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Histochemical and Biochemical Changes in Common Carp (Cyprinus carpio Linnaeus, 1785) Liver after Cypermethrin and Chlorpyrifos Exposure

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Abstract. Nowadays pollution of aquatic ecosystems with pesticides causes acute and chronic poisoning of fish, leading to serious damage to vital organs, such as the liver. Common carp (*Cyprinus carpio* Linnaeus, 1758) is a popular edible fish favored for culture due to its rapid growth, hardiness and reproduction in confined waters. The purpose of the present study was to investigate the negative effects of cypermethrin (CYP) and chlorpyrifos (CPF), based on their maximum allowable concentrations (Directive 2013/39/EU) on histochemical and biochemical biomarkers in the liver of common carp. The histochemical analysis included Periodic acid-Shiff stainig (PAS reaction) and Sudan Black B staining, while in the biochemical study different hepatic enzyme activities such as lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT). The negative effects of the tested pesticides on fish were expressed with liver changes in the amount of glycogen and lipids, and enzyme changes of LDH, ASAT and ALAT, caused by the acute and chronic exposure to cypermethrin and chlorpyrifos under laboratory conditions. The results from such experimental set ups could be used in the legislation of protection water bodies from contamination, in areas near intensive application of plant protection products and also in implementing the Directive 2013/39/EU and Water Frame Directive by using multi-biomarker approaches.

Key words: histochemistry, biochemistry, fish, liver, biomarkers, enzymes, pollution, cypermethrin, chlorpyrifos.

Introduction

Today, more than 1400 different pesticides are used in the environment worldwide, mostly in agriculture. Over the past 50 years, the use of these substances has greatly increased the quantity and improved the quality of food for the growing population of the world (Manuel et al., 2008). A pesticide is a substance or mixture of substances designed to kill, repel or reduce damage from various pests (Eldridge, 2008). Activities, such as agriculture, fishing, forestry, construction, mining, urban development and soil pollution occurring in or near the watershed of a reservoir can lead to disturbance of water quality and fish health (Mustapha, 2009). Pollution of aquatic ecosystems with pesticides causes acute and chronic poisoning of fish, leading to serious damage to vital organs, such as the liver (Omitoyin et al., 2006, Velmurugan et al., 2007). In addition, water pollution, directly or indirectly, can lead to fish kills or increased concentrations of toxic chemicals in edible fish tissue, which could have a negative effect on the health of people consuming such fish (Adedeji & Okocha, 2012).

Common carp (*Cyprinus carpio*) belongs to the order *Cypriniformes* and the family *Cyprinidae*, which is considered to be the largest family of freshwater fish. It inhabits freshwater environments, especially lakes, rivers, and rarely saltwater areas (Barus et al., 2001). Common carp is widespread in almost all countries of the world, but it is very popular in Asia and some European countries (Weber & Brown, 2011; Kloskowski, 2011). Common carp is also a popular edible fish favored for culture due to its rapid growth, hardiness and reproduction in confined waters (Singh, 2014).

Chlorpyrifos (O-O-diethyl-O-(3, 5, 6 trichloro-2-pyridyl)-phosphorothioate) is an organophosphorus insecticide, which is used to control a wide range of pests, such as worms, cockroaches, grubs, fleas, termites, ants and lice. It is applied to crops including cotton, nuts, vegetables and ornamentals (Caceres et al., 2007). The pesticide is a moderately toxic broad-spectrum lipophilic insecticide (Mugni et al., 2016) and was developed in the 1960s to replace other persistent pesticides (Kumar et al., 2017).

Cypermethrin ([cyano-(3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) is a synthetic pyrethroid widely used in agriculture, household and animal husbandry mainly (Valles & Koehler, 1997). Cypermethrin contains chlorine atoms in a vinyl side chain of the compound. The presence of halogens contributes to greater insecticidal activity and high stability, as well as providing better residual activity against insects. Additionally, the presence of halogens leads to a higher potential for negative environmental effects (Bradbury & Coats, 1989).

The combined use of a set of complementary biomarkers that can both signal exposure to pollutants and quantify their impact on living organisms allows for a more comprehensive and integrative assessment of the biochemical and cellular effects induced by environmental pollutants (Linde-Arias et al., 2008; Cazenave et al., 2009).

The fish liver is a key organ that controls many functions and plays an important role in fish physiology, both in

anabolism and catabolism (Bruslé & Anadon, 1996). The activity levels of plasma enzymes (alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH)] and metabolites (cortisol, glucose, cholesterol, total protein, creatinine, blood urea nitrogen and etc.)) are often used as sensitive indicators of the harmful effects of pesticideson vital fish tissues (Canli et al., 2018; Firat & Tutus, 2020). Toxic compounds interfere with key enzymatic processes that are associated with the animal's normal physiology. This includes carbohydrate, protein and lipid metabolism. In this regards, biochemical changes occur before the appearance of tissue pathological symptoms (Ksheerasagar et al., 2011).

Lipids can also be synthesized by the liver (endogenous lipid metabolism). Fish store lipids in various organs, including mesenteric membranes, muscles and liver. Hepatic lipid accumulation is induced by biochemical mechanisms, such as decreased hepatic lipid export, increased hepatic uptake of circulating fatty acids, decreased hepatic beta-oxidation, and increased hepatic fatty acid synthesis (Sheridan, 1988). Abnormal lipid accumulation in the liver has been suggested to be among the most common pathological hepatic responses to chemical exposure (Cave et al., 2011), however it is not that commonly studied in the aquatic toxicology research compared to other biological approaches (enzymes, histopathology, behavior, etc.).

Material and Methods

Certified, juvenile fish were purchased from the Institute of Fisheries and Aquaculture, Plovdiv, where they are bred and raised under strictly controlled conditions. The experimental individuals were of the same size-age group (average length 10.1 ± 0.4 (sd) cm; average weight 11.15 ± 0.6 (sd) g), without external pathological changes. The individuals were transported using polyethylene containers filled with water from the Institute of Fisheries and Aquaculture, Plovdiv, in which oxygen tablets were added. After transportation, the fish were separated into 100 L glass aquaria with pre-dechlorinated water to acclimatize for 2 weeks. During this period, survival and behavior of the fish were also tracked. In addition, during the acclimation period, the fish were fed with specialized carp pellets (CarpCo Excellent Koi Grower, Helmond, The Netherlands) and a twelve-hour light period (12:12) was provided. The fish were not fed for 2 days before the start of the experiment.

The tested concentrations of toxicants for the short-term and long-term laboratory experiment were provided and prepared by expert chemists. The concentrations were prepared according to Bulgarian legislation (Directive 2013/39/EU), representing 100%, 50%, 30 % of MAC-EQS (0.1 μg/L - CPF; 0.0006 μg/L - CYP) for each test chemical. The reason for choosing lower concentrations than MAC-EQS was that in real conditions biota are usually subjected to the chronic action of various toxicants that are presented in rather low concentrations. Moreover, the concentrations according MPC-EQS were applied as the experiment was conducted once. The pesticides were dissolved in methanol and an aquarium with clean, tap,

dechlorinated water was used as a control.

The test fish were randomly divided into 15 individuals in the different concentrations of the pesticides, including a control group (no added chemicals) and were treated under static conditions for 96 hours and 30 days (acute, short-term and chronic, long-term exposure) with nominal and ecologically relevant concentrations of CPF and CYP (Yancheva et al., 2019). During the experiment, the fish were observed for macroscopic and behavioral changes (APHA, 2005). A methanol control group was not used because it was considered that its significantly low concentration would have no observed effects. The test was conducted in a static environment with no water changes for 96 hours and 30 days (Modesto & Martinez, 2010 a,b; Santos & Martinez, 2012; Filho et al., 2017; Sánchez et al., 2018). We also applied this model in our previous study on the subject (Georgieva et al., 2021; Yancheva et al., 2022).

The physico-chemical parameters of water: pH , temperature, °C, electrical conductivity, µS/cm, dissolved oxygen, mg/L were measured at the 96th hour and the 30th day using a combined instrument (MultiLine® Multi 3510 IDS, WTW-Xylem Analytics, Weilheim, Germany) and published in our previous research (Georgieva et al., 2021; Yancheva et al., 2022).

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 the material. They were stained for lipids by Sudan Black B staining according to Daddy's (1896) method and PAS-reaction (Schiff-Iodic acid) according to McManus (1948) for polysaccharides (liver glycogen). The histochemical changes in the liver of experimental individuals were presented semiquantitatively according to our modified scale of Mishra & Mohanty (2008), as follows: (–) – negative reaction of histochemical staining; (+/–) – very weak positive histochemical reaction; (+) – weak positive histochemical reaction; (++) – moderate positive reaction to histochemical staining; (+++) – strong positive histochemical reaction in the hepatocytes.

The biochemical study of liver enzymes - LDH and the aminotransferases ASAT and ALAT, was carried out at the Technology Center at the Faculty of Biology, Plovdiv University "Paisii Hilendarski". The liver samples from both exposures were thawed on ice and homogenized with 50 mM, 300 mM NaCl (pH=7.4). The homogenates were centrifuged at 4°C for 15 min at 9000 rpm using a refrigeration centrifuge (MPW, Poland). After centrifugation, the supernatant was separated into new Eppendorf tubes and frozen at -80°C to measure the activity of the above enzymes. Lactate dehydronese activity of the liver extracts was determined using commercially available enzymatic kit (LDH FL DGKC, Cat. LD F245 CH, Chema Diagnostica, Italy). Alanine aminotransferase activity of the liver extracts was determined using commercially available enzymatic kit (GPT/ALT FL IFCC, Cat. LD F400 CH, Chema Diagnostica, Italy). Aspartate aminotransferase activity of the liver extracts was determined using commercially available enzymatic kit (GOT/AST FL IFCC, Cat. GO F400 CH,

Chema Diagnostica, Italy). All the reactions of LDH, ALAT and ASAT were prepared according to the instructions of the manufacturer of the kits and. The decrease of the light absorbance of the reactions at 340 nm, due to the oxidation of NADH to NAD+ during the reactions was determined spectrophotometrically The enzyme activity was measured with a spectrophotometer (Beckman Coulter DU 80 model, Inc., Brea, CA, USA) at 25°C.

The amount of total protein in the tested samples was determined spectrophotometrically at 595 nm according to the method of Bradford (1976) and the protein content was calculated against a standard curve of bovine serum albumin. The obtained values of LDH, ALAT and ASAT and the protein content of the samples were used to calculate the specific enzyme activity (U/mg protein) of the enzymes of interest.

All the analyses of LDH, ALAT, ASAT and protein content were performed in triplicate and the obtained average values of the measurements were used to calculate the acticities of the corresponding enzymes including ±SD.

Results and Discussion

The PAS-reaction is a staining method used to detect polysaccharides, such as glycogen in tissues, and the staining is mainly purple-magenta in color (Ngokere et al., 2016). It is mainly used in medicine, but can be applied along with other combined biomarkers in aquatic toxicology to study the negative effect of various toxicants for better results. The PAS-reaction works on the basis of the oxidation by the periodic acid (or its salts) of 1,2-glycol groups contained in these compounds to aldehydes, which are detected with the help of Schiff's reagent. The staining with Sudan Black B detects the presence of fatty degeneration in the cells, with the cytoplasmic lipids stained dark gray or black.

As a result of the histochemical analysis by applying the PAS-reaction, we observed an increase in the amount of glycogen in the hepatocytes of the experimental individuals. We found an increase in glycogen according to the proposed scale both in CPF exposure and in all tested CYP concentrations. With a similar degree of expression, we observed an accumulation of glycogen in the cytoplasm of hepatocytes in after 30 days in both exposures (Table 1; Fig. 1, 3).

When monitoring lipid inclusions in the hepatocytes, we observed an increase in the degree of Sudan Black B staining. In the short-term exposure, the degree of staining was similar for both toxicants. When following the changes that occurred after the long-term exposure, we found an increase in the degree of lipid inclusions, again with a similar degree of expression for both test toxicants. The obtained results confirm the observed fatty degeneration with increased lipid inclusions in hepatocytes through the histopathological analysis, as well as its degree of expression (Table 2; Fig. 2, 4).

Table 1. *Degree of PAS-reaction in the liver of common carp.*

Exposure time	Control	0.03 µg/L CPF	0.05 µg/L CPF	0.01 µg/L CPF	0.0002 µg/L CYP	0.0003 µg/L CYP	0.0006 µg/L CYP
96th hour	+/-	+/-	+	+	+/-	+	++
30th day	+/-	+/-	+	+	+/-	+	++

Table 2. *Degree of Sudan Black B staining in carp (Cyprinus carpio) liver.*

Exposure time	Control	0.03 µg/L CPF	0.05 µg/L CPF	0.01 µg/L CPF	0.0002 µg/L CYP	0.0003 µg/L CYP	0.0006 µg/L CYP
96th hour	+	+	+	++	+	++	++
30th day	+	+	+	++	+	++	++

Similarly, to us, Shrivastava (2007) considered that changes in the amount of glycogen in the liver indicate changes in carbohydrate metabolism under the influence of various pesticides. Changes associated with an increase in the amount of glycogen in hepatocytes compared to the control group of fish may be due to changes in the amount of pyruvate, and this may affect the processes of glycogenesis, glycogenolysis and gluconeogenesis.

According to Yogesh & Venkateshwarlu (2022), significant changes in the levels of glucose, glycogen, total protein and free amino acids, as well as changes in the activities of the enzymes lactate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, protease, aspartate aminotransferase and alanine aminotransferase were observed in common carp tissues and the changes developed progressively with exposure time. In addition, changes in the carbohydrate metabolic enzymes indicate that pesticides cause metabolic disturbances and change aerobic metabolism to anaerobiosis, while the changes in protein metabolic enzymes explain that pesticides may interact with peptide sequences in the fish directly or indirectly.

An increase in the lipid inclusions in the cytoplasm of hepatocytes is due to fatty degeneration in the liver cells, which was also found in the histopathological examination of the liver (Georgieva et al., 2021; Yancheva et al., 2022). Increased synthesis of fatty acids leads to increased synthesis of triglycerides and hyperlipidemia associated with fatty infiltration in hepatocytes. In agreement with Ayoola (2008), we believe that the changes related to the alterations in the amount of glycogen and lipids in the liver of the experimental individuals can generally be due to a change in the processes of glycolysis, which depends on the administered concentrations of the toxicant, the duration of action or its chemical nature.

Lactate dehydrogenase (LDH, ES 1.1.1.27), which is an important biomarker enzyme, 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 has a negative effect on its activity (Mommsen, 2000). In general, however, less is known about carbohydrate metabolism in the liver of fish. It has been studied mainly in their muscles, and the studies in the available literature, in which it is considered under the action of heavy metals and various organic toxicants on liver, are considerably less (Norton et al., 2000; Almeida et al., 2002; Cooper et al., 2002; Napierska & Podolska, 2008; Oliva et al., 2012).

The results from the analysis showed a decrease in LDH activity at the 96-hour exposure, and we observed a stronger inhibition at the CYP exposure (Fig. 5). Similar to the short-term test, at the 30-day exposure we found inhibition of the specific enzyme activity, observing a similar trend comparing the two toxicants (Fig. 6).

LDH is a tetrameric glycolytic enzyme that has been used as a potential marker of tissue damage (Diamantino et al., 2001). Viarengo (1985) proved that the action of various poisons can lead to a change in the activity of LDH by binding them to some functional groups. This enzyme is involved in the carbohydrate metabolism, but also serves as an indicator of chemical pollution of the natural environment. The study of

LDH activity can provide useful information about the cellular metabolism in organisms that are subjected to the action of different toxicants (Monteiro et al., 2006). We agree with Das et al. (2004 a,b) and Yousafzai & Shakoori (2011) that alterations in the LDH activity signal changes in cellular energy metabolism as a result of water pollution. For example, the increased activity of LDH can serve as an enzyme biomarker for the diagnosis of various disorders in tissues of many vertebrate organisms, which is often the result of oxygen depletion after the exposure to contaminants. This forces the organisms to start using energy produced through anaerobic processes. On the other hand, a decrease in the LDH activity can serve as a biomarker for increased glycolysis as a result of chemically induced stress in the body. A decrease in the activity of LDH as a result of chemically induced stress and an increased amount of free radicals leads to intensive processes of gluconeogenesis and glycogen accumulation, which is also proven by the histochemical analysis using the PAS reaction in our study. Along with the processes of gluconeogenesis, an enhanced process of glycolysis probably takes place, which in turn could lead to the accumulation of pyruvate, hence of Acetyl CoA and, as a result, to hyperlipidemia, which is again proven by the histochemical analysis with Sudan Black B in the current experiment. The stronger inhibition of the enzyme after the 30-day exposure we could also connect with the higher degree of the observed fatty degeneration through the histological and histochemical analysis, which again shows intense processes of glycolysis and hyperlipidemia.

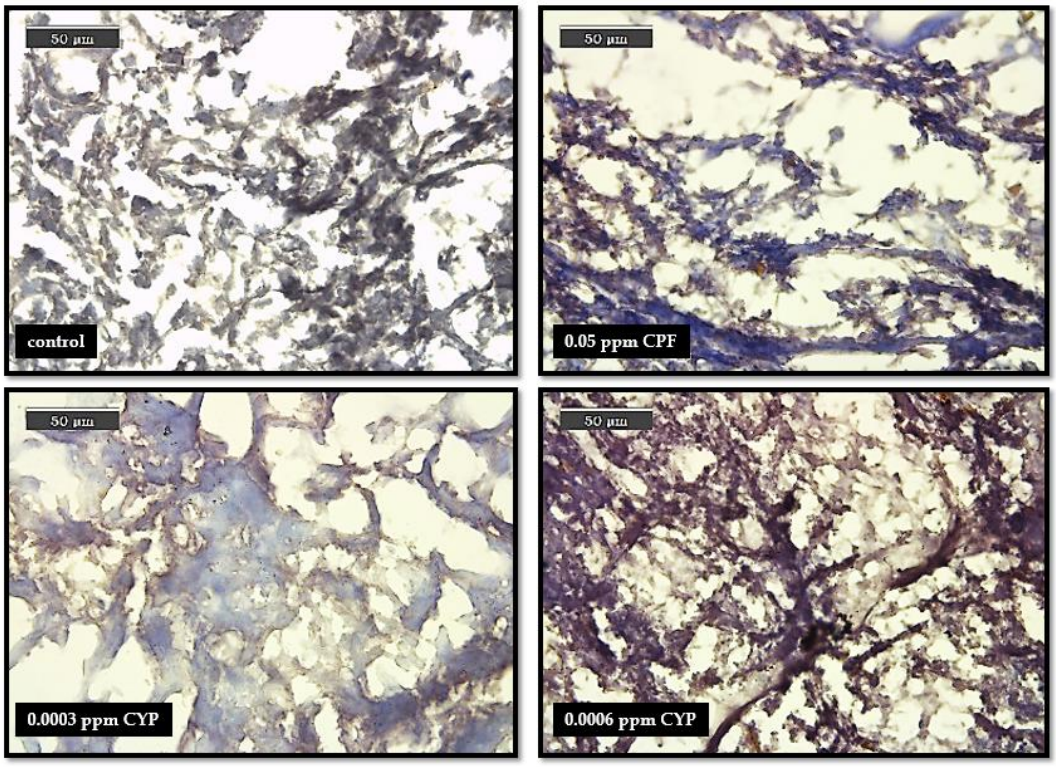

Fig. 1. Intensity of PAS-reaction in carp liver after 96-hour exposure.

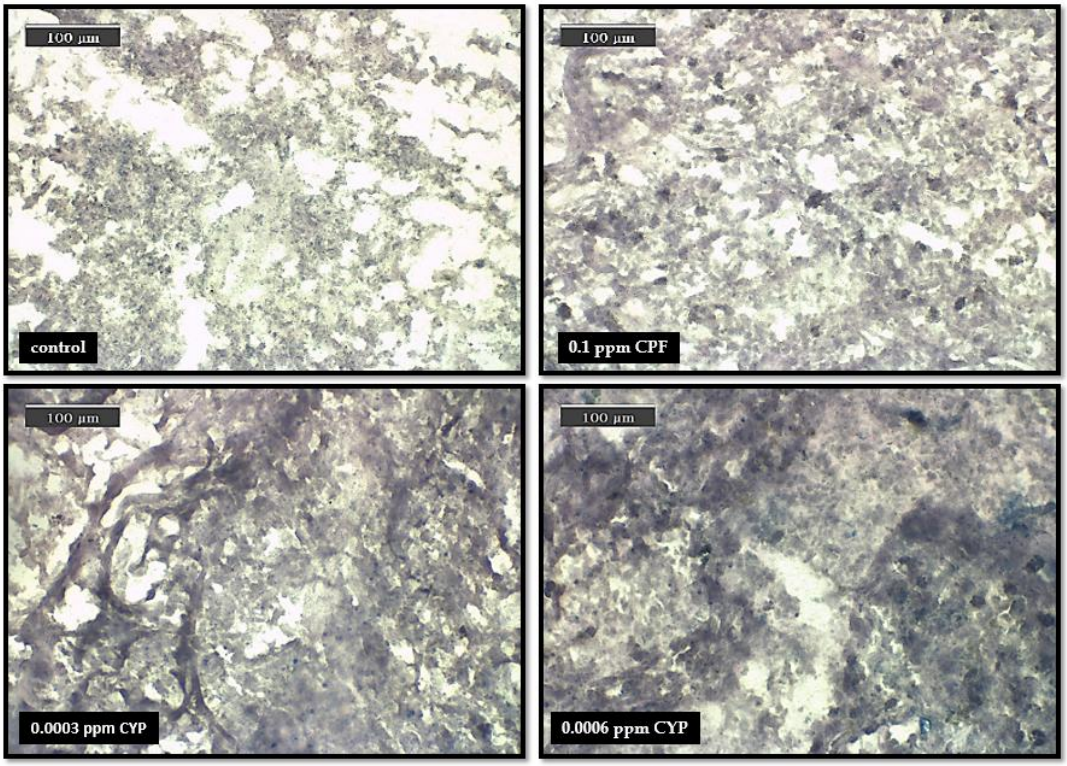

Fig. 2. Intensity of Sudan Black B staining in carp liver after 96-hour exposure.

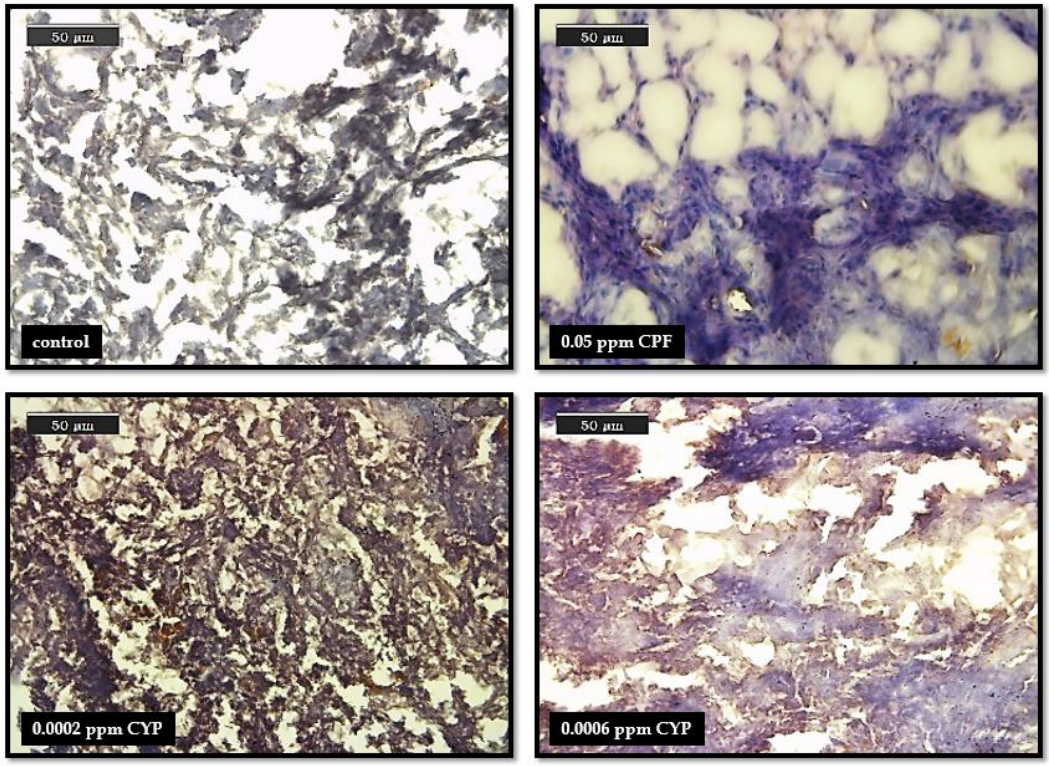

Fig. 3. Intensity of PAS-reaction in carp liver after 30-day exposure.

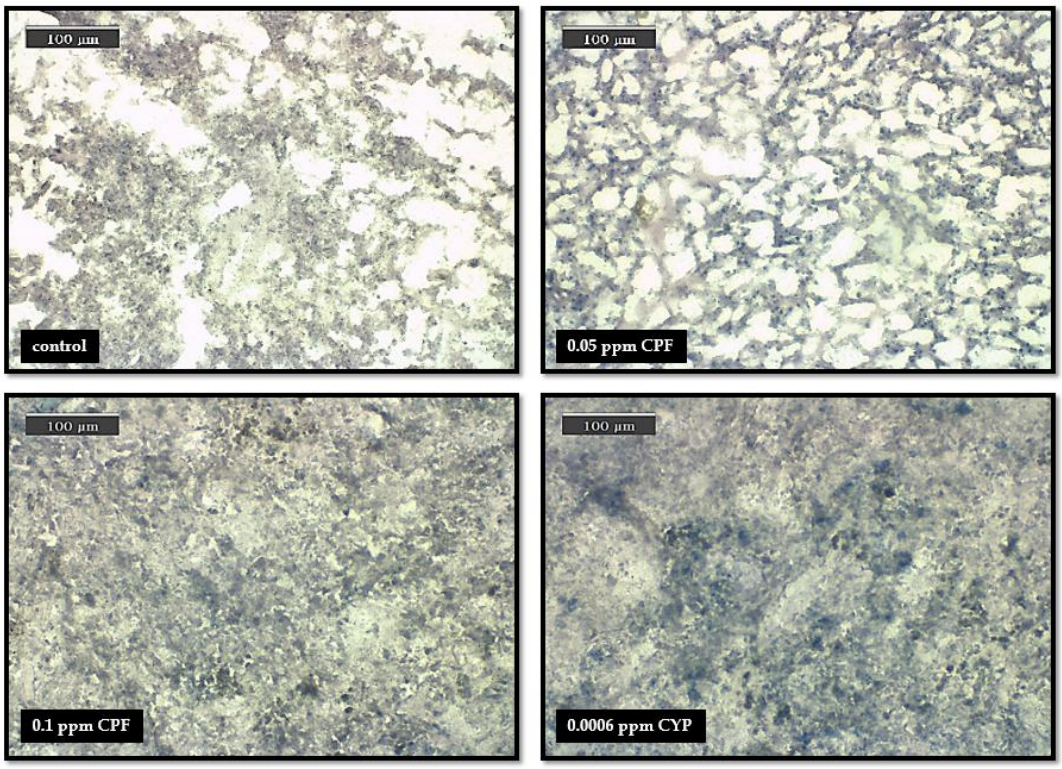

Fig. 4. Intensity of Sudan Black B staining in carp liver after 30-day exposure.

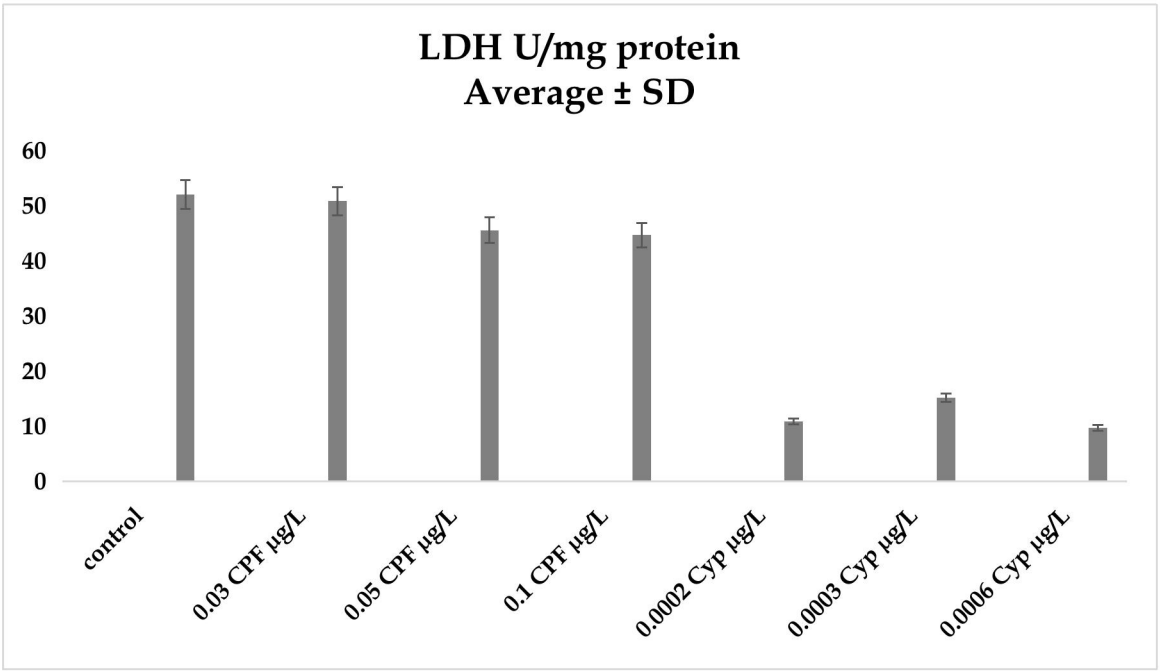

Fig. 5. Changes in the specific enzyme activity of LDH after 96-hour exposure to CPF and CYP.

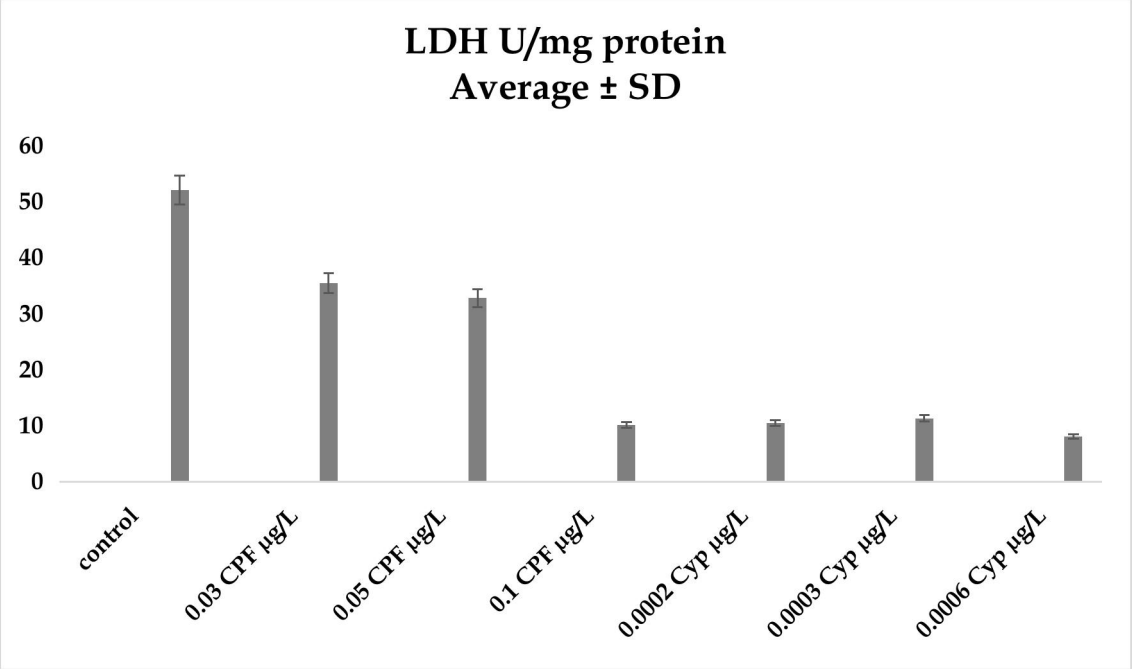

Fig. 6. Changes in the specific enzyme activity of LDH after 30-day exposure to CPF and CYP.

In addition, Rao (2006) found in his experiments an increase in the activity of LDH in the fish brain and gills, and a decrease in the activity of LDH in the liver under the influence of various pesticides, which clearly indicates different damage to the studied tissue.

Furthermore, Abhijith et al. (2016) found a decrease in the LDH activity in the gills and liver of *Catla catla* under the influence of methyl parathion (MP). According to the authors, the results show a higher degree of glycolysis under pesticide stress. According to Abhijith et al. (2016) MP can inhibit the aerobic and anaerobic metabolism of fish, leading to a decrease in the LDH activity, which was confirmed with the results obtained in the present study. Tripathi & Shasmal (2011) found reduction of the LDH activity in the gill, liver, brain and muscle of *Heteropneustes fossilis* exposed to CPF, which may be due to binding of pesticides or their metabolites to the enzyme molecule.

According to El-Shehawi et al. (2007) in ecotoxicological studies, the increase or decrease of ALAT and ASAT levels in the blood and other examined organs and tissues of fish can be used as a successful indicator of water pollution. ASAT is also considered a key enzyme for nitrogen metabolism and energy mobilization in invertebrates, is often used as a biochemical indicator of stress (Shobha et al., 2001). According to El-Shehawi et al. (2007) aminotransferases are widely used to diagnose the damage that toxins do to the liver in fish and some other organs, such as gills and muscles. In addition, transaminases (aminotransferases), have been widely used as sensitive markers of possible tissue damage, particularly liver toxicity, for many years in aquatic toxicology (Ramaiah, 2007).

The results on the changes in the aminotransferase activity showed inhibition of the specific enzyme activity in both the short-term and long-term exposure (Fig. 7, Fig. 8, Fig. 9, Fig. 10). We observed a stronger decrease in the specific activity after the 30-day exposure (Fig. 8, Fig. 10).

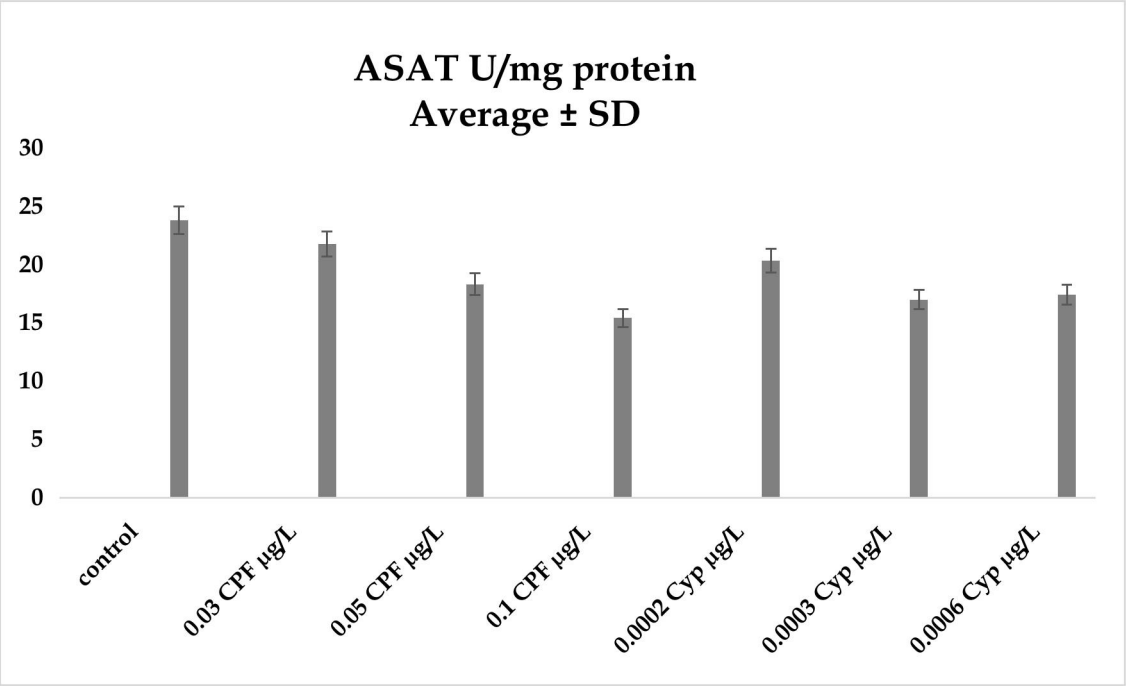

Fig. 7. Changes in the specific enzyme activity of ASAT after 96-hour exposure to CPF and CYP.

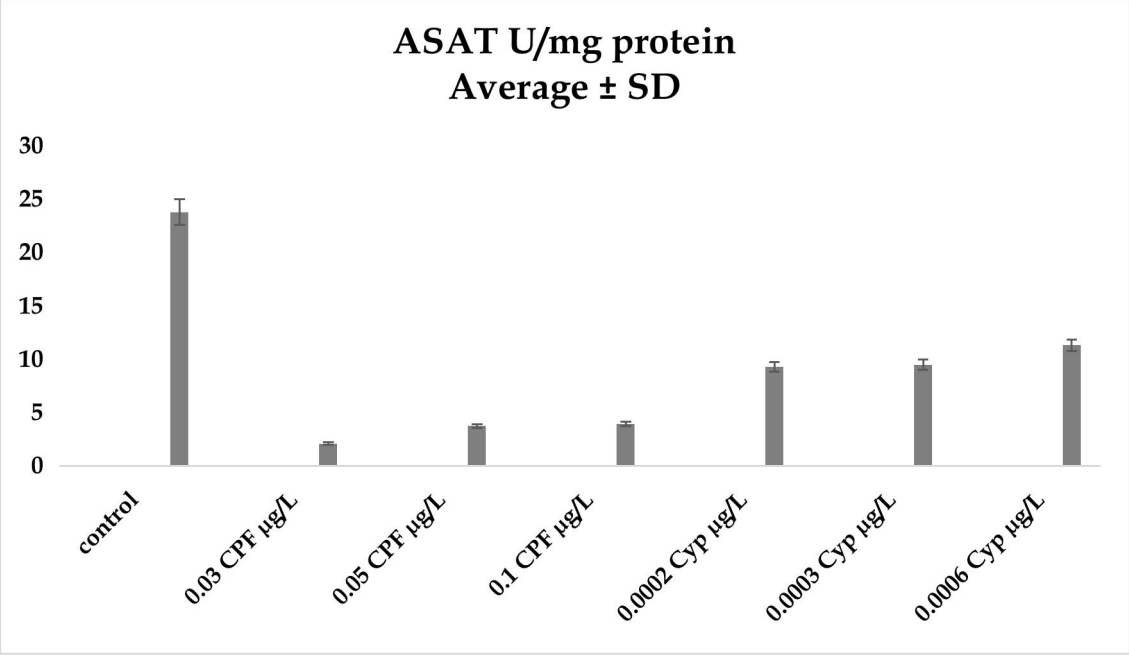

Fig. 8. Changes in the specific enzyme activity of ASAT after 30-day exposure to CPF and CYP.

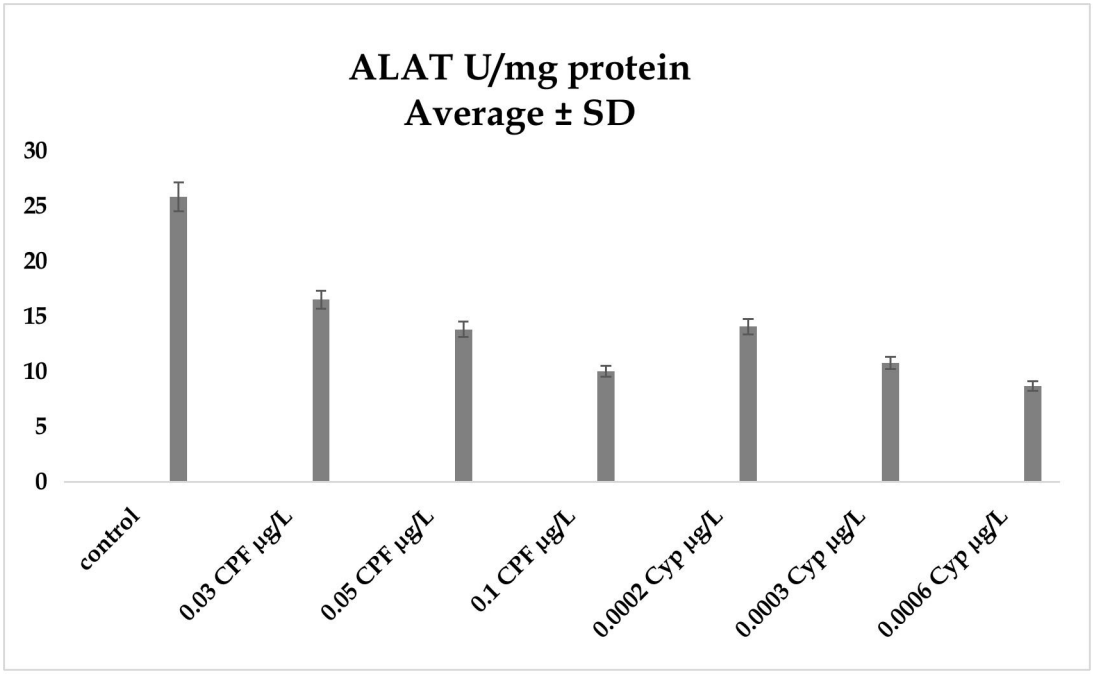

Fig. 9. Changes in the specific enzyme activity of ALAT after 96-hour exposure to CPF and CYP.

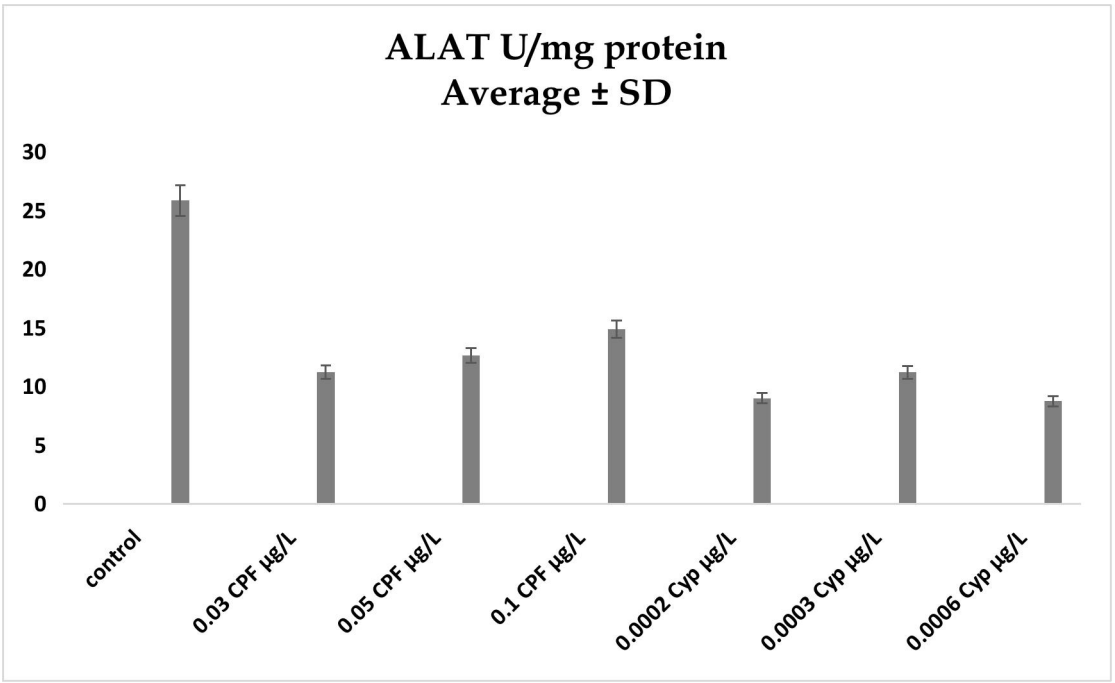

Fig. 10. Changes in the specific enzyme activity of ALAT after 30-day exposure to CPF and CYP.

A number of authors have followed the activity of aminotransferases in various organs of carp fish under the influence of different poisons. For example, Karan et al. (1998) measured a substantial increase in the ALAT activity, Velmurugan et al. (2008)

found an increase in the activity of aminotransferases in gills, brain and muscles of fish, Zikić et al. (2001) and Heydarnejad et al. (2013) indicated that the activity of aminotransferases increased in blood serum. Guo et al. (2002) reported an increase in the aminotransferase activity in liver under the influence of ytterbium.

Moreover, Al-Ghanim (2014) found an increase in ALAT, ASAT and GDH activity in all organs of fish exposed to cypermethrin. This suggests the active trans-deamination of amino acids to incorporate keto acids into the Krebs cycle to release the energy needed for new protein synthesis (Sivaramakrishna & Radhakrishnaiah, 1998). The elevation of these enzymes generally indicates amino acid utilization, while the elevation of transaminases suggests a strong efflux of metabolites under cypermethrin stress, as stress conditions generally induce an increase in the transamination pathway (Awasthi et al., 1984). Involvement of alternative pathways, such as aminotransferase reactions, is also possible due to inhibition of oxidative enzymes, such as isocitrate dehydrogenase and cytochrome C oxidase. Changes in the aminotransferase activity are often reflected in nitrogen metabolism and interdependent biochemical reactions. Elevated aminotransferase levels may be due to tissue damage after intoxication (Raju & Ramna, 1985). Amino acids appear to be mobilized to undergo transamination to 2-keto acids for use in the production of energy-rich compounds (Shobha et al., 2001)

Rao (2006) reported that the reduction of aminotransferase activity in the liver of *Oreochromis mossambicus* under the influence of organophosphorus pesticides may be due to liver damage. We agree with Abhijith et al. (2016) that the detoxification mechanism may not be efficient enough to prevent the toxicity and effect of the toxicant in the body. According to Tripathi & Shasmal (2011), 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. Neelima et al. (2013) also reported a decrease in the ALAT and ASAT activity when treating fish with cypermethrin.

The established decrease in the enzymatic activity of LDH, ASAT and ALAT after the long-term exposure with the test pesticides compared to short-term exposure could be also associated with the increased necrosis in the hepatocytes (Georgieva et al., 2021; Yancheva et al., 2022), which in turn indicates degenerative changes in the hepatic cells.

Conclusion

In sum, all concentrations of the tested pesticides cypermethrin and chlopyrifos in the short-term and long-term exposure negatively affected the histochemical and biochemical values in liver of common carp. The negative effects were expressed with liver changes in the amount of glycogen and lipids, and enzyme alterations in the specific activity of LDH, ASAT and ALAT. We found that these alterations affect the processes of glycogenesis, glycogenolysis, gluconeogenesis, carbohydrate and nitrogen metabolism. The results from such experimental set ups could be used in the legislation of protection water bodies from contamination in areas near intensive application of plant protection products and also in implementing the Water Frame Directive by using multi-biomarker approaches, which are not that common, but easy and reliable to apply, such as histochemistry.

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