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Research article

Assessment of the exposure of two pesticides on common carp (*Cyprinus carpio* Linnaeus, 1758): Are the prolonged biomarker responses adaptive or destructive?

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ABSTRACT

Chlorpyrifos (CPF) and cypermethrin (CYP) are two insecticides that have a proven negative effect on non-target aquatic organisms when they enter the surface waters. However, literature on the comparative effects of these pesticides on important aquaculture fish species, such as common carp (*Cyprinus carpio* Linnaeus, 1758) is not yet scientifically detailed, especially over the long-term. The idea of conducting a long-term exposure is to find out how the observed biomarkers would change compared to the short-term exposure. In the natural environment, toxicants are not present alone, but in combination. By monitoring the long-term impact of individual substances, the state of aquatic ecosystems exposed to various toxicants could be predicted. Thus, this study aimed to evaluate the toxicity of different concentrations of CYP (0.0002, 0.0003, and 0.0006 μg/L) and CPF (0.03, 0.05, and 0.10 μg/L) in 50-L glass tanks on *C. carpio*, exposed for 30 days under laboratory conditions. A set of histological and biochemical biomarkers in the gills and liver were applied with the chemical analyses of water and fish organs. Furthermore, the condition and hepatosomatic index were calculated to assess the physiological status of the treated carps. The behavioral responses were also monitored, and the respiration rate was analyzed. The results suggest that CYP had a more prominent effect on the histological structure of fish organs, biochemical responses of anti-oxidant enzymes, behavior, and respiration rate compared to the effect of CPF. In addition, the results also indicate that the liver is more susceptible to chronic and chemically induced cellular stress compared to the gills, with overall destructive changes in the histological biomarkers rather than adaptive. Regardless of the scenario, our results provide novel insights into pesticide exposure and the possible biological impacts on economically important freshwater fish, exposed to lower CYP and CPF concentrations, based on the EU legislation (maximum allowable concentrations, MAC-EQS).

1. Introduction

Pesticides are a group of man-made chemical compounds, invented to protect crops and control pests and diseases that damage them (Chibee et al., 2021; Tharmavaram et al., 2021). Furthermore, there are many classes of pesticides based on their target organisms or chemical composition, including insecticides. After their extensive application, according to Mohaupt et al. (2020) and Fuller et al. (2021), pesticide residues typically enter aquatic systems via agricultural and urban runoff (Somlyai et al., 2019), spray drift or direct application, with concentrations from highly populated areas exceeding toxicity thresholds for resident non-target invertebrate and vertebrate species. Therefore,

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pollution with pesticides is a key environmental problem, which needs to be addressed and solved (Chapman, 2007; Schwarzenbach et al., 2006).

The pyrethroid usage is preferred over other insecticide classes as explained by Lu et al. (2019) and Sharma et al. (2021) because of their high effectiveness, low mammalian toxicity, high pest specificity, and rapid environmental degradation. Moreover, according to Zhang et al. (2021), pyrethroid insecticides are one of the most commonly used pesticides in agriculture because of their broad-spectrum, insecticidal ability, and high efficiency. In addition, synthetic pyrethroids have been dominating the global insecticide market for a long time since the development of third-generation insecticides (Bej et al., 2021; Ghazouani et al., 2020; Manyilizu, 2019). Furthermore, Jaensson et al. (2007), Sharma et al. (2021) and Ullah et al. (2018) added that CYP is a fourth-generation halogenated and highly active synthetic pyrethroid, which is widely applied in agriculture and household pest management, and it has been gaining popularity since the 1970s. Unfortunately, most of the synthetic pyrethroids, including CYP enter the aquatic ecosystems through surface run-off from agricultural fields, seepage through the soil, and airborne transport. This turns out to be a serious threat to non-target aquatic organisms, including fish fauna.

In general, organophosphates (OPs) as explained by Aldridge (1950), can cause their toxic effects in target insects by binding to the enzyme acetylcholinesterase (AChE) and inhibiting the breakdown of acetylcholine in the neuronal synapses, which subsequently leads to neuronal effects and a lethal outcome. Chlorpyrifos (CPF) is an organophosphorus insecticide, which is frequently used for pest control in households and agriculture worldwide (Foong et al., 2020). According to Colovic et al. (2013), as an organophosphate, CPF inhibits the activity of AChE and prohibits the hydrolysis of acetylcholine, which therefore disturbs synaptic transmission. The commercial production of CPF started in 1969 and since then it has been used for various purposes (Halappa and David, 2009). For instance, CPF has been used for both agricultural and urban applications in the United States for over 60 years (USEPA, 2020) and it is often associated with aquatic toxicity after storm events (Bailey et al., 2000). Moreover, CPF pollution in water usually occurs as a result of soil erosion or through rainwater runoff (Grigorszky et al., 2019) and it is one of the ten chemicals with the highest risk to aquatic organisms in the United Kingdom, according to Johnson et al. (2017). CPF has recently been banned in Europe (European Commission, 2020) and it can no longer be produced, however, it can still be applied until it is no longer available for purchase on the market.

According to da Silva Montes et al. (2020), successful monitoring and risk-assessment programs begin with the choice of bioindicator species. Nakajima et al. (2019) explained that the cultivation of common carp (*Cyprinus carpio* Linnaeus, 1758) can be traced back to 8000 years ago. It is also the most reared fish in aquaculture in Bulgaria. Furthermore, it is among the top five of the most cultivated freshwater fish species in the world, along with grass carp (*Ctenopharyngodon idella* Valenciennes, 1844), silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844), and Nile tilapia (*Oreochromis niloticus* Linnaeus 1758), because of its wide distribution, high economic value, and growth traits (Cai et al., 2021; Sobczak et al., 2020; Zhai et al., 2021). Common carp, as explained by Shahi et al. (2022), contributes to 3.4 % of the global fish production and is overall cultured in >100 countries. Common carp also serves as an excellent test organism in ecotoxicological studies because it is relatively resilient to water pollution, including pesticides, which is essential for the selection of bioindicators in laboratory and field experiments (Georgieva et al., 2021; Stoyanova et al., 2020; Sula et al., 2020; Yancheva et al., 2019).

In field and laboratory risk assessment studies and monitoring programs, biomarkers as biological effect tools have been widely and successfully used for years. As stated by Sanchez et al. (2012), a single biomarker cannot provide a true and practical assessment of the toxicity of the pollutant on aquatic organisms, and hence it is recommended to utilize the multi-biomarker approach to better understand the response of an organism to toxic substances (Aliko et al., 2018). Several key biomarkers, such as oxidative stress enzymes - catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and levels of lipid peroxidation, combined with histological alterations in target organs can be conveniently employed to monitor water quality, assess chemical risks and evaluate the organism's susceptibility to specific contaminants (Delahaut et al., 2019; Sharma and Jindal, 2020; Ullah et al., 2018). These biomarkers are usually applied along with condition and hepatosomatic index, behavioral responses, and respiration rate, which are easily performed and relatively inexpensive, compared to chemical analyses.

As explained in detail by Thoré et al. (2021a), the present ecotoxicological assessment focuses mainly on short-term exposure to pollutants, such as pesticides. Nevertheless, there is an apparent shortage of information on the chronic or long-term impact of chemicals (Philippe et al., 2017, 2018b). In addition, according to European Commission (European Commission, 2002), long-term studies are needed if the degradation time (DT_{50}) of a compound indicates persistence in the environment for two days or longer. Also, long-term ecotoxicological studies are primarily carried out on invertebrates because classic vertebrate models require more labor and time, and have a higher cost (Philippe et al., 2017; Thoré et al., 2021b).

The European Environmental Agency has defined two environmental quality standards (EQS), an annual average (AA-EQS) and a maximum allowable concentration (MAC-EQS) - 0.0006 μg/L for CYP and 0.1 μg/L for CPF (EC, 2011). In this regard, many studies (Georgieva et al., 2021; Kafula et al., 2022; Philippe et al., 2018a; Stoyanova et al., 2020; Yancheva et al., 2019) have focused on the acute effects of CYP and CPF on fish, however, the prolonged impact of these pesticides remains poorly understood. Thus, the present study deals with the investigation of the long-term effects of lower CYP and CPF concentrations, based on Directive 2013/39/EU (Commission of the European Communities, 2013), on different biomarkers in common carp gills and liver. Furthermore, for the first time, the impact of short-term (96 h) (Georgieva et al., 2021) and long-term (30 days) exposure to these two pesticides could be compared and further discussed. Overall, we hypothesize that the long-term effects of pesticides will be rather different from the short-term ones; the fish will acclimate to the polluted aquatic environment, however, we expect that the disturbances in the studied biomarkers will be more severe because of the chronic exposure.

2. Materials and methods

2.1. Test chemicals

Since this experiment is a continuation of our previous study (Georgieva et al., 2021), the test chemicals and concentrations, which were used here were the same. $C_9H_{11}Cl_3NO_3PS$ (*O*,*O*-Diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) (CAS Number: 2921-88-2, molecular weight 350.59) and $C_{22}H_{19}Cl_2NO_3$ (cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) (CAS Number 52315-07-8, molecular weight 416.30) were obtained from Merck (Darmstadt, Germany). For CYP - 0.03, 0.015, and 0.01 μg and for CPF - 5, 2.5, and 1.5 μg were applied in 50-L glass tanks. These concentrations were considered environmentally relevant concentrations of CYP and CPF after a thorough review of the scientific literature (Georgieva et al., 2021; Philippe et al., 2018a; Stoyanova et al., 2020; Yancheva et al., 2019).

2.2. Long-term experimental exposure

105 common carp juveniles with normal morphology and no visible histological lesions were purchased from the Institute of Fisheries and Aquaculture in Plovdiv, Bulgaria. The Institute of Fisheries and Aquaculture propagates freshwater fish species, important for aquaculture under physicochemical conditions, which are monitored regularly

(www.ira-plovdiv.bg). The one-winter carps were about the same size class [average total length 10.1 ± 0.4 (SD) cm; average body mass 11.15 ± 0.6 (SD) g]. After transportation to the laboratory and acclimatization for a week in two 100-L containers with chlorine-free (by evaporation) tap water, attached with oxygen pumps (a 12-hour light:12-hour dark cycle), the fish were randomly split into seven tested groups – 3 for CYP and 3 for CPF, including a control (n = 15 in each aquarium). The fish were exposed to the experimental concentrations of CYP and CPF for 30 days and the study was performed once as a pilot study on the negative short-term and long-term effects of CYP and CPF, supported by the National Scientific Fund (Sofia) in Bulgaria. The carps were given commercial pellets (CarpCo Excellent Koi Grower, Helmond, the Netherlands) every day, but the feeding was suspended 48 h before dissection (about 3.15 g per aquarium). The long-term exposure was performed in a semi-static environment, following Kunwar et al. (2021). Faeces and other waste residues were removed daily after the short-term experiment (96 h) had finished, and consequently, 25–30 % of the water in the aquaria was replaced with water, containing the respective amount of pesticides. After the water replacements, the concentrations of chemicals were remeasured. The basic physicochemical characteristics of the tested water (conductivity, dissolved oxygen, pH, and temperature) were measured with a multi-parameter portable meter (MultiLine® Multi 3510 IDS, WTW-Xylem Analytics, Weilheim, Germany) and monitored every day as suggested by APHA (2005). The experimental setup was performed by the approved ethical rules of the Faculty of Biology, Plovdiv University, Bulgaria (No 4/10.09.2019).

2.3. Dissection procedure

The guidelines of Directive 2010/63/EU (European Union, 2010) regarding the protection of animals used for scientific purposes were followed and the fish were euthanized with minimum pain [100 mg/L water of tricaine methane sulfonate (MS222)] (Argent Chemical Laboratories, Redmond, WA, USA) (Stoyanova et al., 2020). Dissection was done according to the protocol given by Rosseland et al. (2003). Every fish (n = 15 from test concentration) was weighed on a scale (to the nearest 0.01 g) and measured with calipers (to the nearest 0.01 cm) for calculation of the condition and hepatosomatic index. Gill and liver samples, wrapped in aluminum foil were collected for bioaccumulation studies, placed in vials with formaldehyde for histological analyses, and in sterile plastic bags for biochemical assays, respectively.

2.4. Chemical analyses, method validation, and bioaccumulation factor (BAF)

For the chemical analyses, water (n = 5 batches per tested container) and fish gills and livers (n = 5 fish per tested container), including the control were collected on day 30. The chemical studies were performed at the regionally accredited laboratory (Plovdiv, Bulgaria). The sample preparation was carried out with a microwave system (MARS 6, Spectrotech, Charlotte, USA). About 10 g of sample was weighed, which was then extracted with organic solvent hexane:acetone (1:1) by microwave decomposition at temperature of 120 °C and retention of 25 min. The resulting extract was concentrated, purified by silica gel, and concentrated again. The water and fish samples were analyzed by gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) with an Agilent 7890B instrument (Thermo Fisher Scientific, Waltham, MA, USA) as previously performed (Mackay and Fraser, 2000). The limits of quantitation (LoQ) for CYP were set at 0.0002 μg/L for water and 0.009 μg/g for fish (wet weight), and for CPF at 0.005 μg/L and 0.15 μg/g, respectively. Certified reference materials – Chlorpyrifos D10 (CAS: 285138-81-0) and Atrazine D5 (CAS: 163165-75-1) (CPAchem, Bogomilovo, Bulgaria), as well as Cypermethrin (CAS number: 52315-07-8) (Merck, Darmstadt, Germany), were also analyzed to monitor the instrumental performance, peak height, and resolution. All the chemical results showed a good agreement with the standards and the recovery ranged between 96 % and 101 % for the water, and between 95 % and 106 % for the fish samples.

The bioaccumulation factor (BAF) was also determined, following Mackay and Fraser (2000), according to the formula below:

$$BAF = C_B/C_{WT},$$

where C_B - chemical concentration in an organism and C_{WT} - total chemical concentration in the water.

2.5. Test biomarkers

In addition to the histological lesions in the gills and liver, and the biochemical alterations in the liver, the behavioral responses were assessed daily, as explained by Bej et al. (2021). The physiological status (i.e., respiration intensity) was studied on day 30, following Tsekov (1989). Furthermore, the condition and hepatosomatic index for the overall health status of the fish were also calculated on day 30. The chemicals used for the selected histological and enzymatic assays were of analytical grade and were used as received, without any further purification (Merck, Darmstadt, Germany). Following Cui et al. (2020), the biochemical analyses included sterile laboratory plastic or glassware, which was also washed with double distilled water before use, and samples in triplicates and blanks run in sequence to check the instrumental performance, contamination, peak identification, and quantification.

2.5.1. Histological analyses

The dissected-out gills and livers were fixed in 10 % neutral buffered formaldehyde for 24 h and then thoroughly washed in tap water. The samples were dehydrated through a graded alcohol series, cleared in xylene, and finally embedded in paraffin wax. The tissue sections were cut at 5 μm, using a rotary microtome (Leica RM 2125 RTS, Leica Microsystems, Wetzlar, Germany) and stained after the hematoxylin-eosin (H&E) method by Gautier (2011). Mounting was done in Canada balsam. The prepared slides (n = 10 from a fish) were blinded and further investigated for histological alterations with a light microscope (Leica DM 2000 LED, Leica Microsystems, Wetzlar, Germany) and photographed at a 40× and 100× magnification with a digital microscope camera (Leica MS170 HD, Leica Microsystems, Wetzlar, Germany).

The histological lesions were assessed, according to the semi-quantitative system suggested by Bernet et al. (1999). A five-degree (0–5) severity gradation scale, which represents the severity of each lesion, according to Saraiva et al. (2015) was also used (0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations). In addition, the organ index values (I_O) were calculated to classify the severity of histological response, using classes based on the scoring scheme proposed by Zimmerli et al. (2007). The prevalence of gill and liver histological alterations was finally calculated as the percentage occurrence within the total number of examined slides per fish per tested pesticide concentration.

2.5.2. Biochemical analyses

The antioxidant defenses were evaluated by measuring the activity of catalase, glutathione peroxidase, and glutathione reductase. The liver samples were defrosted on ice and individually homogenized, using a Pyrex Potter-Elvehjem tissue grinder with a PTFE pestle (Thermo Fisher Scientific, Waltham, MA, USA) in a chilled phosphate buffer (50 mM, 300 mM NaCl, pH = 7.4). The homogenates were centrifuged at 9000*g* and 4 °C for 15 min, using an LMC-4200R centrifuge (Biosan, Riga, Latvia). The obtained supernatants were divided into aliquots and stored at −80 °C for further biochemical assays. The catalase activity (CAT, EC 1.11.1.6) was measured, using the H_2O_2 decomposition for 5 min with a 30 s interval at a 240 nm absorbance, following Beutler (1984). The

glutathione reductase activity (GR, EC 1.8.1.7) was quantified by monitoring the glutathione-dependent oxidation of NADPH at an absorbance of 340 nm (Zhou et al., 2019). The glutathione peroxidase (GPx, EC 1.11.1.9) was determined, using the method after Wendel (1980). A Beckman Coulter Spectrophotometer DU 800 (Beckman Coulter, Inc., Brea, CA, USA) was used and all the enzymatic activities were determined at 25 °C. The total protein levels were determined, according to Bradford (1976) with Coomassie Brilliant Blue G-250, using bovine serum albumin at a 595 nm absorbance. The protein content was obtained in mg/ml and then the activity of the studied enzymes was calculated as specific enzyme activity (U/mg protein).

2.5.3. Physiological responses

2.5.3.1. Respiration rate. The respiration rate intensity by Tsekov (1989) was followed. It was measured at the end of the experiment (30 days). Five fish per treatment were transferred in 30-L glass tanks. The oxygen levels were measured, and the tanks were then covered with plastic foil to eliminate any oxygen transfer. The fish were left for one hour, then, the oxygen levels were again measured. The method is based on the calculation of the difference in the dissolved oxygen levels before and after the tested time:

$$I = Q_2/G, \text{where :}$$

I – respiration rate index; G – the weight of the fish, in grams, Q_2 – oxygen consumed by the fish between the two measurements (the difference between the oxygen levels before and after the 1-hour $Q_2 = Q_{Start} - Q_{1hour}$).

Q was calculated by the following formula:

$$Q = V \times q, \text{where :}$$

Q – total oxygen level in the water tank; V – the volume of the water in the tank, in liters; q – level of dissolved oxygen in 1 L of water (mg/L).

2.5.3.2. Condition and hepatosomatic index. The health status of the fish at each tested concentration was assessed by calculating the relationship between fish weight and length, as well as between the weight of the whole fish and the liver, known as Fulton's condition factor (K) and hepatosomatic index (HSI) at the end of the experiment. Fulton's condition index (K) (Fulton, 1904) was calculated, according to the formula below:

$$K = \left(BW/TL^3\right)*100, \text{where :}$$

BW = total weight (g) and TL = total length – mouth to tail (cm).

The hepatosomatic index was calculated, following Delahaut et al. (2019):

$$HSI = (LW/BW)*100, \text{where :}$$

LW = liver weight (g) and BW = body weight (g).

Fish sex/stage was not assessed and the gonadosomatic index was not calculated in the present study.

2.5.4. Behavior analyses

The changes in fish behavior in all replicates were determined visually 3 times daily (in the morning, midday and evening) in their housing tanks by 3 different observers, following the combined and modified scoring systems by Neves et al. (2020), Bej et al. (2021) and Kunwar et al. (2021), assessed quantitatively: normal (1), none (0), mild (2), moderate (3), strong (4), very strong (5) reaction compared to the control. The behavioral responses included color change (CC), erratic swimming (ES), gill movement (GM), gulping of air (GA), hypoactivity (H), loss of equilibrium (LE), mucous secretion (MS), schooling behavior (SB), touch response (TR) and widening of the mouth (WM). The touch response itself was scored according to Stanley et al. (2009): (1) normal – fish quickly swim away from the source of the touch, move across the length of the aquarium, (2) – fish quickly respond to the touch, includes prolonged periods of swimming and fish act disoriented, (3) – fish are slower to respond to the touch and swim a shorter distance, (4) – fish flick the tail in response to the touch, but do not swim, (5) – paralysis - fish do not move. All behavioral responses were recorded for each individual (n = 15 per treatment) and the average results were presented.

2.6. Statistical analyses

Past 3.03 (Hammer et al., 2001) and GraphPad Prism 7 for Windows (USA) were applied for the statistical analyses in the present study. The results were presented in μg/L for the tested water and μg/g for the bioaccumulated pesticides in the tested fish organs, and U/mg protein for the specific CAT, GPx, and GR activities. The results from all conducted analyses were expressed as mean ± standard deviation (SD). The normal distribution was determined with the Shapiro–Wilk test. The homogeneity of variances was tested with Levene's test. The results were also analyzed for the significance of differences among the control and the replicates by one-way analysis of variance (ANOVA), followed by Tukey's test (means comparison) and by Kruskal-Wallis test, followed by the Mann-Whitney test (medians comparison). The levels of statistical significance were set at $p < 0.05$.

3. Results

3.1. Water physicochemical properties

As presented in Table 1, the basic physicochemical properties of the water during the long-term exposure (30 days) remained relatively constant, without any unexpected changes between the control and the tested tanks (ANOVA, Conductivity CPF: $F = 1.02$, $df = 3; 16$, $p > 0.05$; Conductivity CYP: $F = 1.34$, $df = 3; 16$, $p > 0.05$; O_2 CPF: $F = 1.45$, $df = 3; 16$, $p > 0.05$; O_2 CYP: $F = 1.34$, $df = 3; 16$, $p > 0.05$; pH CPF: $F = 1.64$, $df = 3; 16$, $p > 0.05$; pH CYP: $F = 1.53$, $df = 3; 16$, $p > 0.05$; T CPF: $F = 1.75$, $df = 3; 16$, $p > 0.05$; T CYP: $F = 1.72$, $df = 3; 16$, $p > 0.05$). Similarly, to the short-term exposure (96 h) they will not be further discussed as we consider that the changes in the studied biomarker responses were not associated with changes in the abiotic properties.

3.2. Pesticide concentrations and bioaccumulation factor (BAF)

The results for the content of the two pesticides in the water are presented in Table 2. In the control group, residues of CYP and CPF were not detected in either the water or the fish. However, our results indicate that the concentrations of CYP and CPF in the treated groups were higher than in the control; they were also higher compared to our short-term results (Georgieva et al., 2021). The CPF concentrations differed significantly among the treatment groups of water, gills, and liver, respectively (ANOVA, water: $F = 2980$, $df = 2; 12$, $p < 0.001$; gills: $F = 7.517$, $df = 2; 12$, $p < 0.01$; liver: $F = 10{,}650$, $df = 2; 12$, $p < 0.001$) (Table 2). The concentrations of CYP also differed significantly among the treatment groups of water, gills, and liver, respectively (ANOVA, water: $F = 2488$, $df = 2; 12$, $p < 0.001$; gills: $F = 1157$, $df = 2; 12$, $p < 0.001$; liver: $F = 6662$, $df = 2; 12$, $p < 0.001$) (Table 2).

The BAF values, which were obtained for the fish organs, are also presented in Table 2. The BAF for CYP for all the test concentrations in the fish gills and liver ranged from 3.4190 to 49.2308, while those for CYP ranged from 17.5905 to 619.2308 for the CPF concentrations, respectively. The BAF values for CYP did not differ significantly among the different groups in the case of gills (ANOVA, $F = 2.114$, $df = 2; 12$, $p = 0.1635$). At the same time, there were significant differences among the BAF values for CYP for the liver from the different groups (ANOVA, $F = 2016$, $df = 2; 12$, $p < 0.001$). The BAF values for CPF also differed significantly among the different groups in the case of gills and livers, respectively (ANOVA, gills: $F = 2077$, $df = 2; 12$, $p < 0.001$; liver: $F = 1007$, $df = 2; 12$, $p < 0.001$).

Table 1
Average results on the basic physicochemical properties of the water treated with cypermethrin (CYP) and chlorpyrifos (CPF) for 50-L tanks measured during the long-term exposure (30 days).

Parameters	Control	Concentration of CYP (μg/L)			Concentration of CPF (μg/L)		
		0.0002	0.0003	0.0006	0.03	0.05	0.10
Conductivity (μS/cm)	510	489.0	531.3	545.0	527.0	514.5	532.5
Dissolved oxygen (mg/L)	9.03	8.91	8.81	9.00	9.25	9.25	9.00
pH	7.8	7.53	7.57	7.50	7.50	7.31	7.10
T (°C)	12.5	11.5	11.5	12.1	12.01	12.03	12.02

Table 2
Average results on the concentration of cypermethrin (CYP) and chlorpyrifos (CPF) in the water (μg) and fish organs (μg/g wet weight) measured on day 30 (mean value ± standard deviation), as well as the calculated bioaccumulation factor (BAF).

	Control	Total applied amount of CYP, μg (for 50-L tanks)**		
		0.01	0.015	0.03
Water	n.d.*	0.0013 ± 0.000007[a]	0.0080 ± 0.000071[b]	0.0210 ± 0.000707[c]
Gills	n.d.	0.0083 ± 0.000045[a]	0.0634 ± 0.048747[b]	0.0718 ± 0.000447[b]
Liver	n.d.	0.0640 ± 0.000354[a]	0.1416 ± 0.002302[b]	0.9690 ± 0.005477[c]
BAF, gills	–	6.3615 ± 0.034401[a]	7.9250 ± 6.093413[a]	3.4190 ± 0.021296[a]
BAF, liver	–	49.2308 ± 0.271964[a]	17.7000 ± 0.287772[b]	46.1429 ± 0.260820[c]

	Control	Total applied amount of CPF, μg (for 50-L tanks)		
		1.5	2.5	5
Water	n.d.	0.8050 ± 0.007071[a]	1.8030 ± 0.009747[b]	2.9800 ± 0.083667[c]
Gills	n.d.	0.2472 ± 0.004658[a]	0.3130 ± 0.004472[b]	0.3694 ± 0.002608[c]
Liver	n.d.	0.2120 ± 0.002739[a]	0.2916 ± 0.002302[b]	0.4584 ± 0.004775[c]
BAF, gills	–	619.2308 ± 5.439283[a]	225.3750 ± 1.218349[b]	141.9048 ± 3.984095[c]
BAF, liver	–	190.1538 ± 3.583328[a]	39.1250 ± 0.559017[b]	17.5905 ± 0.124175[c]

a,b,c The values with different letters in the same row are significantly different (Tukey's test, $p < 0.05$).
* n.d.: not detectable.
** The limits of quantitation (LoQ) for CPF in water and fish were set at 0.005 μg/L and 0.15 μg/g and for CYP at 0.0002 μg/L and 0.009 μg/g wet weight, respectively.

3.3. Changes in the histological structure

3.3.1. Gills

The histological results did not reveal any morphological anomalies in the control group. Our ultrastructural analysis of the control was in agreement with Laurent and Perry (1991) and Evans et al. (2005) that the fish gills had a basic structural design (primary and secondary lamellae) with a single epithelium, surrounded by different cell types. In contrast, the fish treated with CYP (Fig. 1) and CPF (Fig. 2) showed severe histological changes in the gill architecture. These histological anomalies were expressed, both in the primary filaments and in the secondary lamellae. The anomalies could be classified into three groups – lesions in the circulatory system of the organ, degenerative and proliferative lesions, following Bernet et al. (1999) (Table 3). After exposure to CPF for 30 days in the gills' circulatory system, we observed mild vasodilation of the blood vessels in the filament and secondary lamellae (Fig. 2C). In parallel with vasodilation, we also found aneurysms, considered to be a more severe histological alteration, in the blood vessels in the secondary lamellae (Fig. 2D, Table 3). The degenerative changes in the gill epithelium were expressed in necrotic tissue, observed in both, the filament and the secondary lamellae, and the degree of expression was very mild (Table 3). Regarding the proliferative changes, after the exposure to CPF, we found the highest degree of fusion of the secondary lamellae, in a moderate degree (Fig. 2B). Edema and proliferation of glandular cells were observed mainly to a very mild degree. The level of lamellar lifting showed an increase, which was dose-dependent (Table 3).

Furthermore, the histological analysis after exposure of 30 days to CYP indicates also changes in the fish circulatory system, which were expressed in vasodilation of the blood vessels. Similar to the CPF exposure, aneurysms were found in the secondary lamellae. Vasodilation in the secondary lamellae and the filament was scored low at all three experimental CYP concentrations. The degenerative changes, affecting the epithelial tissue of the filament and the secondary lamellae were overall mild. However, at a higher level, we detected necrotic tissue in the primary lamellae (Table 3, Fig. 1D). The proliferative changes in the gills under the effect of CYP were observed in both, the filament, and the secondary lamellae (Fig. 1C). These lesions were described mostly as mild. The degree of lamellar lifting was maintained to the same extent at all the three experimental CYP concentrations, while the degree of proliferation decreased compared to the applied concentrations. Edema and fusion were observed mainly in a very mild degree (Fig. 1B). Glandular cell proliferation was found only at the highest CYP concentration in a very mild degree (Table 3).

Comparing the indices of histological lesions in the circulatory system (I_{GC}) for both pesticides we found mainly $I_{GC} = 8$, only at the lowest CPF concentration, we have $I_{GC} = 7$. Regarding the indices for degenerative changes in the gills (I_{GR}), we observed the same degree of these changes at all three applied CYP experimental concentrations, $I_{GR} = 6$. In contrast, after the CYP exposure, the indices showed an increase, associated with the test concentrations. At the other two, lower CYP concentrations we calculated $I_{GR} = 9$, while at the highest concentration the I_{GR} was found to be 12. The proliferative (I_{GP}) indices after exposure for 30 days to CPF and CYP showed a tendency to increase with increasing insecticide concentration to $I_{GP} = 21$ at 0.1 μg CPF. $I_{GP} = 17$ was calculated only at the highest CYP concentration (Table 3). In sum, because of the comparative study of the effects of CYP and CPF, we found relatively identical indices for the changes in the circulatory system. The proliferative change indices had higher values for the CPF exposure, while the indices for the degenerative changes – for the CYP exposure, confirm the higher toxicity of CYP compared to CPF.

In terms of the overall gill index (I_o), associated with the CPF concentrations, the lowest ones fell into the Class III group (index 21–30) – a moderate degree of change in the histological structure (reversible), according to Zimmerli et al. (2007), while the higher tested concentrations fell into Class IV (index 31–40) – severe degree of change in the histological structure (irreversible). All the CYP concentrations fell into Class IV (index 31–40), which once again confirms the more toxic mode of action of CYP. Furthermore, regarding the CPF toxic character in parallel with the proliferative changes, the inclusion of degenerative changes, as well as changes in the circulatory system of the organ were observed. On the other hand, after the CYP toxic action, a lower degree of proliferative changes was found, at the expense of more active inclusion of degenerative changes, associated with necrotic processes in

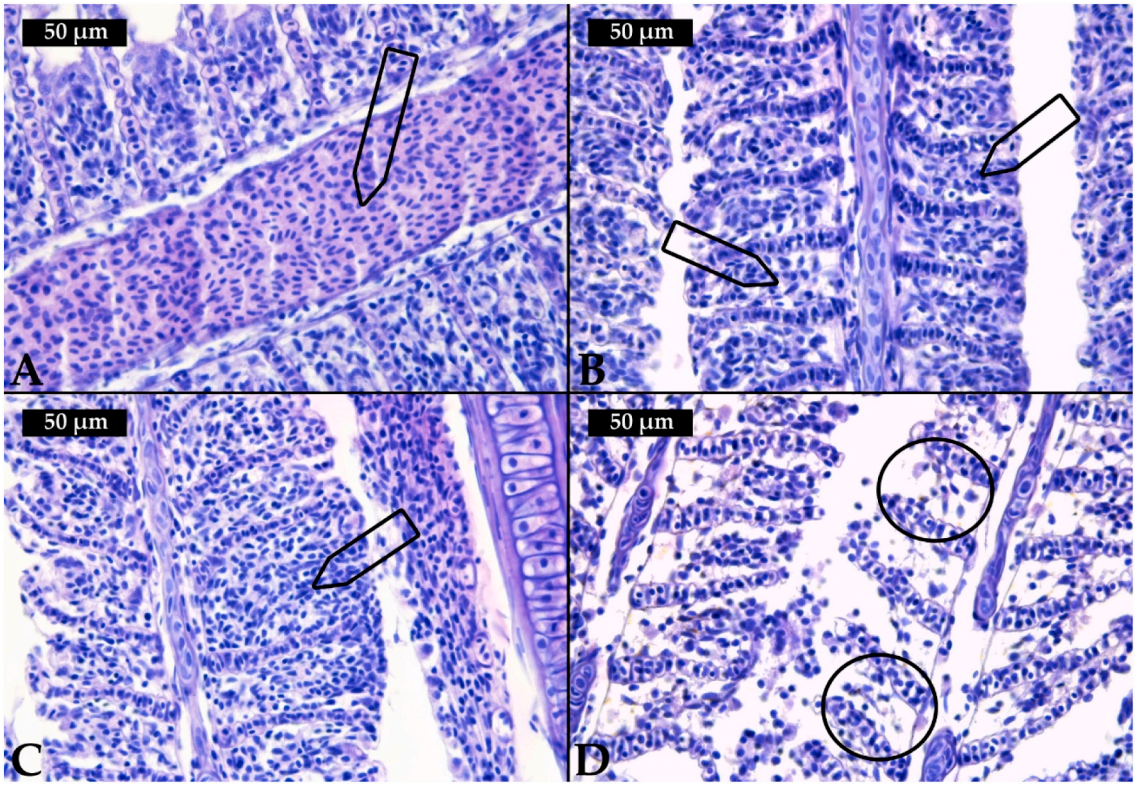

Fig. 1. Histological alterations in the carp gills after cypermethrin (CYP) exposure (H&E): A – vasodilation of central sinus at 0.0002 μg/L; B – fusions of the secondary lamellae at 0.0003 μg/L; C – epithelial proliferation at 0.0006 μg/L; D – necrosis at 0.0006 μg/L.

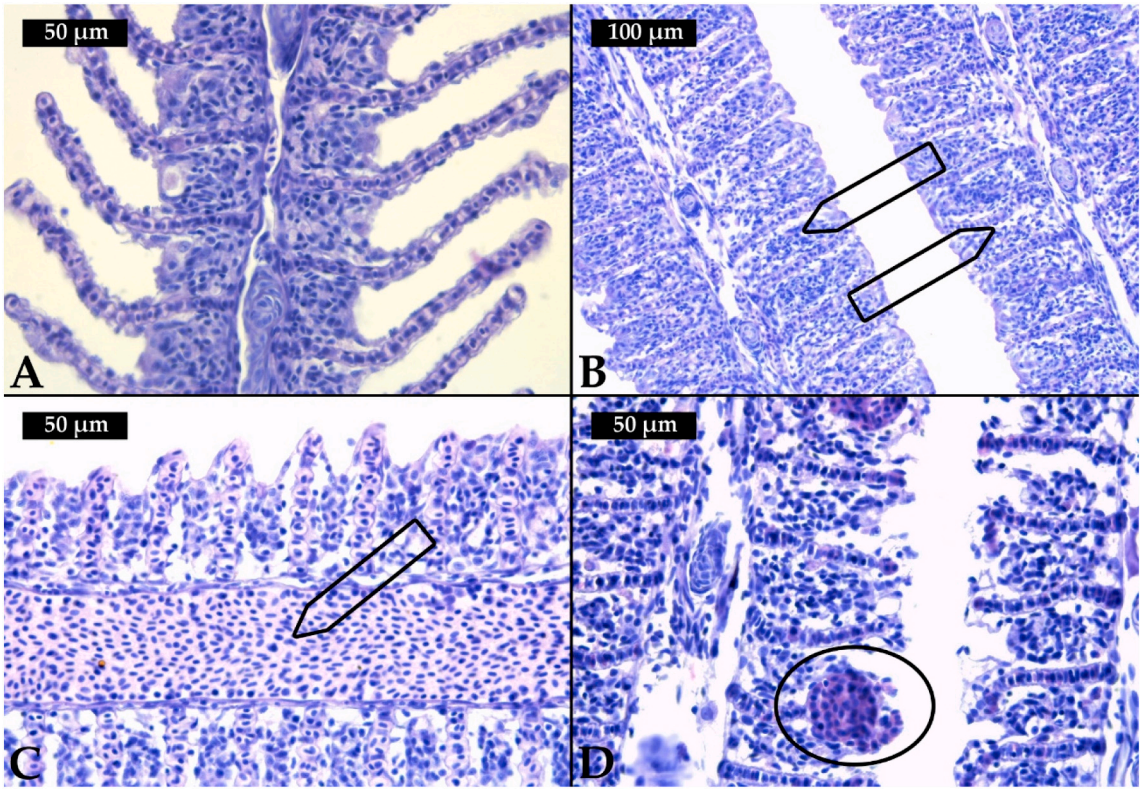

Fig. 2. Histological alterations in the carp gills after chlorpyrifos (CPF) exposure (H&E): A – control gills; B – fusion of the secondary lamellae at 0.03 μg/L; C – vasodilation of central sinus at 0.05 μg/L; D – aneurysms in the secondary lamellae at 0.1 μg/L.

Table 3
Histological lesions in the gills of common carp after a 30-day exposure to cypermethrin (CYP) and chlorpyrifos (CPF) (n = 10 fish per treatment).

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value - Concentrations of CYP (μg/L)				ANOVA F value	Index for each group (0.0002; 0.0003; 0.0006 μg/L)
				Control	0.0002	0.0003	0.0006		
Circulatory disturbances	Blood vessels of secondary lamellae	Vasodilation	$W_{GC1} = 1$	0^{A}	2^{B}	2^{B}	2^{B}	71.89	$I_{GC} = 8$ $I_{GC} = 8$
	Blood vessels of secondary lamellae	Aneurysms	$W_{GC2} = 2$	0^{A}	1^{B}	1^{B}	1^{B}	25.52	$I_{GC} = 8$
	Blood vessels of primary lamellae	Vasodilation	$W_{GC3} = 2$	0^{A}	2^{B}	2^{C}	2^{C}	48.97	
Regressive lesions	Epithelium of secondary lamellae	Degeneration (necrosis)	$W_{GR1} = 3$	0^{A}	1^{B}	1^{B}	2^{C}	50.32	$I_{GR} = 9$ $I_{GR} = 9$
	Epithelium of primary lamellae	Degeneration (necrosis)	$W_{GR2} = 3$	0^{A}	2^{B}	2^{B}	2^{B}	49.37	$I_{GR} = 12$
Progressive lesions	Epithelium of secondary lamellae	Lamellar lifting	$W_{GP1} = 1$	0^{A}	2^{B}	2^{B}	2^{B}	41.15	$I_{GP} = 14$
		Proliferation	$W_{GP2} = 2$	0^{A}	2^{BC}	2^{B}	1^{C}	40.12	$I_{GP} = 14$
	Epithelium of primary lamellae	Edema	$W_{GP3} = 1$	0^{A}	1^{B}	1^{B}	2^{C}	46.37	$I_{GP} = 17$
		Proliferation of stratified epithelium	$W_{GP4} = 2$	0^{A}	2^{B}	2^{B}	2^{B}	55.03	
		Proliferation of glandular cells	$W_{GP5} = 1$	0^{A}	0^{A}	0^{A}	1^{B}	34.52	
		Fusion	$W_{GP6} = 3$	0^{A}	1^{B}	1^{B}	2^{C}	55.35	
Index organ				$I_C = 0^{A}$	$I_{0.0002} = 31^{B}$	$I_{0.0003} = 31^{B}$	$I_{0.0006} = 37^{C}$	201.8	

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value - Concentrations of CPF (μg/L)				ANOVA F value	Index for each group (0.03; 0.05; 0.1 μg/L)
				Control	0.03	0.05	0.10		
Circulatory disturbances	Blood vessels of secondary lamellae	Vasodilation	$W_{GC1} = 1$	0^{A}	1^{B}	2^{C}	2^{C}	56.35	$I_{GC} = 7$ $I_{GC} = 8$
	Blood vessels of secondary lamellae	Aneurysms	$W_{GC2} = 2$	0^{A}	1^{B}	1^{B}	2^{C}	32.77	$I_{GC} = 10$
	Blood vessels of primary lamellae	Vasodilation	$W_{GC3} = 2$	0^{A}	2^{B}	2^{BC}	2^{C}	77.21	
Regressive lesions	Epithelium of secondary lamellae	Degeneration (necrosis)	$W_{GR1} = 3$	0^{A}	1^{B}	1^{BC}	1^{C}	17.95	$I_{GR} = 6$ $I_{GR} = 6$
	Epithelium of primary lamellae	Degeneration (necrosis)	$W_{GR2} = 3$	0^{A}	1^{B}	1^{BC}	1^{C}	25.10	$I_{GR} = 6$
Progressive lesions	Epithelium of secondary lamellae	Lamellar lifting	$W_{GP1} = 1$	0^{A}	1^{B}	2^{C}	2^{C}	33.49	$I_{GP} = 16$
		Proliferation	$W_{GP2} = 2$	0^{A}	0^{A}	1^{B}	2^{C}	35.47	$I_{GP} = 19$
	Epithelium of primary lamellae	Edema	$W_{GP3} = 1$	0^{A}	1^{B}	1^{B}	1^{B}	21.88	$I_{GP} = 21$
		Proliferation of stratified epithelium	$W_{GP4} = 2$	0^{A}	2^{B}	2^{BC}	2^{C}	76.34	
		Proliferation of glandular cells	$W_{GP5} = 1$	0^{A}	1^{B}	1^{BC}	1^{C}	15.52	
		Fusion	$W_{GP6} = 3$	0^{A}	3^{B}	3^{BC}	3^{C}	113.8	
Index organ				$I_C = 0^{A}$	$I_{0.03} = 29^{B}$	$I_{0.05} = 33^{C}$	$I_{0.1} = 37^{D}$	150.8	

0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations. Class I (index < 10)—normal tissue structure with slight histological alterations; Class II (index 11–20)—normal structure with moderate histological alterations; Class III (index 21–30)—moderate modifications of normal tissue; Class IV (index 31–40)—pronounced histological alterations; Class V (index > 41)—severe histological alterations.
a,b,c The values with different letters in the same row are significantly different (Tukey's test, $p < 0.05$).

the gills.

The overall gill indices differed significantly among the control and treatments, exposed to CYP (ANOVA, $F = 201.8$, $df = 3$; 436, $p < 0.001$) and CPF (Kruskal-Wallis, $H = 150.8$, $df = 3$; 436, $p < 0.001$).

3.3.2. Liver

The histological analysis demonstrated that the control fish group had a relatively normal liver morphology (Table 4, Fig. 4A). Our results are in line with Ghayyur et al. (2021) that the healthy fish had polygonal hepatocytes, containing spherical, large, and centrally located nuclei with homogenous cytoplasm. Moreover, the sinusoids were described as communication channels between the hepatocytes, containing blood cells and giving chord-like structure to the liver parenchyma (Ghayyur et al., 2021). On the other hand, the prolonged treatment with both pesticides caused severe histological alterations in the liver. Regarding the semi-quantitative system of Bernet et al. (1999), we categorized once again the histological lesions into four main groups: circulatory, regressive, and progressive lesions, including inflammation (Table 4).

After CPF exposure for 30 days, we observed hyperemia of the blood vessels, which showed a tendency to increase with the increasing concentration of the toxicant (Fig. 4C). At the higher concentrations, hyperemia was mild, similar to the results obtained from our short-term experiment (Georgieva et al., 2021). The degenerative changes in the liver parenchyma included granular, fatty, and vacuolar degeneration, which were the most prominent lesions in the hepatic structure (Table 4). Granular and vacuolar degeneration was observed mostly in a moderate and severe degree (Table 4, Fig. 4B, D), while after the short-term exposure of 96 h these alterations were not pronounced (Georgieva et al., 2021). Regarding the necrotic changes in hepatocytes, we observed karyorrhexis, karyopyknosis, and karyolysis to a very mild degree. In line with our findings from the acute test (Georgieva et al., 2021), necrobiosis was found to be mild only at the highest CPF

Table 4
Histological lesions in the liver of common carp after a 30-day exposure to cypermethrin (CYP) and chlorpyrifos (CPF) (n = 10 fish per treatment).

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value - Concentrations of CYP (μg/L)				ANOVA F value	Index for each group (0.0002; 0.0003; 0.0006 μg/L)
				Control	0.0002	0.0003	0.0006		
Circulatory disturbances	Liver	Hyperemia	$W_{LC1} = 1$	0^A	2^B	2^C	3^D	100.2	$I_{LC} = 2$
	Liver	Intercellular edema	$W_{LC2} = 1$	0^A	0^A	0^A	0^A	–	$I_{LC} = 2$
									$I_{LC} = 3$
Regressive lesions	Liver	Granular degeneration	$W_{LR1} = 1$	1^A	2^B	2^B	2^C	61.58	$I_{LR} = 17$
	Liver	Deposits (lipids)	$W_{LR2} = 1$	0^A	2^B	2^B	3^C	58.3	$I_{LR} = 22$
	Liver	Nuclear alterations	$W_{LR3} = 2$	0^A	2^B	2^B	3^C	99.24	$I_{LR} = 25$
	Liver	Necrosis	$W_{LR4} = 3$	0^A	1^B	2^C	2^D	62.72	
	Liver	Vacuolar degeneration	$W_{LR5} = 2$	0^A	3^B	4^C	4^C	271	
	Interstitial tissue	Architectural and structural alterations	$W_{LR6} = 1$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Deposits	$W_{LR7} = 1$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Nuclear alterations	$W_{LR8} = 2$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Necrosis	$W_{LR9} = 3$	0^A	0^A	0^A	0^A	–	
Progressive lesions	Liver	Hypertrophy	$W_{LP1} = 1$	0^A	1^B	2^C	2^D	62.72	$I_{LP} = 1$
	Interstitial tissue	Hypertrophy	$W_{LP2} = 1$	0^A	0^A	0^A	0^A	–	$I_{LP} = 2$
									$I_{LP} = 2$
Inflammation	Liver	Activation of RES	$W_{LI1} = 1$	0^A	0^A	0^A	0^A	–	$I_{LI} = 2$
	Liver	Infiltration	$W_{LI2} = 2$	0^A	1^B	2^C	3^D	124.6	$I_{LI} = 4$
									$I_{LI} = 6$
Index organ				$I_C = 1^A$	$I_{0.0002} = 22^B$	$I_{0.0003} = 30^C$	$I_{0.0006} = 36^D$	52.24	

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value - Concentrations of CPF (μg/L)				ANOVA F value	Index for each group (0.03; 0.05; 0.1 μg/L)
				Control	0.03	0.05	0.1		
Circulatory disturbances	Liver	Hyperemia	$W_{LC1} = 1$	0^A	1^B	2^C	2^C	40.6	$I_{LC} = 1$
	Liver	Intercellular edema	$W_{LC2} = 1$	0^A	0^A	0^A	0^A	–	$I_{LC} = 2$
									$I_{LC} = 2$
Regressive lesions	Liver	Granular degeneration	$W_{LR1} = 1$	1^A	3^B	3^{BC}	3^C	144.8	$I_{LR} = 16$
	Liver	Deposits (lipids)	$W_{LR2} = 1$	0^A	2^B	2^B	3^C	73.86	$I_{LR} = 16$
	Liver	Nuclear alterations	$W_{LR3} = 2$	0^A	1^B	1^B	2^C	44.53	$I_{LR} = 21$
	Liver	Necrosis	$W_{LR4} = 3$	0^A	1^B	1^B	2^C	26.79	
	Liver	Vacuolar degeneration	$W_{LR5} = 2$	0^A	3^B	3^C	4^C	149.8	
	Interstitial tissue	Architectural and structural alterations	$W_{LR6} = 1$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Deposits	$W_{LR7} = 1$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Nuclear alterations	$W_{LR8} = 2$	0^A	0^A	0^A	0^A	–	
	Interstitial tissue	Necrosis	$W_{LR9} = 3$	0^A	0^A	0^A	0^A	–	
Progressive lesions	Liver	Hypertrophy	$W_{LP1} = 1$	0^A	1^B	1^{BC}	1^C	16.04	$I_{LP} = 1$
	Interstitial tissue	Hypertrophy	$W_{LP2} = 1$	0^A	0^A	0^A	0^A	–	$I_{LP} = 1$
									$I_{LP} = 1$
Inflammation	Liver	Activation of RES	$W_{LI1} = 1$	0^A	0^A	0^A	0^A	–	$I_{LI} = 2$
	Liver	Infiltration	$W_{LI2} = 2$	0^A	1^B	1^B	3^C	97.79	$I_{LI} = 2$
									$I_{LI} = 6$
Index organ				$I_C = 1^A$	$I_{0.03} = 20^B$	$I_{0.05} = 21^B$	$I_{0.1} = 33^C$	44.1	

0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations. Class I (index < 10)—normal tissue structure with slight histological alterations; Class II (index 11–20)—normal structure with moderate histological alterations; Class III (index 21–30)—moderate modifications of normal tissue; Class IV (index 31–40)—pronounced histological alterations; Class V (index > 41)—severe histological alterations.
a,b,c The values with different letters in the same row are significantly different (Tukey's test, $p < 0.05$).

concentration (Table 4). Necrosis was observed only in individual hepatic areas, affecting several liver cells. Following Bernet et al. (1999) the scores for this histological lesion changed in the different treatments and it was highest at 0.1 μg/L CPF. Similar results were found for hypertrophy in the hepatocytes after exposure to all three experimental CPF concentrations. As a result of the toxic exposure to CPF, according to the proposed rating scale, we found lymphocytic infiltration in a very mild degree for the lower concentrations, while at the highest - the level of expression was moderate.

Regarding the effect of CYP, we found mild hyperemia at the lower concentrations, while at the highest concentration the degree was scored as moderate (Table 4). The degenerative changes in the hepatic parenchyma with the highest level of expression included fatty and vacuolar degeneration (Fig. 3A, B, Table 4). Slight granular degeneration was detected at all three applied CYP concentrations. The necrobiotic lesions, such as karyorrhexis, karyopyknosis, and karyolysis (Fig. 3C) showed a tendency to increase the rate of manifestation (moderate) compared to the short-term exposure (Georgieva et al., 2021). Necrotic areas were also found (Fig. 3D). The proliferative changes were mainly mild. Lastly, after the CYP exposure, we observed lymphocyte proliferation, ranging from very mild at the lowest tested concentration to moderate at the highest (Table 4).

Comparing the indices of histological alterations in the circulatory system (I_{LC}), the CPF exposure showed $I_{LC} = 2$ at the highest and middle experimental concentrations, while for CYP the value was higher – $I_{LC} = 3$. Regarding the indices of degenerative changes (I_{LR}) in the liver parenchyma (I_{LR}), we determined higher values again for the CYP exposure at all three tested concentrations (Table 4). The indices of inflammation

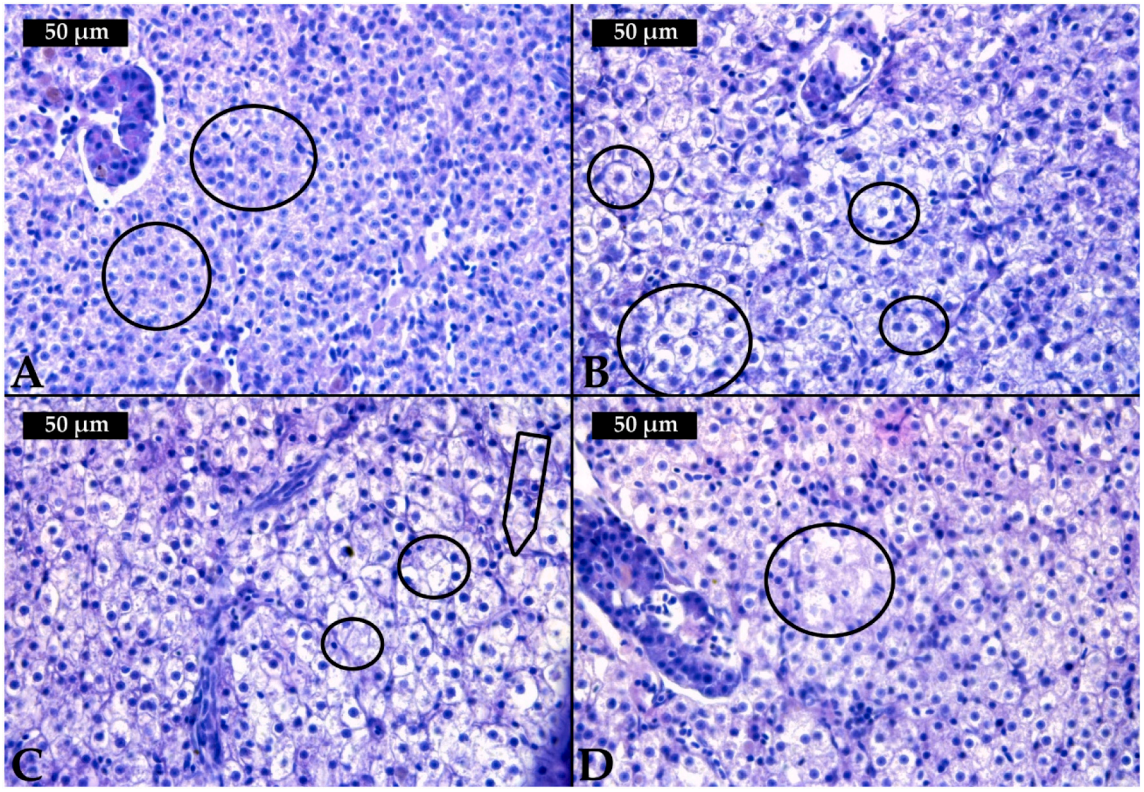

Fig. 3. Histological alterations in the carp liver after cypermethrin (CYP) exposure (H&E): A – granular degeneration at 0.0002 μg/L; B – vacuolar degeneration at 0.0003 μg/L; C – karyolysis and karyopyknosis (arrow) in the hepatocytes (3) at 0.0006 μg/L; D – necrosis at 0.0006 μg/L.

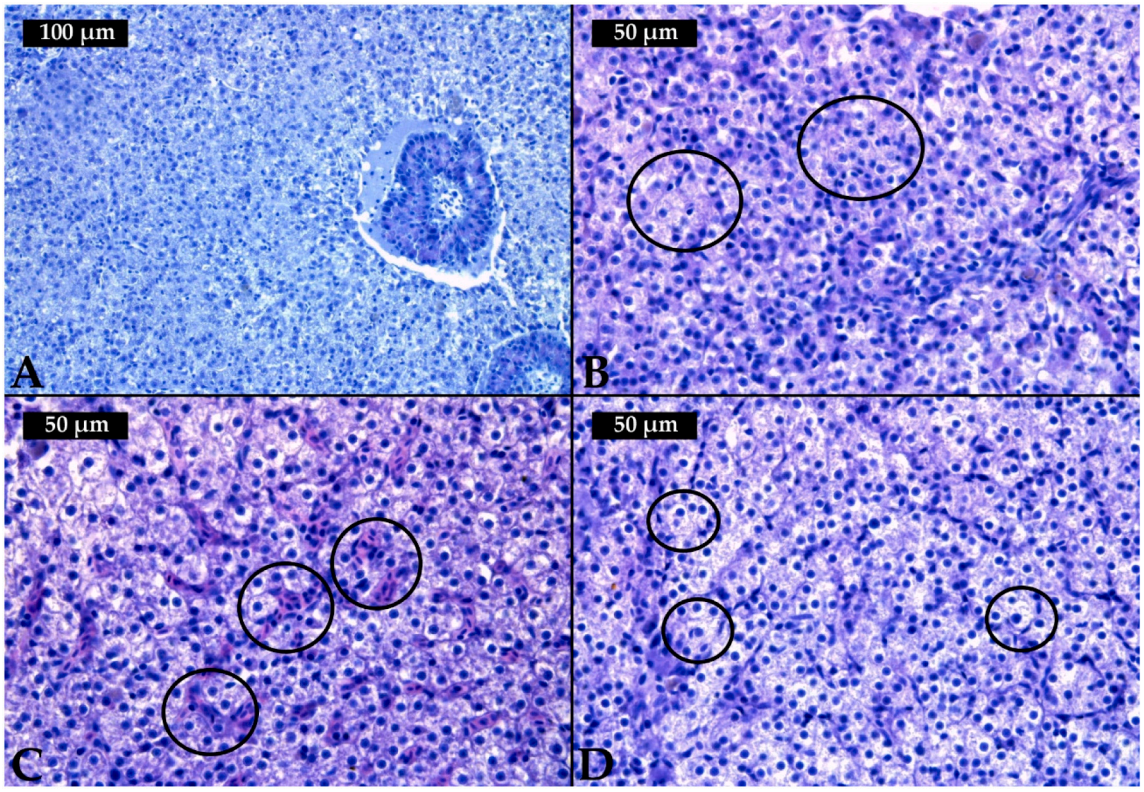

Fig. 4. Histological alterations in the carp liver after chlorpyrifos (CPF) exposure (H&E): A – control group; B – granular degeneration at 0.03 μg/L; C – hyperemia at 0.05 μg/L; D – vacuolar degeneration at 0.1 μg/L.

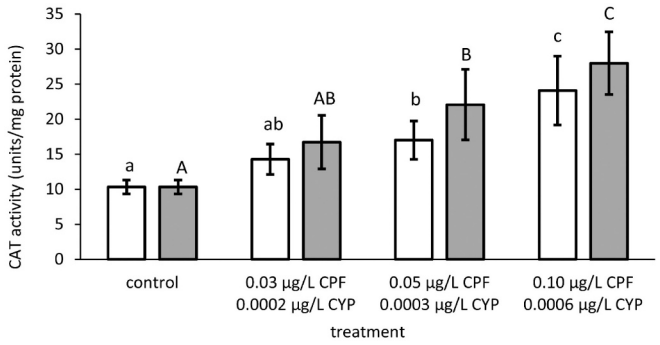

Fig. 5. Catalase (CAT) activity in the liver of common carp under different cypermethrin (CYP) (grey bars) and chlorpyrifos (CPF) (white bars) and exposures. Bars represent the means ± SD of the control and experimental groups, measured on day 30. Different letters indicate significant differences among treatments ($p < 0.05$, lowercase for CPF, capital letters for CYP treatments).

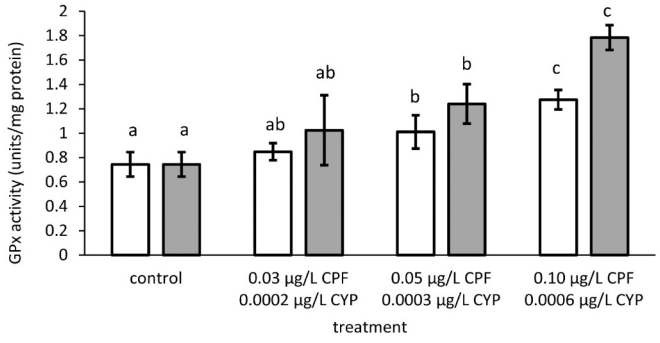

Fig. 6. Glutathione peroxidase (GPx) activity in the liver of common carp under different cypermethrin (CYP) (grey bars) and chlorpyrifos (CPF) (white bars) exposures. Bars represent the means ± SD of the control and experimental groups, measured on day 30. Different letters indicate significant differences among treatments ($p < 0.05$, lowercase for CPF, capital letters for CYP treatments).

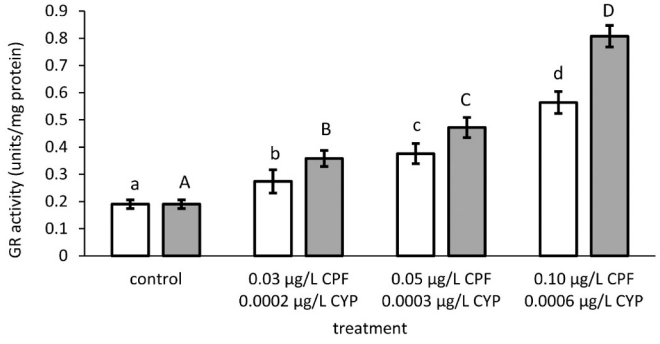

Fig. 7. Glutathione reductase (GR) activity in the liver of common carp under different cypermethrin (CYP) (grey bars) and chlorpyrifos (CPF) (white bars) exposures. Bars represent the means ± SD of the control and experimental groups, measured on day 30. Different letters indicate significant differences among treatments ($p < 0.05$, lowercase for CPF, capital letters for CYP treatments).

(I_{LI}) and proliferative changes (I_{LP}) showed higher values for CYP compared to CPF, which is evidence that the CYP toxicity on common carp is more pronounced compared to CPF (Table 4).

According to Zimmerli et al. (2007) for the lowest CPF concentration, the liver index fell into the Class II group (index 11–20) – a normal histological structure with moderate pathological changes (reversible), while the values calculated for the higher concentrations fell into Class III (index 21–30) – a moderate degree of change in the histological structure (reversible) and Class IV (index 31–40) – pronounced histological alterations. For the CYP exposure, the lowest and middle concentrations also fell into the Class III group and the highest one – into the Class IV group (pronounced histological alterations), however, their values were higher compared to CPF.

The overall liver indices differed significantly among the control and treatments exposed to CYP (ANOVA, $F = 52.24$, $df = 3; 596$, $p < 0.001$) and CPF (ANOVA, $F = 44.1$, $df = 3; 596$, $p < 0.001$).

3.4. Changes in the antioxidant activities

Our results illustrated prominent biochemical alterations in the fish exposed to CYP and CPF, even at the lower applied concentrations. Moreover, the changes in the activity of the tested enzymes after the pesticides' exposure varied with the different concentrations. Overall, the dose of CYP and CPF was important as all treated groups showed significant ($p < 0.05$) differences in the specific CAT, GR, and GPx activities compared to the control (Figs. 5–7). These activities were increased compared to the control, as well as compared to the results, which we obtained in our short-term experiment (Georgieva et al., 2021).

The CAT activity was higher in the pesticide-exposed fish compared to the control fish, depending on the applied CPF and CYP concentrations (Fig. 5). In addition, the CAT activity differed significantly among the control and the different groups treated with CPF and CYP, respectively (ANOVA CPF: $F = 18.09$, $df = 3$, $p < 0.001$; CYP: $F = 18.66$, $df = 3$, $p < 0.001$) (Fig. 5).

Similarly to the CAT activities, the GPx activity increased compared to the control and it was dependent on the concentrations of the pesticides (Fig. 6). In addition, the specific GPx activities of the groups also differed significantly in the case of CPF and CYP exposure (ANOVA CPF: $F = 26.68$, $df = 3; 16$, $p < 0.001$; CYP: $F = 30.15$, $df = 3; 16$, $p < 0.001$) (Fig. 6).

The same tendency was found in the case of the GR activity, which increased compared to the control, depending on the applied CPF and CYP concentrations (Fig. 7). Furthermore, the GPx activities of the different groups also differed significantly in the case of CPF and CYP exposure (ANOVA CPF: $F = 26.68$, $df = 3; 16$, $p < 0.001$; CYP: $F = 30.15$, $df = 3; 16$, $p < 0.001$) (Fig. 7).

There were significant differences among effects of CPF and those of CYP on every investigated enzyme's activity (ANOVA, CAT: $F = 8.53$, $df = 5; 24$, $p < 0.001$; GPX: $F = 21.76$, $df = 5; 24$, $p < 0.001$; GR: $F = 126.7$, $df = 5; 24$, $p < 0.001$). These results showed that CYP had significantly greater effects on all investigated enzyme's activities than those of CPF after 30 days of exposure.

3.5. Respiration rate

The results of the long-term study of respiration intensity show that after 30 days of exposure, the fish from all CPF and CYP treatments had lower respiration intensities than those of the controls, with the same trend of lower intensity at the highest applied pesticide concentrations (ANOVA CYP: $F = 184$, $df = 3; 16$, $p < 0.001$; CPF: $F = 18.07$, $df = 3; 16$, $p < 0.001$) (Table 5).

3.6. Changes in the condition and hepatosomatic index

In contrast to our short-term experiment (Georgieva et al., 2021), in the long-term, we further calculated the indices listed in Table 6. Lower values were calculated for K on day 30 compared to the control; the lowest values were for the fish treated with the highest CYP and CPF concentrations. This trend was not valid for the HSI values as we got increased values compared to the control. It is noteworthy that overall, for both K and HSI, the values were relatively similar among the treated

Table 5
Respiration rate (mean ± S.D.) in common carp, exposed to different cypermethrin (CYP) and chlorpyrifos (CPF) concentrations for 30 days (n = 5 fish per treatment).

	Control	Concentration of CYP, μg/L		
		0.0002	0.0003	0.0006
Respiration rate	0.049 ± 0.007[a]	0.036 ± 0.004[b]	0.005 ± 0.001[c]	0.004 ± 0.002[c]

	Control	Concentration of CPF, μg/L		
		0.03	0.05	0.10
Respiration rate	0.049 ± 0.007[a]	0.027 ± 0.003[b]	0.021 ± 0.004[b]	0.016 ± 0.003[b]

a,b,c The values with different letters in the same row are significantly different (Tukey's test, $p < 0.05$).

Table 6
Mean results for condition (K) and hepatosomatic index (HIS) for common carp, treated with cypermethrin (CYP) and chlorpyrifos (CYP) concentrations after 30 days (n = 15 fish per treatment).

	Control	Concentration of CYP (μg/L)		
		0.0002	0.0003	0.0006
K	1.22[a]	0.91[a]	0.90[a]	0.80[b]
HIS	2.00[a]	2.56[a]	2.79[a]	3.63[b]

	Control	Concentration of CPF (μg/L)		
		0.03	0.05	0.1
K	1.22[a]	1.10[a]	1.11[a]	1.05[a]
HIS	2.00[a]	2.42[a]	2.65[a]	3.08[b]

Different letters indicate significant differences among treatments ($p < 0.05$).

groups and in the most cases, there were no significant differences, except for the highest pesticide concentrations (ANOVA CYP K: $F = 15.04$, $df = 3; 16$, $p < 0.05$; CYP HSI: $F = 18.23$, $df = 3; 16$, $p < 0.05$; CPF K: $F = 6.54$, $df = 3; 16$, $p > 0.05$; CPF HSI: $F = 17.11$, $df = 3; 16$, $p < 0.05$) (Table 6).

3.7. Changes in the behavioral responses

The control fish showed normal behavior during the exposure of 30 days. As we already explained, in our acute experiment the changes in the behavioral responses of the fish exposed to both tested pesticides started on the first day after dosing and continued until the experiment stopped. However, some of them gradually disappeared. The fish treated with the lower CYP and CPF concentrations showed a behavior similar to that of the control group. Furthermore, the fish exposed to the highest experimental CYP and CPF concentrations presented changes in their behavior, as presented in Table 7. The fish behavioral responses treated by the highest concentrations of CYP and CPF differed significantly than those of control and other groups (CYP: ANOVA, $F = 3.784$, $df = 3; 36$, $p < 0.05$; CPF: $F = 2.864$, $df = 3; 36$ $p < 0.05$). These behavioral alterations were normalized at the end of the treatment. Moreover, we also observed intense mucus secretion, which lasted until day 30. Even though, the fish were subjected to toxic stress, no mortality was observed during the long-term experiment.

4. Discussion

Compared to our previous results (Georgieva et al., 2021), we also observed changes in the fish behavior and physiological responses (K, HSI, and respiration rate), which are difficult to detect in short-term

Table 7
Mean results for fish behavioral responses (n = 15 fish per treatment) of common carp, treated with cypermethrin (CYP) and chlorpyrifos (CPF) concentrations after 30 days.

Parameter	Control	Concentration of CYP (μg/L)		
		0.0002	0.0003	0.0006
CC	0	0	0	0
ES	0	2	3	5
GM	0	2	3	4
GA	0	0	2	3
H	0	0	2	0
LE	0	2	2	5
MS	2	3	3	5
SB	1	0	0	0
TR	1	3	0	0
WM	0	2	3	5

Parameter	Control	Concentration of CPF (μg/L)		
		0.03	0.05	0.1
CC	0	0	0	0
ES	0	2	2	4
GM	1	2	2	3
GA	0	0	2	3
H	0	0	0	0
LE	0	2	2	3
MS	2	2	3	4
SB	1	0	0	0
TR	1	2	2	0
WM	0	2	3	4

Color change (CC), erratic swimming (ES), gill movement (GM), gulping of air (GA), hypoactivity (H), loss of equilibrium (LE), mucous secretion (MS), schooling behavior (SB), touch response (TR) and widening of the mouth (WM) – normal (1), none (0), mild (2), moderate (3), strong (4), very strong (5).

exposures. Moreover, we found evidence for our hypothesis that the alterations in the studied biomarkers were classified mainly as degenerative, displacing the compensatory-adaptive ones because of the chronic treatments. However, we have to clarify that we had no true replication, but pseudoreplication (Moermond et al., 2016). Thus, we compared our results to the control group (Aliko et al., 2019).

4.1. Pesticide concentrations

According to Kumar et al. (2007) and Jaensson et al. (2007), the concentration of CYP in surface water usually ranges from 0.100 to 1.000 μg/L, but could also be up to 2.8 μg/L. The USEPA (2020) and National water-quality assessment program in the USA (NAWQA) reported CPF concentrations, ranging from 0.026 μg/L to 0.400 μg/L from a field study on a total of 1530 agricultural streams and 604 urban streams, which were investigated. Furthermore, Bhattacharjee et al. (2012) reported CYP at concentrations of 0.605 μg/L from paddy fields, and according to House et al. (1997), the CYP concentrations in surface waters overall do not exceed 1 μg/L. In Ireland, a pilot study detected CYP concentrations up to 0.0034 μg/L as reported by Regan et al. (2018). Also, according to UKEA (2019) in the UK, 26 out of 280 freshwater monitoring sites had average concentrations above the annual average concentrations (AA-EQS) of 0.08 ng/L, with site averages ranging from 0.005 to 1.3 ng/L. In continental Europe, Bereswill et al. (2013) and Herrero-Hernández et al. (2020) detected the highest CYP post-runoff concentrations of 0.086 μg/L in Germany and 0.42 μg/L in Spain. CPF was registered in Argentinean streams up to 10 mg/L in waters and 19 mg/kg in sediments and in Indian waters between 0.019 and 2.73 μg/L (Bonifacio et al., 2017; Brodeur et al., 2011; Nag et al., 2020). Therefore, because of their proven toxicity and lipophilicity both pesticides have been categorized as priority substances within the Water Framework Directive of the European Union (Commission of the European Communities, 2000) and we considered our applied test

concentrations as environmentally relevant and not only based on the EU legislation.

As explained before, pesticides could break down into other by-products and the rate of evaporation from water surfaces is expected to be reduced due to the adsorption of the pesticides on the suspended matter, and sediment in the aquatic environment. The rate of hydrolysis for CPF increases with temperature and alkalinity. Half-lives ranging from 35 to 78 days have been reported in water with a pH of 7 and a temperature of 25 °C, but volatilization of CPF from water is the most likely route of CPF loss, with volatilization half-lives of 3.5 and 20 days estimated for pond water (Kamrin, 1997). In addition, as stated by Kamrin (1997) during midsummer, the photolysis half-life of CPF in water is between three and four weeks. In water, CYP hydrolyzes slowly, with hydrolysis being more rapid at a pH of 9. However, according to USEPA under normal environmental temperatures and pH, CYP is stable to hydrolysis with a half-life of >50 days, as well as to photodegradation with a half-life of >100 days.

In our previous experiment, on day 4 we measured the concentrations of CYP and CPF. In the present study, the results showed values of both pesticides in the water similar to our short-term exposure (Georgieva et al., 2021). On the other hand, the bioaccumulation results for the fish organs showed higher values. Moreover, the concentrations of CYP and CPF, which we measured in the liver were higher than in the gills, which was the opposite case in our prior study (Georgieva et al., 2021). This result deserves attention because it can be related to the long-term mechanisms of action of pesticides, which in this case were selected to be deposited at higher concentrations in the liver. The liver is particularly susceptible to chemical toxicity because it is the main site of toxicant filtration and toxicant metabolic breakdown. The liver is also known as an important site for toxicant breakdown and is often referred to as the “metabolic clearinghouse” of the body. As pesticides are quite often broken down in the liver, the byproducts of the pesticide's metabolism can also be toxic. Lastly, another noteworthy result is that we confirmed the findings of Datta and Kaviraj (2003) who explained how pesticides, owing to their toxic properties, lipophilicity, etc., are easily absorbed, even at much lower than the permitted concentrations, via the fish gills and then they end up in other organs, such as the liver.

BAFs are considered significant when exceeding 100 or more, according to USEPA (1991). In terms of the BAF values, we can consider that both pesticides, despite their concentrations, which were lower than the maximum permissible levels, according to Directive 2013/39/EU (Commission of the European Communities, 2013), have a great potential for bioaccumulation. A notable result is also that the BAF values for CYP were higher in the liver, while the CPF showed higher values in the gills. These data may be important, as some metals are already known to have preferences for certain organs, although toxicants can generally disrupt any organ and function. For example, mercury accumulates mainly in the brain and leads to the bones (Jaishankar et al., 2014). In addition, the deposition or localization, and in some cases metabolism, of pesticides depend on the characteristics of particular tissues and organs and may influence toxicity as explained by Timbrell (2000). We confirmed the results of Di et al. (2017) that tissue-specific accumulation of pesticides can be a key indicator of chronic exposure, and hence our results may indicate that the different pesticides have strong affinities, but for different tissues or organs in fish.

4.2. Histology

We agree with Trivedi et al. (2021) that water pollution may cause oxidative stress-associated molecular damage, which in turn reflects in the impairment of tissue and organ architecture. The histological analysis revealed different affection degrees in each target organ (liver > gills) of common carp exposed to CYP and CPF, which was probably due to the specific function and sensitivity of each organ as noted by Costa et al. (2013) and Cuevas et al. (2016).

The gills are the main respiratory organ in fish and play important roles in osmotic and ionic regulation, acid-base equilibrium, and nitrogen excretion (Evans et al., 2005; Laurent, 1984). Furthermore, as explained by Fernandes (2019), the gills are one of the most important sites for the entry of dissolved pollutants, because of their very large surface and their morphological characteristics. In addition, their large surface area and very thin water-blood diffusion distance support the contaminants uptake in fish due to the great water volume, which flows onto the gill lamellae to obtain the needed oxygen for aerobic metabolism (Fernandes and Moron, 2020). Moreover, pyrethroids are considered to be up to 1000 times more toxic in fish compared to birds or mammals because of their high absorption in the gills, however, the susceptibility to these pesticides is also fish species-specific (Edwardst et al., 1987; Srivastav et al., 1997).

The liver is an organ, which participates in many essential functions, such as homeostasis and metabolism, and enzyme production. Therefore it also serves as a reliable organ in histological biomarker studies (Ghayyur et al., 2021; Sharma et al., 2019). It is also the main depot for the bioaccumulation of different toxicants, including pesticides, and their detoxification as well, as previously mentioned.

In our short-term exposure with CPF (Georgieva et al., 2021), a high degree of manifestation of compensatory-adaptive mechanisms, associated with epithelial tissue proliferation processes was observed, while degenerative processes were observed to a lesser degree. The long-term exposure showed that in parallel with the processes of proliferation, the inclusion of degenerative processes also occurred due to the prolonged exposure to the toxicant, although to a lesser extent. In contrast, CYP showed a reduction in the proliferation processes after 96 h (Georgieva et al., 2021) at the expense of those of necrosis in the current study (30 days). Our results are in line with previous studies (Alexopoulos et al., 2003; Sula et al., 2020), which show that the proliferative lesions in fish gills could serve as a mechanism of defense because separation of the epithelial lamellae increases the distance between the pollutants and the bloodstream in the organ. Therefore, these histological alterations are considered not only mild but also compensatory-adaptive in the field of fish histology.

The histological changes in the liver also showed a higher degree of manifestation compared to the conducted short-term experiment (Georgieva et al., 2021). In terms of the CPF exposure, more pronounced degenerative changes, mainly associated with vacuolar degeneration and accumulation of lipids in the cytoplasm of hepatocytes were observed. At the same time, there was an increase in inflammatory processes at the highest experimental concentration, associated with lymphocytic infiltration. Compared to the short-term exposure to CYP (Georgieva et al., 2021), the 30 days' exposure showed an increase in the processes of vacuolar and fatty degeneration again, but in contrast to the exposures with CPF, there was an increase in cells with necrobiotic changes, mainly related to karyolysis, as well as necrotic areas in the hepatic parenchyma. The established necrosis during the short-term exposure affected single parts of the histostructure of the organs (several cells), an indicator of the toxic effect of the applied substances. With long-term exposure, the degree of necrobiotic and necrotic changes increased, in parallel with the manifestation of proliferative changes. The degree of necrosis was generally moderate, with no high or severe degree due to the toxicant concentrations administered. The results showed that higher values are observed for the exposure to CYP, which also indicates higher toxicity of this insecticide compared to CPF. Therefore, our results could serve as evidence of the stronger negative impact of CYP, as well as the fact that the liver is more severely affected by the long-term pesticide exposure.

Moreover, based on the displayed findings, we can also consider that the effects of the tested pesticides were severe, with more severe effects of CYP on the morphological structure of both, gills and liver, even at lower than the permissible concentrations. These results are coherent with our previous study (Georgieva et al., 2021). However, in the present experiment, the histological changes were described as destructive with observed histological degeneration, rather than being

compensatory-adaptive. We consider that this was a direct result of the impaired ability of the fish body to cope with the toxic and chronic effects of CYP and CPF.

In the case of chronic exposure, the proliferative changes (compensatory-adaptive) in the gills were gradually replaced by degenerative ones, which is a natural environment and a combination of toxicants, can significantly affect the histological structure of the gills and thus hinder their functions related to gas exchange processes. In addition, the liver was found to have mainly degenerative changes, manifested in varying degrees. The tendency to increase the degree of necrotic changes was preserved, and their degree was mainly moderate. Again, in the natural environment and the complex action of toxicants, albeit in lower concentrations, can lead to an increase in the incidence of necrotic changes, and hence a decrease in the survival of fish living in contaminated ecosystems. As indicated, in the short-term exposure, although with established single areas with necrobiotic changes, compensatory adaptive processes in the organs against the influence of the applied toxicants had prevailed. In the case of chronic exposure, equalization of the degree of manifestation of the compensatory-adaptive and proliferative changes was observed, and at the highest applied concentrations, although in a moderate degree of manifestation, necrotic changes related to destructive processes in the organs were found.

In sum, we can summarize that in our short-term experiment (Georgieva et al., 2021), the gills were more severely affected, as a prime and target organ, which was in direct contact with toxicants when the pollution occurred through the aquatic environment. Our results indicate that because of the significant impact of the two pesticides, which reached the liver through the bloodstream, the prolonged effects were more pronounced in this organ. This statement is in line with van der Oost et al. (2003) who explained that in the gills the chemicals could be accumulated, metabolized, and transferred to the bloodstream, reaching other internal organs. We agree with Banik et al. (2017) who considered that the prevalence of histological alterations in the liver could be associated with greater perturbations in metabolically active organs, involved in bioaccumulation and detoxifications of xenobiotics.

Our results also correspond to the findings of other authors who examined the effects of organophosphorus and pyrethroid insecticides on the gills and liver of various fish species. For instance, the alterations, which we detected were similar to those reported by Khan et al. (2019), Albañil Sánchez et al. (2019), Dawood et al. (2020), Günal et al. (2020), and Sharma and Jindal (2020) in fish after pesticide exposure under laboratory conditions, as well as by McHugh et al. (2011), Cuevas et al. (2016) and Kostić et al. (2017) who studied the biomarker responses in fish gills and liver from polluted with various toxicants sites, including pesticides.

4.3. Biochemistry

Long-term exposure to pesticides results in oxidative stress, which is associated with the overproduction of reactive oxygen species (ROS), such as hydrogen ions (HO^-), hydrogen peroxides (H_2O_2), and superoxide anions ($^{\bullet}O_2^-$) (Trivedi et al., 2021; Yang et al., 2020). In this regard, OP pesticides impact various cellular responses in organisms, including fish (Laxmi et al., 2019). Furthermore, fish have low levels of carboxylesterases that can hydrolyze pyrethroids (Glickman and Casida, 1982). The sensitivity of fish to pyrethroids is thought to be due to the slow metabolism of these substances (Glickman and Lech, 1982). Therefore, our results are coherent with Yang et al. (2020) that pyrethroids in fish are shown to act on different organs, including the liver.

The antioxidant defense system responds to neutralize oxidative stress, which leads to damaged DNA, proteins, and lipid peroxidation (Fang et al., 2002; Lesser, 2006). Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD) are the first-line antioxidant enzymes, the glutathione s-transferase (GST) participates as the biotransformation enzyme of phase II and the glutathione (GSH) system is essential for the cellular defense against ROS as explained by Nataraj et al. (2017) and Ighodaro and Akinloye (2018). Catalase (CAT) is located in the peroxisomes where it decomposes H_2O_2 to water and O_2 through the reaction of the enzyme's porphyrin heme groups with H_2O_2 and is widely distributed in the fish tissues (Carvalho et al., 2012). Glutathione peroxidase (GPx), which also detoxifies hydroperoxides, and glutathione reductase (GR), which is needed for the reduction of glutathione disulfide (GSSG) to glutathione (GSH) are the two essential enzymes, required to protect the fish cells from oxidative stress (Gonçalves et al., 2021; Oruç and Usta, 2007; Tekman et al., 2008).

Our results highlight that the antioxidant mechanisms of common carp were provoked by the chemical stress to prevent oxidative damage. The activity of all three antioxidant enzymes was increased compared to the control. In contrast, in our short-term experiment (Georgieva et al., 2021) the activities of GPx and GR were significantly reduced compared to the control. We link these results to different cellular response mechanisms of the exposed fish to the short and long-term pesticide effects. For instance, similarly to Cheung et al. (2004), we think that the decreased activity of GPx and GR may be related to the reduction in GSH levels required to reduce the effects of ROS at the 96-hour exposure. On the other hand, in chronic exposure, we observed the inclusion of CAT, GPx, and GR, which probably shows the activation of compensatory-adaptive mechanisms in the fish to neutralize free radicals as a result of chemically induced stress.

Furthermore, our results confirm the findings of Trivedi et al. (2021) who studied the effects of dichlorvos on the biochemical and histological alterations in the liver of *Channa punctatus* (Bloch, 1793), which were time and dose-dependent. Our opinion is in agreement with Trivedi et al. (2021) that the increased activities of CAT probably suggest higher concentrations of ROS in the hepatic tissue of common carp, as well as the faster conversion of ROS – H_2O_2 and $^{\bullet}O_2^-$. Moreover, Nataraj et al. (2017) reported significantly increased CAT activities in fish exposed to the organophosphorus pesticide profenofos after 7 days of exposure, suggesting adaptive responses of the fish and compensatory mechanisms to defend against oxidative stress. In contrast, the study of Rossi et al. (2020) showed that the CAT activities were inhibited in two Tetra fish species (*Markiana nigripinnis* Perugia, 1891 and *Astyanax lacustris* Lütken, 1875), inhabiting rice fields after pesticide treatment. In the study of Nataraj et al. (2017) after 21 days of exposure, the CAT activities were also decreased. The authors explained this reduction with peroxidative damage in the liver.

We consider that antioxidant mechanisms in our case did not completely fail to prevent oxidative damage, however, the effects of CYP and CPF were evident because of the severe histological alterations in the hepatic architecture. In this sense, we agree with da Silva Montes et al. (2020) that the histological lesions are usually combined with biochemical responses in fish, subjected to environmental stress even if the chemical pollutants are presented at low concentrations. Furthermore, our findings are consistent with Sachi et al. (2021) that the direct action of toxic substances in the liver and the changes in antioxidant systems may induce histological lesions, which may affect vital functions of the organ. Our results also confirmed the findings of Salamat and Zarie (2012) that the fish liver is one of the prime targeted organs for various types of water contaminants, including pesticides due to its involvement in the removal and detoxification of toxic substances, which results in biochemical alterations along with the histological ones.

4.4. Respiration rate

The rate of respiration reflects the metabolic activity of fish and the responses due to changes in the surrounding environment could be an indicator of adjustment capacity (Yancheva et al., 2017). As explained by Killen et al. (2021) interest in the measurement of metabolic rates is growing rapidly, because of the importance to understand the organismal physiology, behavior, evolution and responses to environmental

changes, including pollution. Fish can reflect immediate responses to toxic substances in the surrounding water by changes in their physiological responses, such as respiration rate intensity. Our results are in line with the results of Dobreva et al. (2008) and Todorova et al. (2015) who examined the negative effects of wastewater and heavy metals on this parameter and Cyprinid species. Dobreva et al. (2008) studied the intensity of respiration in Prussian carp (*Carassius gibelio* Bloch, 1782) after exposure to copper, finding a 30–45 % decrease in the intensity of respiration of fish with increasing the respective metal concentration. Ivanova et al. (2008) conducted a study on the effects of wastewater from wood processing activities on the respiration rate in common carp. Similarly to us, the authors found that the treated individuals had reduced oxygen deficiency compared to the control group. In general, our results could be related to respiration impairment, caused by limited access to oxygen in the fish gills, which may therefore lead to oxygen stress and the formation of free radicals, which is the subject of future studies.

4.5. Condition and hepatosomatic index

Biological indices, such as condition factor (CF)/Fulton's factor of condition and hepatosomatic index (HSI) are also used to assess the general physiological state of individual organisms in a given population (Salvanes et al., 2018). We agree with Barišić et al. (2018) to whom these indices cannot be considered as appropriate biomarkers of water pollution due to their dependence on a seasonal cycle, food availability, fish physiology, etc. However, we think that under controlled conditions and along with histological and biochemical analyses, they can be successfully applied with regard to the multi-biomarker approach.

In general, we found lower values for K and higher values for HIS for all the experimental treatments. However, we found statistically significant lower values for K compared to the control only for the highest CYP concentration, which in terms of this tested biological tool also confirmed the higher toxicity of the pesticide. Our results are in agreement with other authors (Cazenave et al., 2014; Delahaut et al., 2019) regarding the lower values of Kf of fish from prolonged exposures to xenobiotics and one possible explanation is the energy budget; the body tries to invest more energy in detoxifying processes than in other metabolic processes, such as growth. As explained by Delahaut et al. (2019) the liver stores energy as glycogen, fat, and protein molecules, thus it is expected that the healthier fish possess larger livers, and thus the HIS will be characterized by higher values. Our results did not follow this trend; in terms of HIS, we found higher values for all the CYP and CPF treatments compared to the control. This result is consistent with other ecotoxicological studies (Delahaut et al., 2019;Sanchez et al., 2007) reporting an enlarged fish liver. The higher HSI values are representative of negative effects from the exposure to contaminants since this requires a higher detoxification capacity of the liver. In our case, we consider that this result can be also related to the hepatic histological lesions, which were reported above, i.e. hypertrophy.

4.6. Behavior

According to Melvin and Wilson (2013) and Peterson et al. (2017), behavioral endpoints are generally 10–1000 times more sensitive compared to classic endpoints, such as mortality. Some of our results on the behavior of common carp exposed to both pesticides are in agreement with Bonansea et al. (2016), Redondo-López et al. (2022), Thoré et al. (2021a), and Özok et al. (2018) who reported similar changes. All the behavioral traits were affected by the pesticides throughout the experiment, and behavioral responses became more distinct with increasing pesticide concentrations. In addition, stronger responses were observed in the fish treated with CYP, which once again confirmed the more severe effects of this chemical compared to CPF. The alterations in fish behavior after exposure to the pesticides were normalized at the end of the 30 days treatment. This probably is evidence that the organisms had started acclimating to chronically polluted water, however, the effects on the histological structure of the gills and liver were severe. Furthermore, we observed intense mucus secretion. Our results agree with Bej et al. (2021) that excessive mucous secretion could be the result of defensive and avoiding responses to minimize the irritation caused by the tested pesticides. According to Uchenna et al. (2022), mucous cells can be efficient in seizing toxic agents and they assist in preventing the entrance of harmful substances into the gills, thereby the detachment can reduce the entrance of various toxic compounds into the circulatory system.

5. Conclusions

In the present study, an attempt was made to study the chronic effects of sub-lethal concentrations of two common pesticides, based on EU legislation on common carp after 30 days, which we considered environmentally relevant. We also tried to compare the prolonged biomarker responses to our short-term (96 hours) results (Georgieva et al., 2021). Our findings demonstrated significant changes in the histological structure of fish gills and liver, which were overall destructive rather than adaptive due to the long-term exposure and action of the toxicants. In addition, according to the qualitative results, the liver was more severely affected compared to the gills. What is more, our results on the other biomarkers, including antioxidant enzymes, respiration rate, and behavioral responses showed that CYP is more toxic compared to CPF. Such experiments need to be further performed to gain a more thorough knowledge on the long-term effects of pesticides on fish species, which is important to take targeted action to reduce or prevent the negative impact of chronic pesticide exposure on non-target species. Lastly, we suggest the use of the results obtained from similar experiments in the legal regulations on the application of pesticides and environmental conservation.

CRediT authorship contribution statement

Conceptualization, V.Y., S.S.; Methodology, E.G., V.Y., S.S., I.I., T.V., V.B., B.T.; Formal analysis, K.N.; Supervision, E.G., I.V.; Writing—review and editing, V.Y., S.S., K.N., L.A.; Funding acquisition, V.Y., E.G., I. B.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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