | 2* |
| | | Proliferation of stratified epithelium | $W_{GP6} = 2$ | 0 | 4* | 3* |
| ***Index for proliferative changes*** | | | | **$I_{GP}$=0** | **$I_{GP}$=36** | **$I_{GP}$=30** |
| ***Index for organ $I_G$*** | | | | **$I_G$=0** | **$I_G$=44** | **$I_G$=36** |

* $p<0.05$

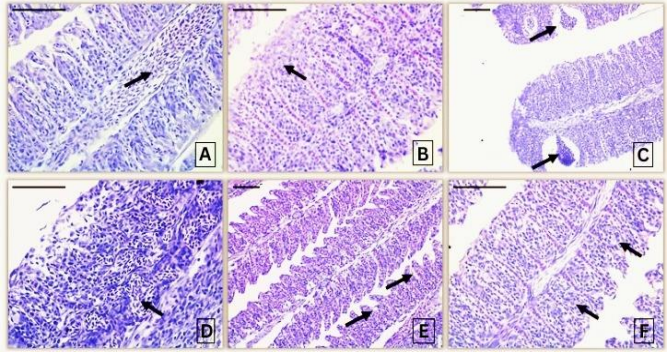


**Figure 3. Histopathological alterations in gills of *Cyprinus carpio* L. after acute (96 h) exposure with propamocarb hydrochloride (H&E): A** – vasodilatation in the filament (40 µg/L), x400; **B** – fusion of the secondary lamellae (40 µg/L), x 400; **C** – aneurysms of the secondary lamellae (40 µg/L),x200; **D** – vasodilatation in the secondary lamellae (80 µg/L),x400; **E** – lamellar lifting (80 µg/L), x 200; **F** – proliferation of stratified epithelium in the filament (80 µg/L), x 400.

## 1.4. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute exposure with 2,4-D

**Table 3. Histopathological alterations in gills of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with 2,4-D**

| Reaction pattern | Functional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | 2, 4 - D | |
| | | | | | 50 µg/L | 100 µg/L |
| **Changes in the circulatory system** | *Filament* | Vasodilation | $W_{GC1} = 1$ | 0 | 2* | 3* |
| | *Secondary lamellae* | Vasodilation | $W_{GC2} = 2$ | 0 | 2* | 3* |
| | *Secondary lamellae* | Aneurysms | $W_{GC3} = 2$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{GC}$ =0** | **$I_{GC}$=6** | **$I_{GC}$=9** |
| **Degenerative changes** | *Gill epithelium (filament)* | Некроза | $W_{GR1} = 3$ | 0 | 1 | 0 |
| | *Gill epithelium (secondary lamellae)* | Некроза | $W_{GR2} = 3$ | 0 | 0 | 1 |
| ***Index for degenerative changes*** | | | | **$I_{GR}$ =0** | **$I_{GR}$=3** | **$I_{GR}$=3** |
| **Proliferative changes** | *Gill epithelium (filament)* | Edema | $W_{GP1} = 1$ | 0 | 2* | 2* |
| | | Proliferation of stratified epithelium | $W_{GP2} = 2$ | 0 | 3* | 4* |
| | | Proliferation of glandular cells | $W_{GP3} = 1$ | 0 | 1 | 0 |
| | | Fusion | $W_{GP4} = 3$ | 0 | 3* | 3* |
| | *Gill epithelium (secondary lamellae)* | Lamellar lifting | $W_{GP5} = 1$ | 0 | 2* | 3* |
| | | Proliferation of stratified epithelium | $W_{GP6} = 2$ | 0 | 2* | 2* |
| ***Index for proliferative changes*** | | | | **$I_{GP}$=0** | **$I_{GP}$=24** | **$I_{GP}$=26** |
| ***Index for organ $I_G$*** | | | | **$I_G$=0** | **$I_G$=33** | **$I_G$=38** |

* $p<0.05$

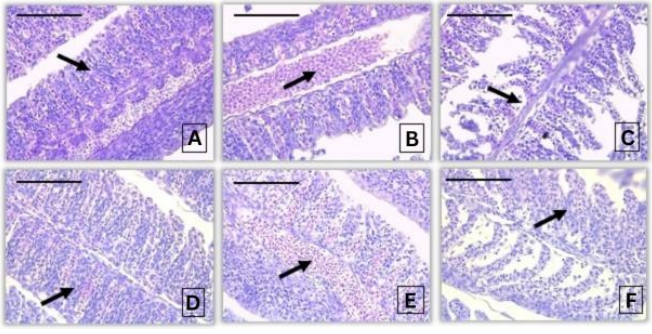


**Figure 4. Histopathological alterations in gills of *Cyprinus carpio* L. after acute (96 h) exposure with 2,4-D (H&E), x400**: **A** – proliferation of stratified epithelium in the filament (50 µg/L); **B** – vasodilatation in the filament (50 µg/L); **C** – degenerative changes (50 µg/L) 2; **D** – fusion of the secondary lamellae (100 µg/L); **E** – vasodilatation in the filament (100 µg/L); **F** – proliferation of stratified epithelium (100 µg/L).

Based on the obtained results and the classification scheme, the gill index ($I_G$) for pirimiphos-methyl at a concentration of 10 µg/L is 33, and at 60 µg/L it is 40. The values fall into class IV - a severe degree of irreversible change (**Table 1, Figure 2**). For propamocarb hydrochloride, the $I_G$ value is 44 (at 40 µg/L) and falls into class V - very severe histopathological irreversible changes in gill tissue. At 80 µg/L, the $I_G$ is 36 and falls into class IV - a severe degree of histopathological changes, also of an irreversible nature (**Table 2, Figure 3**). After exposure to 2,4-D, the calculated $I_G$ is 33 at 50 µg/L of applied herbicide and 38 at 100 µg/L. Both concentrations fall into class IV, indicating the presence of severe irreversible histopathological changes (**Table 3, Figure 4**).

Our results are similar to those reported by Xing et al. (2012), Al-Mamoori et al. (2014), Devi & Mishra (2013), Kunjamma et al. (2008), Makinde et al. (2015), Vigario & Saboia-Morais (2014), Ortiz et al. (2003), Rocha et al. (2015) and others. In contrast to the authors, the present study established the degree of histological changes and the index of pathological changes in the organ. This allows, based on the obtained results, to determine the degree of the negative impact of the toxicant and to make an objective comparative assessment.

Based on the comparative analysis, the degree of toxicity of the experimental pesticides can be summarized in the following descending order (**Figure 5**):

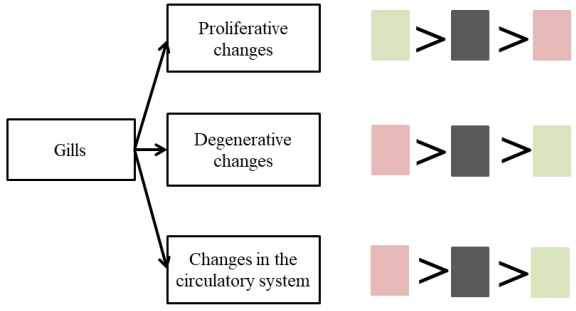


**Figure 5. Comparative analysis of the toxic effects of the used pesticides on the histological structure of common carp gills (*Cyprinus carpio* L.)**

## 2. Histopathological alterations in carp liver after acute exposure with the tested pesticides

### 2.1. Control group

The results show normal morphology of the liver histological structure in the control group (**Table 4** and **Figure 6)**.

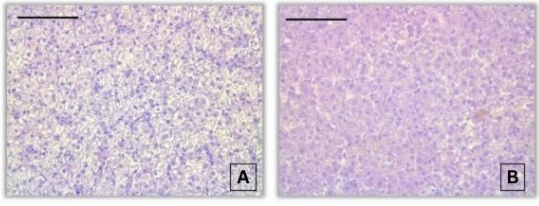


**Figure 6. Normal morphology of the liver histological structure in the control group, x400, H&E**

## 2.2. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute exposure with pirimiphos-methyl

**Table 4. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with pirimiphos-methyl**

| Reaction pattern | Functional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | Pirimiphos-methyl | |
| | | | | | 10 µg/L | 60 µg/L |
| **Changes in the circulatory system** | *Liver* | Hyperaemia | $W_{LC1} = 1$ | 0 | 3* | 3* |
| | | Intracellular edema | $W_{LC2} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{LC} = 0$** | **$I_{LC} = 3$** | **$I_{LC}$=3** |
| **Degenerative changes** | *Liver* | Granular degeneration | $W_{LR1} = 1$ | 0 | 5* | 4* |
| | | Vacuolar degeneration | $W_{LR2} = 2$ | 0 | 4* | 4* |
| | | Necrobiosis | $W_{LR3} = 2$ | 0 | 1 | 1 |
| | | Necrosis | $W_{LR4} = 3$ | 0 | 1 | 2* |
| | | Fatty degeneration | $W_{LR2} = 1$ | 0 | 2* | 3* |
| ***Index for degenerative changes*** | | | | **$I_{LR}$=0** | **$I_{LR}$=20** | **$I_{LR}$=23** |
| **Proliferative changes** | *Liver* | Hypertrophy | $W_{LP1} = 1$ | 0 | 0 | 0 |
| ***Index for proliferative changes*** | | | | **$I_{LP}$=0** | **$I_{LP}$=0** | **$I_{LP}$=0** |
| **Inflammation** | *Liver* | Activation of RES | $W_{LI1} = 1$ | 0 | 0 | 0 |
| | | Lymphocyte infiltration | $W_{LI2} = 2$ | 0 | 0 | 0 |
| ***Index for inflammatory processes*** | | | | **$I_{LI}$=0** | **$I_{LI}$=0** | **$I_{LI}$=0** |
| ***Index for organ $I_L$*** | | | | **$I_L$=0** | **$I_L$=23** | **$I_L$=26** |

* $p<0.05$

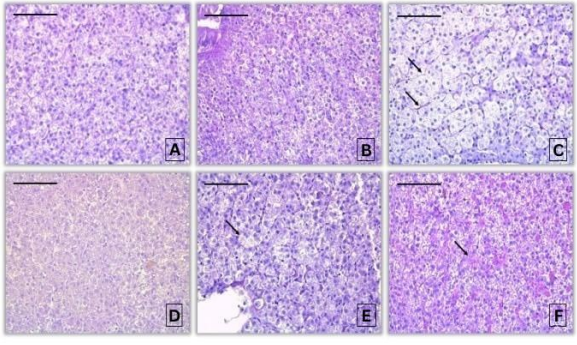


**Figure 7. Histopathological alterations in liver of *Cyprinus carpio* L. after acute (96 h) exposure with pirimiphos-methyl (H&E), x400: A, B** – granular degeneration (10 µg/L); **C** – vacuolar degeneration (10 µg/L); **D** – granular degeneration (60 µg/L); **E** – karyolysis (60 µg/L); **F** – hyperaemia (60 µg/L)

## 2.3. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute exposure with propamocarb hydrochloride

**Table 5. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with propamocarb hydrochloride**

| Reaction pattern | Functional unit of the tissue | 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 | 4* | 4* |
| | | Intracellular edema | $W_{LC2} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{LC} = 0$** | **$I_{LC}$=4** | **$I_{LC}$=4** |
| **Degenerative changes** | *Liver* | Granular degeneration | $W_{LR1} = 1$ | 0 | 5* | 5* |
| | | Vacuolar degeneration | $W_{LR2} = 2$ | 0 | 4* | 5* |
| | | Necrobiosis | $W_{LR3} = 2$ | 0 | 1 | 1 |
| | | Necrosis | $W_{LR4} = 3$ | 0 | 2* | 2* |
| | | Fatty degenerarion | $W_{LR2} = 1$ | 0 | 2* | 4* |
| ***Index fordegenerative changes*** | | | | **$I_{LR}$=0** | **$I_{LR}$=23** | **$I_{LR}$=27** |
| **Proliferative changes** | *Liver* | Hypertrophy | $W_{LP1} = 1$ | 0 | 0 | 0 |
| ***Index for proliferative changes*** | | | | **$I_{LP}$=0** | **$I_{LP}$=0** | **$I_{LP}$=0** |
| **Inflammation** | *Liver* | Activation of RES | $W_{LI1} = 1$ | 0 | 0 | 0 |
| | | Lymphocyte infiltration | $W_{LI2} = 2$ | 0 | 1 | 1 |
| ***Index for inflammatory processes*** | | | | **$I_{LI}$=0** | **$I_{LI}$=2** | **$I_{LI}$=2** |
| ***Index for organ $I_L$*** | | | | **$I_L = 0$** | **$I_L$=29** | **$I_L$=33** |

* $p<0.05$

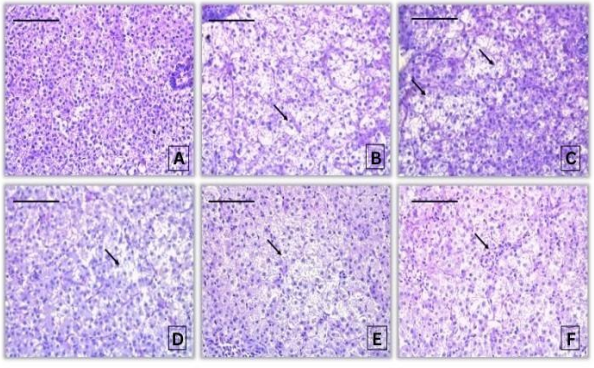


**Figure 8. Histopathological alterations in liver of *Cyprinus carpio* L. after acute (96 h) exposure with propamocarb hydrochloride (H&E), x400: A** – granular degeneration (40 µg/L); **B, C** – vacuolar degeneration (40 µg/L); **D** – karyolysis (80 µg/L); **E** – fatty degeneration (80 µg/L); **F** – hyperaemia (80 µg/L)

## 2.4. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute exposure with 2,4-D

**Table 6. Histopathological alterations in liver of common carp (*Cyprinus carpio* L.) after acute (96 h) exposure with 2,4-D**

| Reaction patter | Functional unit of the tissue | 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 | 4* | 4* |
| | | Intracellular edema | $W_{LC2} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{LC}$=0** | **$I_{LC}$=4** | **$I_{LC}$=4** |
| **Degenerative changes** | *Liver* | Granular degeneration | $W_{LR1} = 1$ | 0 | 4* | 5* |
| | | Vacuolar degeneration | $W_{LR2} = 2$ | 0 | 4* | 5* |
| | | Necrobiosis | $W_{LR3} = 2$ | 0 | 1 | 1 |
| | | Necrosis | $W_{LR4} = 3$ | 0 | 2* | 2* |
| | | Fatty degeneration | $W_{LR2} = 1$ | 0 | 2* | 5* |
| ***Index for degenerative changes*** | | | | **$I_{LR}$=0** | **$I_{LR}$=22** | **$I_{LR}$=28** |
| **Proliferative changes** | *Liver* | Hypertrophy | $W_{LP1} = 1$ | 0 | 0 | 0 |
| ***Index for proliferative changes*** | | | | **$I_{LP}$ = 0** | **$I_{LP}$ =0** | **$I_{LP}$ =0** |
| **Inflammation** | *Liver* | Activation of RES | $W_{LI1} = 1$ | 0 | 0 | 0 |
| | | Lymphocyte infiltration | $W_{LI2} = 2$ | 0 | 1 | 1 |
| ***Index for inflammatory processes*** | | | | **$I_{LI}$ = 0** | **$I_{LI}$ =2** | **$I_{LI}$ =2** |
| ***Index for organ $I_L$*** | | | | **$I_L$ = 0** | **$I_L$=28** | **$I_L$=34** |

* $p<0.05$

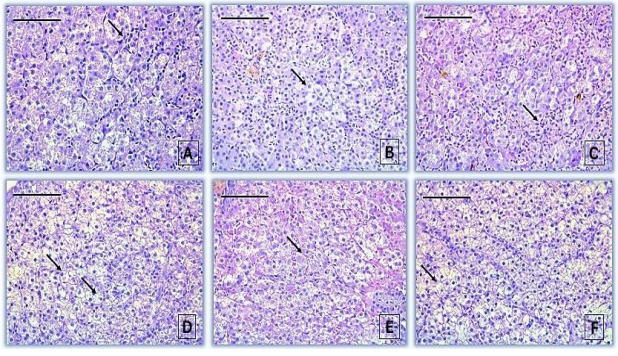


**Figure 9. Histopathological alterations in liver of *Cyprinus carpio* L. after acute (96 h) exposure with 2,4-D (H&E), x400: A** – hyperaemia (50 µg/L); **B** – vacuolar degeneration (50 µg/L); **C** – hyperaemia (50 µg/L); **D** – fatty degeneration (100 µg/L); **E** – hyperaemia (100 µg/L); **F** – vacuolar degeneration (100 µg/L)

Based on the obtained results, the liver index ($I_L$) for pirimiphos-methyl falls into class III for both concentrations, indicating a moderate degree of histological structure change, and the processes are reversible (**Table 4, Figure 7**). Propamocarb hydrochloride $I_L$ at 40 μg/L is 29 and falls into class III with a moderate degree of reversible changes. $I_L$ at 80 μg/L is with a value of 33 and falls into class IV, with a severe degree of histological structure change, and the processes are irreversible (**Table 5, Figure 8**). 2,4-D $I_L$ at 50 μg/L falls into class III, indicating that there is a moderate degree of histological change. $I_L$ at 100 μg/L falls into class IV, with a severe degree of irreversible histological changes. (**Table 6, Figure 9**).

A number of authors found similar histopathological changes in the liver of fish treated with toxicants (Subburaj et al., 2020; Farhan et al., 2021; Ezemonye & Ogbomida, 2010; Al-Otaibi et al., 2019; Nagaraju & Rathnamma, 2014; Babatunde et al., 2014; Cattaneo et al., 2008, Vigario et al., 2014 and others). Along with this, the results found regarding the $I_L$ for the degree of toxicity of the three applied pesticides allow establishing an objective comparative assessment. The summarized results in relation to the established disorders in the four categories of liver changes, the degree of toxicity of the tested pesticides can be summarized in the following descending order (**Figure 10**):

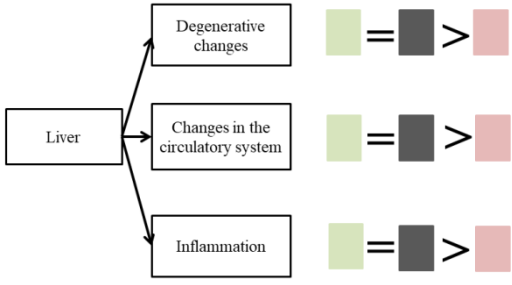


**Figure 10. Comparative analysis of the toxic effects of applied pesticides on the histological structure of common carp liver (*Cyprinus carpio* L.)**

## 3. Histopathological alterations in carp kidney after acute exposure with the tested pesticides

### 3.1. Control group

The results show normal morphology of the kidney histological structure in the control group (**Table 7** и **Figure 11)**.

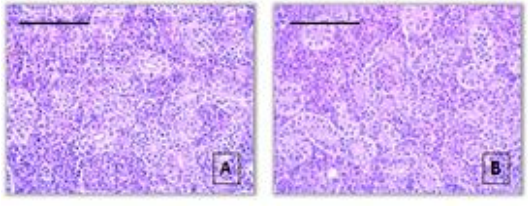


**Figure 11. Normal morphology of the kidney histological structure in the control group, x400, H&E**

## 3.2. Histopathological alterations in kidney of common carp (*Cyprinus carpio* L.) after acute exposure with pirimiphos-methyl

**Table 7. Histopathological alterations in kidney ot common carp (*Cyprinus carpio* L.) after acute exposure with pirimiphos-methyl**

| Reaction pattern | Funtional unit of the tissue | Ateration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | Pirimiphos-methyl | |
| | | | | | 10 µg/L | 60 µg/L |
| **Changes in the circulatory system** | *Kidney* | Haemorrhage | $W_{KC1} = 1$ | 0 | 0 | 0 |
| | | Hyperaemia | $W_{KC2} = 1$ | 0 | 0 | 0 |
| | | Aneurysms | $W_{KC3} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{KC}$ =0** | **$I_{KC}$ =0** | **$I_{KC}$ =0** |
| **Degenerative changes** | *Tubule* | Vacuolar degeneration | $W_{KR1} = 1$ | 0 | 2* | 2* |
| | | Hyaline degeneration | $W_{KR2} = 1$ | 0 | 0 | 1 |
| | | Necrobiosis | $W_{KR3} = 2$ | 0 | 0 | 1 |
| | | Necrosis | $W_{KR4} = 3$ | 0 | 0 | 1 |
| | *Glomerulus* | Dilatation of the Bowman's capsule | $W_{KR5} = 1$ | 0 | 2* | 3* |
| | | Contraction | $W_{KR6} = 1$ | 0 | 1 | 1 |
| | | Necrobiosis | $W_{KR7} = 2$ | 0 | 0 | 0 |
| | | Necrosis | $W_{KR8} = 3$ | 0 | 0 | 0 |
| | *Interstitial tissue* | Necrosis | $W_{KR9} = 3$ | 0 | 0 | 0 |
| ***Index for degenerative changes*** | | | | **$I_{KR}$ = 0** | **$I_{KR}$=5** | **$I_{KR}$=12** |
| **Proliferative changes** | *Tubule* | Hypertrophy | $W_{KP1} = 1$ | 0 | 2* | 2* |
| | | Hyperplasia | $W_{KP2} = 2$ | 0 | 0 | 0 |
| | *Glomerulus* | Hypertrophy | $W_{KP3} = 1$ | 0 | 0 | 0 |
| | | Hyperplasia | $W_{KP4} = 2$ | 0 | 0 | 0 |
| | | Thickening of Bowman's capsular membrane | $W_{KP5} = 2$ | 0 | 0 | 1 |
| | *Interstitial tissue* | Hypertrophy | $W_{KP6} = 1$ | 0 | 3* | 3* |
| | | Edema | $W_{KP7} = 2$ | 0 | 1 | 1 |
| ***Index for proliferative changes*** | | | | **$I_{KP}$ = 0** | **$I_{KP}$ = 7** | **$I_{KP}$ = 9** |
| **Inflammation** | *Kidney* | Infiltration | $W_{KI1} = 2$ | 0 | 0 | 0 |
| | | Activation of melanomacrophages | $W_{KI2} = 2$ | 0 | 3* | 3* |
| ***Index for inflammatory processes*** | | | | **$I_{KI}$ = 0** | **$I_{KI}$ =6** | **$I_{KI}$ =6** |
| ***Index for organ $I_K$*** | | | | **$I_K$ = 0** | **$I_K$=18** | **$I_K$=27** |

* $p<0.05$

## 3.3. Histopathological alterations in kidney of common carp (*Cyprinus carpio* L.) after acute exposure with propamocarb hydrochloride

**Table 8. Histopathological alterations in kidney of common carp (*Cyprinus carpio* L.) after acute exposure with propamocarb hydrochloride**

| Reaction pattern | Functional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | Propamocarb hydrochloride | |
| | | | | | 40 µg/L | 80 µg/L |
| **Changes in the circulatory system** | *Kidney* | Haemorrhage | $W_{KC1} = 1$ | 0 | 0 | 0 |
| | | Hyperaemia | $W_{KC2} = 1$ | 0 | 0 | 0 |
| | | Aneurysms | $W_{KC3} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{KC}$ =0** | **$I_{KC}$ =0** | **$I_{KC}$ =0** |
| **Degenerative changes** | *Tubule* | Vacuolar degeneration | $W_{KR1} = 1$ | 0 | 1 | 3* |
| | | Hyaline degeneration | $W_{KR2} = 1$ | 0 | 1 | 2* |
| | | Necrobiosis | $W_{KR3} = 2$ | 0 | 0 | 0 |
| | | Necrosis | $W_{KR4} = 3$ | 0 | 0 | 0 |
| | *Glomerulus* | Dilatation of the Bowman's capsule | $W_{KR5} = 1$ | 0 | 2* | 2* |
| | | Contraction | $W_{KR6} = 1$ | 0 | 0 | 0 |
| | | Necrobiosis | $W_{KR7} = 2$ | 0 | 0 | 0 |
| | | Necrosis | $W_{KR8} = 3$ | 0 | 0 | 0 |
| | *Interstitial tissue* | Necrosis | $W_{KR9} = 3$ | 0 | 0 | 0 |
| ***Index for degenerative changes*** | | | | **$I_{KR}$ = 0** | **$I_{KR}$=4** | **$I_{KR}$=7** |
| **Proliferative changes** | *Tubule* | Hypertrophy | $W_{KP1} = 1$ | 0 | 1 | 1 |
| | | Hyperplasia | $W_{KP2} = 2$ | 0 | 0 | 0 |
| | *Glomerulus* | Hypertrophy | $W_{KP3} = 1$ | 0 | 1 | 1 |
| | | Hyperplasia | $W_{KP4} = 2$ | 0 | 0 | 0 |
| | | Dilatation of the Bowman's capsule | $W_{KP5} = 2$ | 0 | 0 | 1 |
| | *Interstitial tissue* | Hypertrophy | $W_{KP6} = 1$ | 0 | 0 | 0 |
| | | Edema | $W_{KP7} = 2$ | 0 | 0 | 0 |
| ***Index for proliferative changes*** | | | | **$I_{KP}$ = 0** | **$I_{KP}$ =2** | **$I_{KP}$ =4** |
| **Inflammation** | *Kidney* | Infiltration | $W_{KI1} = 2$ | 0 | 0 | 0 |
| | | Activation of melano-macrophages | $W_{KI2} = 2$ | 0 | 1 | 2* |
| ***Index for inflammatory processes*** | | | | **$I_{KI}$ = 0** | **$I_{KI}$ =2** | **$I_{KI}$ =4** |
| ***Index for organ $I_K$*** | | | | **$I_K$ = 0** | **$I_K$=8** | **$I_K$=15** |

* $p<0.05$

## 3.4. Histopathological alterations in kidney of common carp (*Cyprinus carpio* L.) after acute exposure with 2,4-D

**Table 9. Histopathological alterations in kidney of common carp (Cyprinus carpio L.) after acute exposure with 2,4-D**

| Reaction pattern | Functional unit of the tissue | Alteration | Importance factor | Score value | | |
|---|---|---|---|---|---|---|
| | | | | Control | 2, 4 - D | |
| | | | | | 50 µg/L | 100 µg/L |
| **Changes in the circulatory system** | *Kidney* | Haemorrhage | $W_{KC1} = 1$ | 0 | 0 | 0 |
| | | Hyperaemia | $W_{KC2} = 1$ | 0 | 0 | 0 |
| | | Aneurysms | $W_{KC3} = 1$ | 0 | 0 | 0 |
| ***Index for changes in the circulatory system*** | | | | **$I_{KC}$ =0** | **$I_{KC}$ =0** | **$I_{KC}$ =0** |
| **Degenerative changes** | *Tubule* | Vacuolar degeneration | $W_{KR1} = 1$ | 0 | 2* | 4* |
| | | Hyaline degeneration | $W_{KR2} = 1$ | 0 | 0 | 0 |
| | | Necrobiosis | $W_{KR3} = 2$ | 0 | 0 | 0 |
| | | Necrosis | $W_{KR4} = 3$ | 0 | 0 | 0 |
| | *Glomerulus* | Dilatation of the Bowman's capsule | $W_{KR5} = 1$ | 0 | 1 | 2* |
| | | Contraction | $W_{KR6} = 1$ | 0 | 0 | 1 |
| | | Necrobiosis | $W_{KR7} = 2$ | 0 | 1 | 1 |
| | | Necrosis | $W_{KR8} = 3$ | 0 | 1 | 1 |
| | *Interstitial tissue* | Necrosis | $W_{KR9} = 3$ | 0 | 0 | 1 |
| ***Index for degenerative changes*** | | | | **$I_{KR}$ = 0** | **$I_{KR}$=5** | **$I_{KR}$=15** |
| **Proliferative changes** | *Tubule* | Hypertrophy | $W_{KP1} = 1$ | 0 | 0 | 1 |
| | | Hyperplasia | $W_{KP2} = 2$ | 0 | 0 | 0 |
| | *Glomerulus* | Hypertrophy | $W_{KP3} = 1$ | 0 | 1 | 3* |
| | | Hyperplasia | $W_{KP4} = 2$ | 0 | 0 | 1 |
| | | Dilatation of the Bowman's capsule | $W_{KP5} = 2$ | 0 | 0 | 0 |
| | *Interstitial tissue* | Hypertrophy | $W_{KP6} = 1$ | 0 | 1 | 1 |
| | | Edema | $W_{KP7} = 2$ | 0 | 2* | 4* |
| ***Index for proliferative changes*** | | | | **$I_{KP}$ = 0** | **$I_{KP}$ =6** | **$I_{KP}$=15** |
| **Inflammation** | *Kidney* | Infiltration | $W_{KI1} = 2$ | 0 | 0 | 0 |
| | | Activation of melano-macrophages | $W_{KI2} = 2$ | 0 | 2* | 2* |
| ***Index for inflammatory processes*** | | | | **$I_{KI}$ = 0** | **$I_{KI}$ =4** | **$I_{KI}$ =4** |
| ***Index for organ $I_K$*** | | | | **$I_K$ = 0** | **$I_K$=15** | **$I_K$=34** |

* $p<0.05$

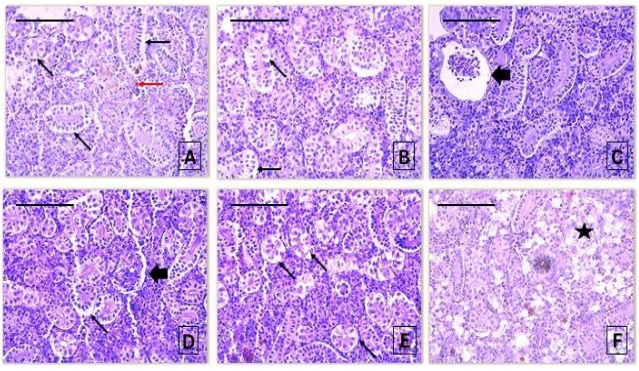


**Figure 12. Histopathological alterations in kidney of common carp after acute (96 h) exposure with pirimiphos-methyl (H&E):** **A** – activation of melanomacrophages (⟶) and vacuolar degeneration (⟶) (10 µg/L), x400; **B** – vacuolar degeneration (⟶) (10 µg/L), x400; **C** – dilatation of Bowman's capsule (➡) (10 µg/L), x400; **D**- dilatation of Bowman's capsule (➡) (60 µg/L), x400; **E** - vacuolar degeneration (⟶) (60 µg/L), x400; **F** – hypertrophy of interstitial tissue (★) (60 µg/L), x400

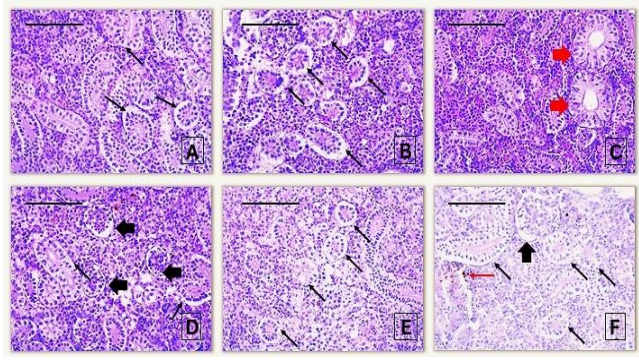


**Figure 13. Histopathological alterations in kidney of common carp after acute (96 h) exposure with propamocarb hydrochloride (H&E):** **A, B** – vacuolar degeneration (⟶) при (40 µg/L), x400; **C** – hypertrophy of the tubule (➡) (40 µg/L), x400; **D**- dilatation of Bowman's capsule (➡) and vacuolar degeneration (⟶) (80 µg/L), x400; **E** - vacuolar degeneration (⟶) (80 µg/L), x400; **F** – vacuolar degeneration (⟶), dilatation of Bowman's capsule (➡) and activation of melanomacrophages (⟶) (80 µg/L), x400

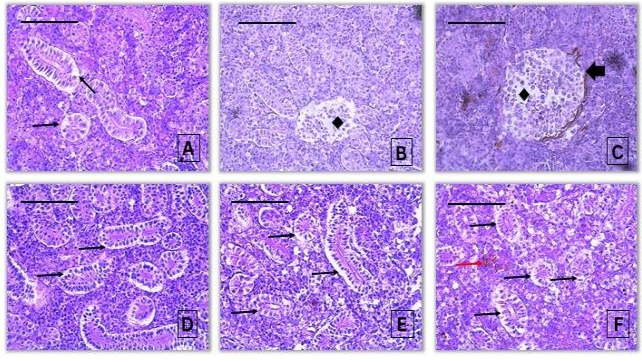


**Figure 14. Histopathological alterations in kidney of common carp after acute (96 h) exposure with 2,4 - D (H&E):** **A** – vacuolar degeneration (⟶) (50 µg/L), x400; **B** – necrosis of glomerulus (◆) (50 µg/L), x400; **C** – necrosis of glomerulus (◆) and dilatation of Bowman's capsule (➡) (50 µg/L), x400; **D, E** - vacuolar degeneration (⟶) (100 µg/L), x400; **F** – vacuolar degeneration (⟶) and activation of melanomacrophages (⟶) (100 µg/L), x400

The kidney index ($I_K$) of fish treated with pirimiphos-methyl at 10 µg/L is 18 and falls into class II, and in those treated with 60 µg/L $I_K$ is 27 and falls into class III. In both classes, the changes are reversible (**Table 7, Figure 12**). Propamocarb hydrochloride $I_K$ at 40 µg/L is 8 and

falls into Class I, while at 40 µg/L the $I_K$ is 15 and falls into Class II. In both classes, the changes are reversible (**Table 8, Figure 13**). 2,4-D $I_K$ at 50 µg/L is 15 and falls into class II, where the changes were reversible, and at 100 µg/L it is 34 and falls into class IV, with a severe degree of irreversible histological changes (**Table 9, Figure 14**).

Our results are also confirmed in studies by Mostakim et al. (2015), Al-Otaibi et al. (2019), Boran et al. (2012), Sharmin et al. (2021), Okogwu et al. (2016) and others.

After conducting a comparative analysis based on the results obtained, the degree of toxicity of the experimental pesticides can be summarized in the following descending order (**Figure 15**):

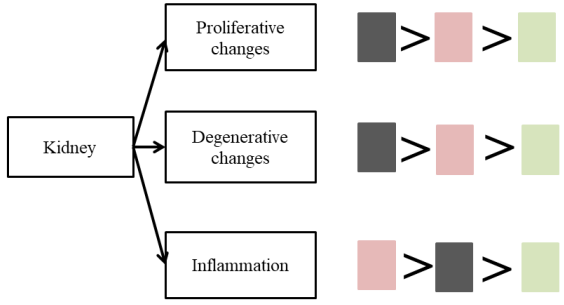


**Figure 15. Comparative analysis of the toxic effect of applied pesticides on the histological structure of a common carp (*Cyprinus carpio* L.) kidney.**

## 4. Histochemical alterations in liver of common carp *(Cyprinus carpio* L.) after acute exposure with the tested pesticides

**Table 10** and **Table 11** present the results of the histochemical analysis.

**Table 10. Histochemical alterations in liver of common carp (*Cyprinus carpio* L.) after acute (96-h) exposure with pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, established by PAS-reaction**

| Intensity of the PAS-reaction | Pirimiphos-methyl | | | Propamocarb hydrochloride | | | 2,4 - D | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration µg/L | К | 10 µg/L | 60 µg/L | К | 40 µg/L | 80 µg/L | К | 50 µg/L | 100 µg/L |
| *Cyprinus carpio* | +/- | ++ | + | +/- | ++ | ++ | +/- | ++ | ++ |

(-) – negative reaction to histochemical staining; (+/-) – very weak histochemical reaction; (+) – weak histochemical reaction; (++) – moderate histochemical staining reaction; (+++) – severe histochemical reaction

**Table 11. Histochemical alterations in liver of common carp (Cyprinus carpio L.) after acute (96-h) exposure with pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, established by Sudan Black B staining**

| Intensity of Sudan Black B staining | Pirimiphos-methyl | | | Propamocarb hydrochloride | | | 2,4 - D | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration µg/L | К | 10 µg/L | 60 µg/L | К | 40 µg/L | 80 µg/L | К | 50 µg/L | 100 µg/L |
| *Cyprinus carpio* | +/- | +++ | +/- | +/- | ++ | ++ | +/- | + | + |

(-) – negative reaction to histochemical staining; (+/-) – very weak histochemical reaction; (+) – weak histochemical reaction; (++) – moderate histochemical staining reaction; (+++) – severe histochemical reaction

## 4.1. Histochemical alterations in liver of common carp (*Cyprinus carpio* L.) after exposure with pirimiphos-methyl

At a concentration of 10 µg/L pirimiphos-methyl, a moderate increase in the level of glycogen is observed compared to the control. At a concentration of 60 µg/L, there is a slight increase in the level of glycogen compared to the control, but a decrease compared to the lower concentration of applied pesticide (**Table 10, Figure 16**).

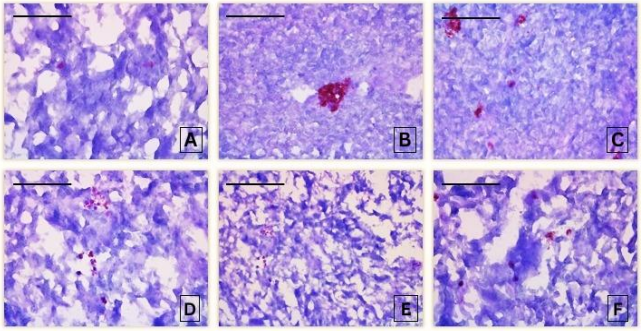


**Figure 16. Intensity of the PAS-reaction in liver of *Cyprinus carpio* L. after exposure with pirimiphos-methyl: A** – control, x400; **B, C** – 10 µg/L, x400; **D, E, F** – 60 µg/L, x400

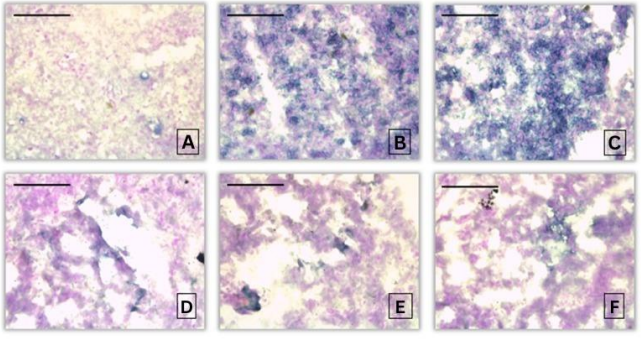


**Figure 17. Intensity of the Sudan Black B staining in liver of *Cyprinus carpio* L. after exposure with pirimiphos-methyl: A** – control, x400; **B, C** – 10 µg/L, x400; **D, E, F** – 60 µg/L, x400

On the other hand, at a concentration of 10 μg/L, a strong increase in lipid stores in the liver is observed, while at 60 μg/L, no change in the lipid profile is observed compared to the control (**Table 11, Figure 17**).

## 4.2. Histochemical alterations in liver of common carp (*Cyprinus carpio* L.) after exposure with propamocarb hydrochloride

The fungicide propamocarb hydrochloride increased the amount of glycogen and lipids compared to the controls to a moderate extent, similarly at both concentrations tested (**Table 10, Table 11, Figure 18, Figure 19**).

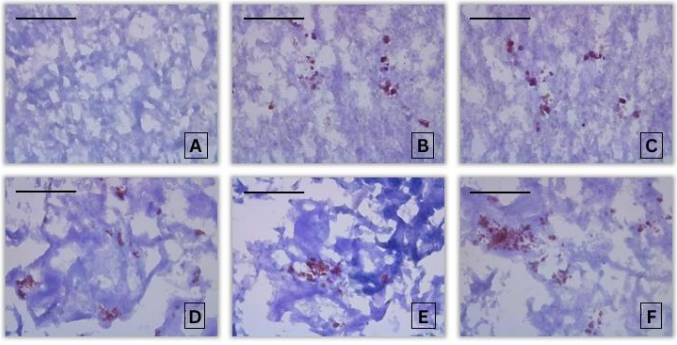


**Figure 18. Intensity of the PAS-reaction in liver of *Cyprinus carpio* L. after exposure with propamocarb hydrochloride: A** – control, x400; **B, C** –40 μg/L, x400; **D, E, F** –80 μg/L, x400

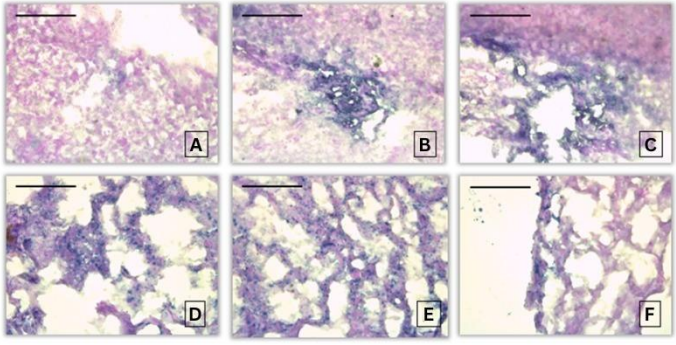


**Figure 19. Intensity of the Sudan Black B staining in liver of Cyprinus carpio L. after exposure with propamocarb hydrochloride: A** – control, x400; **B, C** –40 μg/L, x400; **D, E, F** –80 μg/L, x400

## 4.3. Histochemical alterations in liver of common carp (*Cyprinus carpio* L.) after exposure with 2,4 – D

Moderate accumulation of glycogen and low accumulation of lipids are found in herbicide 2,4-D treatment compared to the control group in a similar manner, at both concentrations. (**Table 10, Table 11, Figure 20, Figure 21**).

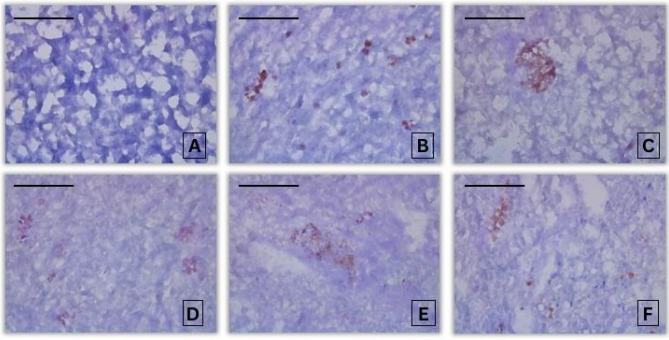


**Figure 20. Intensity of the PAS-reaction in liver of *Cyprinus carpio* L. after exposure with 2,4-D: A** – control, x400; **B, C** –50 µg/L, x400; **D, E, F** –100 µg/L, x400.

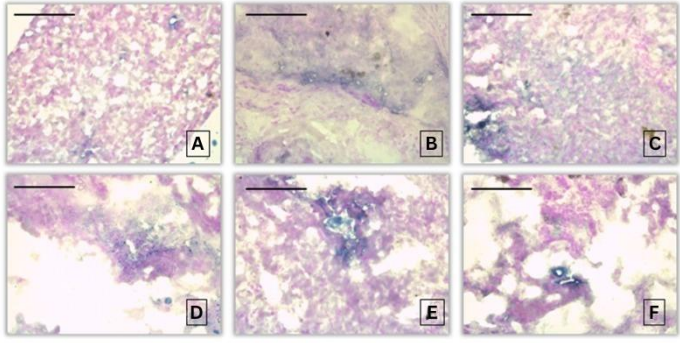


**Figure 21. Intensity of the Sudan Black B staining in liver of Cyprinus carpio L. after exposure with 2,4-D: A** – control, x400; **B, C** – 50 µg/L, x400; **D, E, F** – 100 µg/L, x400.

Based on the results obtained and according to the semi-quantitative scale used, the toxicity of the tested pesticides can be summarized in the following descending order (**Figure 22**):

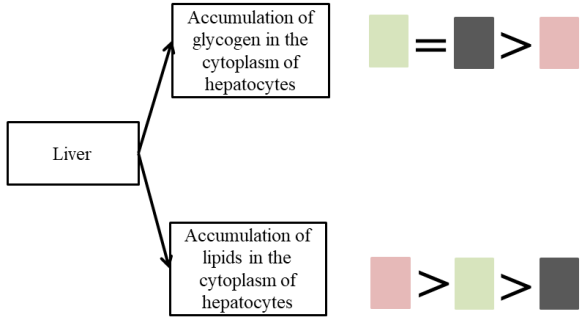


**Figure 22. Comparative analysis of the results of the histochemical analysis of the liver of common carp (*Cyprinus carpio* L.) under the effect of the applied pesticides**

Pirimiphos-methyl  Propamocarb hydrochloride  2,4-D

From the comparative analysis, propamocarb hydrochloride shows the highest degree of accumulation of glycogen in hepatocytes, while for lipids - pirimiphos-methyl. Similar to Ayoola (2008), we believe that the changes related to the alteration in the amount of glycogen and lipids in liver of the experimental individuals may be due to a change in the processes of glycolysis, which in turn depends on the administered concentrations of the toxicant, the duration of action or its chemical nature.

## 5. Biochemical alterations in liver of common carp (*Cyprinus carpio* L.) after acute exposure with the tested pesticides

### 5.1. LDH

The results of the analyzes show a decrease in LDH activity at all exposures of the applied pesticides (**Figure 23**). Compared to the control, the most significant decrease in the specific enzyme activity of LDH is recorded at 50 µg/L 2,4-D, and the least at 40 µg/L propamocarb hydrochloride.

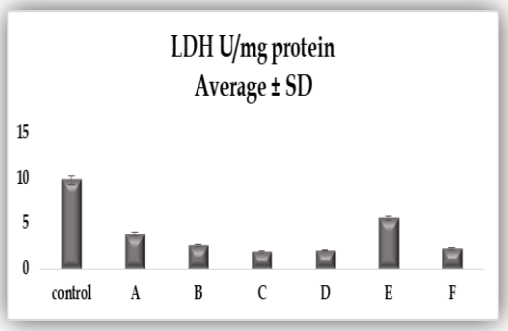


**Figure 23. Change in specific enzyme activity of LDH in liver of *Cyprinus carpio* L. after 96 hours of exposure: A** – 10 µg/L pirimiphos-methyl ($p<0.05$); **B** – 60 µg/L pirimiphos-methyl ($p<0.05$); **C** – 40 µg/L propamocarb hydrochloride ($p<0.05$); **D** – 80 µg/L propamocarb hydrochloride ($p<0.05$); **E** – 50 µg/L 2,4-D ($p<0.05$); **F** – 100 µg/L 2,4-D ($p<0.05$)

### 5.2. ASAT и ALAT

Compared to the control, the specific enzyme activity of ASAT is decreased most significantly at 40 µg/L propamocarb hydrochloride, and least at 100 µg/L 2,4-D. The specific enzyme activity of ALAT is most significantly inhibited at 40 µg/L propamocarb hydrochloride and least at 60 µg/L pirimiphos-methyl (**Figure 24, Figure 25**).

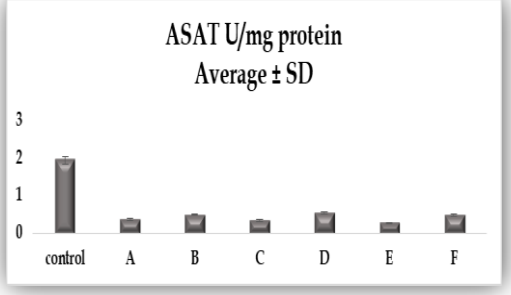


**Figure 24. Change in specific enzyme activity of ASAT in liver of *Cyprinus carpio* L. after 96 hours of exposure: A** – 10 µg/L pirimiphos-methyl ($p<0.05$); **B** – 60 µg/L pirimiphos-methyl ($p<0.05$); **C** – 40 µg/L propamocarb hydrochloride ($p<0.05$); **D** – 80 µg/L propamocarb hydrochloride ($p<0.05$); **E** – 50 µg/L 2,4-D ($p<0.05$); **F** – 100 µg/L 2,4-D ($p<0.05$)

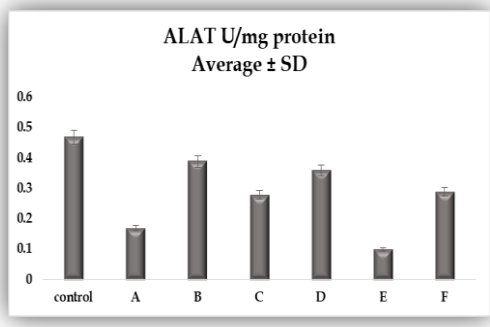


**Figure 25. Change in specific enzyme activity of ALAT in liver of *Cyprinus carpio* L. after 96 hours of exposure: A** – 10 μg/L pirimiphos-methyl ($p<0.05$); **B** – 60 μg/L pirimiphos-methyl ($p<0.05$); **C** – 40 μg/L propamocarb hydrochloride ($p<0.05$); **D** – 80 μg/L propamocarb hydrochloride ($p<0.05$); **E** – 50 μg/L 2,4-D ($p<0.05$); **F** – 100 μg/L 2,4-D ($p<0.05$)

## 5.3. ChE

The strongest decrease in specific enzyme activity is observed at 80 μg/L propamocarb hydrochloride, and the weakest at 10 μg/L pirimiphos-methyl (**Figure 26**).

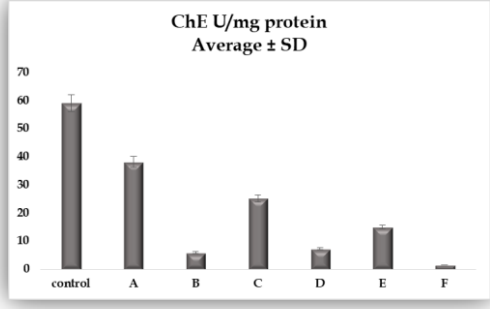


**Figure 26. Change in specific enzyme activity of ChE in liver of *Cyprinus carpio* L. after 96 hours of exposure: A** – 10 μg/L pirimiphos-methyl ($p<0.05$); **B** – 60 μg/L pirimiphos-methyl ($p<0.05$); **C** – 40 μg/L propamocarb hydrochloride ($p<0.05$); **D** – 80 μg/L propamocarb hydrochloride ($p<0.05$); **E** – 50 μg/L 2,4-D ($p<0.05$); **F** – 100 μg/L 2,4-D ($p<0.05$)

## 5.4. CAT

The strongest decrease in specific enzyme activity was observed at 60 μg/L pirimiphos-methyl, and the weakest at 80 μg/L propamocarb hydrochloride (**Figure 27**).

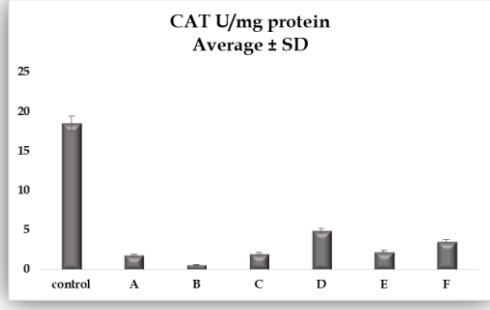


**Figure 27. Change in specific enzyme activity of CAT in liver of *Cyprinus carpio* L. after 96 hours of exposure: A** – 10 μg/L pirimiphos-methyl ($p<0.05$); **B** – 60 μg/L pirimiphos-methyl ($p<0.05$); **C** – 40 μg/L propamocarb hydrochloride ($p<0.05$); **D** – 80 μg/L propamocarb hydrochloride ($p<0.05$); **E** – 50 μg/L 2,4-D ($p<0.05$); **F** – 100 μg/L 2,4-D ($p<0.05$)

According to the degree of inhibition of the specific enzyme activity, the comparative analysis for the degree of toxicity of the experimental pesticides can be summarized in the following descending order (**Figure 28**):

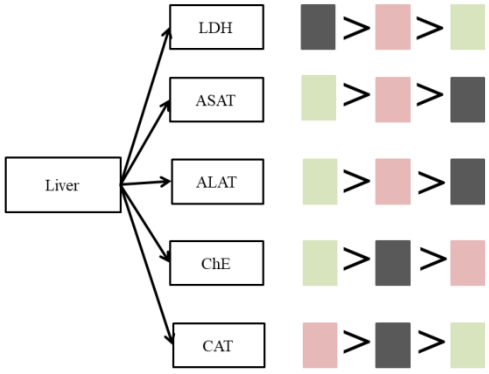


**Figure 28. Comparative analysis of the biochemical examination of the liver of common carp (*Cyprinus carpio* L.) under the influence of applied pesticides**

Similar to the results of the present experiment, Abhijith et al. (2016) found for LDH, Rao (2006) for aminotransferases, Bonansea et al. (2016), Da Cuña et al. (2011), Tian et al. (2018) and Vieira et al. (2018) for ChE, Seidel et al. (2001) and Al-Ghanim et al. (2020) for CAT. In addition, a comparative analysis is carried out, according to which the degree of toxicity of the applied pesticides on the specific enzyme activity is determined.

## IV. CONCLUSION AND INFERENCES

### CONCLUSION

In summary, we can conclude that the applied pesticides pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, each tested at two concentrations that are many times lower than the $LC_{50}$, negatively affected the histological structure of the gills, liver and kidney from the studied individuals common carp (*C. carpio* L.). Along with this, compensatory-adaptive mechanisms are activated in the body, which affects the functionality of the carp's organs and can inevitably lead to a deterioration of the health of the entire organism. There is a tendency to increase the morphological changes and the degree of their occurrence in proportion to the increasing concentrations of the applied pesticides. In addition, the results of the conducted histochemical study show that all tested concentrations of the applied pesticides cause a change in the carbohydrate and lipid profile of the bioindicator species. A decrease in the specific activity of the monitored liver enzymes is found in varying degrees, depending on the applied pesticide and its concentration. Changes in the activity of liver enzymes, like other applied analyses, can be used as a diagnostic marker for the effects of toxicants in aquatic ecosystems. Based on the data from the conducted research, comparative analyzes are also carried out, providing an objective assessment of the toxicity degree of the pesticides.

The results of the present study for establishing histopathological, histochemical and biochemical biomarkers in *Cyprinus carpio* L. could be used to determine maximum permissible concentrations of organic pollutants in biota, as well as for ecological biomonitoring purposes, applying an assessment model based on correlational dependencies between established biomarkers.

## INFERENCES

1. After exposure to pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, histopathological alterations, including proliferative and degenerative changes, changes in the circulatory system of the organ and inflammation are found in the examined gills, liver and kidney of common carp (*Cyprinus carpio* L.).

- ❖ The index of histopathological changes in gills is highest when treated with the fungicide propamocarb hydrochloride.
- ❖ The index of histopathological changes in the liver is highest with exposure to the herbicide 2,4-D.
- ❖ The index of histopathological changes in the kidney is highest when treated with the herbicide 2,4-D.
- ❖ When comparing the indices of histopathological changes of the three organs, it is found that they are highest in gills, followed by liver and lowest in kidney.
- ❖ All changes vary in degree of manifestation in relation to the applied concentrations of pesticides, with a tendency to increase the degree of manifestation in direct proportion to the concentration of the toxicant.

2. After exposure to pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, histochemical changes including glycogen and lipid accumulation are found in the liver of common carp (*Cyprinus carpio* L.).

- ❖ The amount of glycogen and lipids in the liver with all three tested pesticides increased compared to the control group. The highest degree of accumulation of glycogen is found in propamocarb hydrochloride and 2,4-D, while the highest degree of accumulation of lipids is found in pirimiphos-methyl treatment.

3. After exposure to pirimiphos-methyl, propamocarb hydrochloride and 2,4-D, biochemical changes are found, including a alterations in the specific enzyme activity of LDH, ASAT, ALAT, ChE and CAT in the liver of common carp (*Cyprinus carpio* L.) . The specific enzyme activity of LDH is most strongly inhibited by 2,4-D, that of ASAT, ALAT and ChE – by propamocarb hydrochloride, and that of CAT – by pirimiphos-methyl.

4. The established biochemical changes under the influence of the experimental pesticides confirm the observed histological and histochemical ones, which is an indicator of a disturbance in the functions of the liver, related to the processes of glycolysis, glyconeogenesis and lipogenesis.

## V. CONTROBUTIONS

***1. Of original scientific character:***

1.1. The present study on the effect of pirimiphos-methyl and propamocarb hydrochloride on common carp (*Cyprinus carpio* L.) is the first of its kind.

1.2. In connection with the applied pesticides, the first complex morphophysiological study was carried out in laboratory conditions, including histopathological analysis of gills, liver and kidney, as well as histochemical and biochemical analysis of liver, to clarify the degree of toxicity of three active ingredients of PPP on common carp (*Cyprinus carpio* L.).

1.3. The performed histochemical analysis of the liver of common carp (*Cyprinus carpio* L.) for the influence of the toxicity of the herbicide 2,4-D is the first of its kind.

***2. Confirmatory:***

2.1. Common carp (*Cyprinus carpio* L.) can be used as a good bioindicator for pesticide contamination of aquatic ecosystems.

2.2. The followind histopathological changes were found in gills: proliferative - lamellar lifting, edema, proliferation of the covering epithelium and glandular cells in it and fusion; degenerative in the epithelial tissue of the filament and secondary lamellae; in the circulatory system – vasodilatation of the filament and secondary lamellae, and aneurysms.

2.3. The following histopathological changes were found in the liver: proliferative mainly concerning the hepatocytes - hypertrophy; degenerative - granular, vacuolar and fatty degeneration, necrobiosis and necrosis; in the circulatory system – hyperemia; inflammation - lymphocytic infiltration.

2.4. The following histopathological changes were found in the kidney: proliferative - hypertrophy and hyperplasia in the epithelial cells of the renal tubules and glomerulus, thickening of the membrane of Baumann's capsule, hypertrophy and edema of interstitial hematopoietic tissue; degenerative - vacuolar and hyaline-droplet degeneration of the epithelial cells of the renal tubules, necrobiosis and necrosis of the renal tubules and glomerulus, dilatation of Bauman's capsule, shrinkage of the renal corpuscles, necrosis of the interstitial tissue; inflammation – activation of melanoma macrophages.

2.5. Histopathological and histochemical changes represent compensatory-adaptive mechanisms for the survival of fish in pesticide-contaminated water.

2.6. Depending on the concentration of applied pesticides, the activity of liver enzymes in fish changes (inhibits or activates).

***3. With a methodical and applied nature:***

3.1. The advantages of the scale used to determine the degree of changes in the histological structure of the gills, liver and kidney are indicated.

3.3. Histopathological, histochemical and biochemical analyzes can serve as tools to assess the effect of various toxicants on bioindicator species.

3.4. The established changes in gill, liver and kidney can be successfully applied as biomarkers and included in a model for the assessment of contamination of aquatic ecosystems with pesticides, with the aim of preparing an adequate normative base regarding the presence of organic pollutants in aquatic ecosystems.

***Publications:***

1. Georgieva, E., Yancheva, V., Velcheva, I., Stoyanova, S., Iliev, I., Vasileva, T., Bivolarski, V., **Petkova, E.,** László, B., Nyeste, K., Antal, L. “Which one is more toxic? - Evaluation of chlorpyrifos and cypermethrin toxic effects on selected biomarkers in common carp (*Cyprinus carpio*, Linnaeus 1758)”. Toxics. 2021, May 31;9(6):125. doi: 10.3390/toxics9060125. IF=4.6, Q1.

2. **Kovacheva, E.,** Georgieva, E., Velcheva, I., Nikolova, M., 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, Vol. 14, Issue 2, pp 143-159., Q4.

3. **Kovacheva, E.,** Georgieva, E., Velcheva, I., Nikolova, M., Todorova, B., Todorova-Bambaldokova, D., Yancheva, V., Stoyanova, S., Tomov, S. „Histochemical and biochemical changes in Common carp (*Cyprinus carpio* Linnaeus, 1785) liver after cypermethrin and chlorpyrifos treatment". Ecologia Balkanica, 2022, Vol. 14, Issue 2, pp 123-141., Q4.

4. Georgieva, E., **Kovacheva, E.,** Yancheva, V., Velcheva, I., Hrischev, P., Atanassova, P., Tomov, S., Stoyanova, S. "Pesticides induce fatty degeneration in liver of *Cyprinus carpio* (Linnaeus 1758) after acute exposure". Ecologia Balkanica, 2023, Vol. 15, Issue 2, pp 77-82., Q4.

***Participation in conferences with a report:***

1. Histopathological measures in gills of Common carp, *Cyprinus carpio* (Linnaeus, 1875) after xenobiotics exposure, **Eleonora Petkova**, Stela Stoyanova, Iliana Velcheva, Vesela Yancheva, Elenka Georgieva, The 5th Balkan Scientific Conference BalkanBio, $15^{th}$-$16^{th}$ of April 2021, Plovdiv.

***Participation in conferences with posters:***

1. "Acute and chronic assessment of pesticide toxicity on histochemical and biochemical changes in the liver of common carp (*Cyprinus carpio* 1758, Linnaeus)", Georgieva E., Velcheva I., Yancheva V., Stoyanova S., Ivanova A., **Petkova E.,** Iliev I., Vasileva T., Bivolarski, V., presented at the International Seminar on Ecology, Sofia, 2021.

2. „Acute toxicity of pesticide exposure and histopathological changes in Common carp (*Cyprinus carpio* Linnaeus, 1785)", Elenka Georgieva, Iliana Velcheva, Vesela Yancheva, **Eleonora Petkova**, Dobrinka Todorova-Bambaldokova, Stela Stoyanova, presented at the VIII National Conference with international participation "Morphological Days", which was held from June 10 to 12, 2022 in the city of Sofia.

3. „Effects of microplastics (MPs) on common carp (*Cyprinus carpio* Linnaeus, 1758)", Todorova-Bambaldokova, D., **Petkova, E**., Yancheva, V., Stoyanova, S., Todorova, B., Velcheva, I., Georgieva, E., presented at the Third National Youth Conference on Biology, held on 01.11.2022 in Plovdiv, Bulgaria.

4. „Histopathological effects in *Cyprinus carpio* L. gills caused by propamocarb hydrochloride fungicide", **Petkova, E.,** Todorova-Bambaldokova, D., Yancheva, V., Stoyanova, S., Todorova, B., Velcheva, I., Georgieva, E., presented at the Third National Youth Conference on Biology, held on 01.11.2022 in Plovdiv, Bulgaria.

5. "Histochemical changes in the bioindicator species carp (*Cyprinus carpio* Linnaeus, 1758) under the influence of pesticides", Kovacheva, E., Georgieva, E., Stoyanova, S., Yancheva, V., Velcheva, I., presented at the XIV National Conference on Medical Biology, held on 02.06-04.06.2023 in Varna, Bulgaria.

***Participation in conferences with oral presentations:***

1. „Multi-biomarker approach for PBDEs effects in Common carp (*Cyprinus carpio* Linnaeus, 1785)“, Stoyanova, S, Velcheva, I., Yancheva, V., Todorova, B., **Petkova, E.**, Todorova-Bambaldokova, D., Georgieva, E., presented at the International Seminar on Ecology, which was held in an electronic environment in the period 29.09.-30.09. 2022

2. „Toxicological effects of commonly applied pesticides in Common carp (*Cyprinus carpio* Linnaeus, 1785)“, Georgieva, E., Velcheva, I., Yancheva, V., Todorova, B., **Petkova, E.,** Todorova-Bambaldokova, D., Stoyanova, S., presented at the International Seminar on Ecology, which was held in an electronic environment in the period 29.09.-30.09. 2022

***Participation in projects:***

1. **СП 19 БФ 010 (2019-2020)** Investigating the *ex situ* effect of water pollution with pesticides on the biological indicator *Cyprinus carpio* (Linnaeus, 1785) using multi-biomarkers. Funded by the Scientific Research Fund at the PU "P. Hilendarski".

2. **МУ19-БФ-014 (2019-2020)** Evaluation of the impact of Cypemethrin and Chlorpyrifos on the zebra mussel (*Dressena polymorpha* Pallas, 1771) by applying complex biological approaches according to DIRECTIVE 2013/39/EU. Funded by the Scientific Research Fund at the PU "P. Hilendarski".

3. **ФП21-БФ-008 (2021-2022)** *Ex situ* assessment of contamination of aquatic ecosystems with xenobiotics by application of complex biomarkers in selected bioindicator species. Funded by the Scientific Research Fund at the PU "P. Hilendarski".

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