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Acute Histopathological Changes in Common carp (Cyprinus carpio Linnaeus, 1785) Gills: Pirimiphos-methyl, 2, 4 - Dichlorophenoxyacetic Acid and Propamocarb Hydrochloride Effects

Eleonora Kovacheva[1], Elenka Georgieva[1*], Iliana Velcheva[2], Mima Nikolova[3], Pepa Atanassova[5], Borislava Todorova[2], Dobrinka Todorova-Bambaldokova[2], Vesela Yancheva[2], Stela Stoyanova[1], Stoil Tomov[4]

[1]University of Plovdiv, Faculty of Biology, Department of Developmental Biology, Tzar Assen Str. 24, Plovdiv, 4000, BULGARIA
[2]University of Plovdiv, Faculty of Biology, Department of Ecology and Environmental Conservation, Tzar Assen Str. 24, Plovdiv, 4000, BULGARIA
[3]University of Plovdiv, Faculty of Biology, Department of Human Anatomy and Physiology, Tzar Assen Str. 24, Plovdiv, 4000, BULGARIA
[4]Medical University-Plovdiv, Department of General Medicine and Urology, Vassil Aprilov Blvd. 15A, Plovdiv, 4002, BULGARIA
[5]Medical University-Plovdiv, Department of Anatomy, Histology and Embryology, Vassil Aprilov Blvd. 15A, Plovdiv, 4002, BULGARIA
[*]Corresponding author: elenkageorgieva@uni-plovdiv.bg

Abstract. A number of characteristics make fish excellent experimental models in toxicological research, especially for the contamination of aquatic systems. The main aim of the present study was to investigate the negative effects of different classes of pesticides (insecticide, herbicide and fungicide), based on their LC_{50} on the gills histological architecture of common carp (*Cyprinus carpio* Linnaeus, 1758). The effects of the tested pesticides on fish gills were expressed with histopathological alterations, such as proliferative, degenerative and changes in the circulatory system. Based on our results, the test insecticide showed higher toxicity with more severe irreversible necrotic changes in common carp gills compared to the herbicide and fungicide exposure. The identified histopathological changes in the fish gills can be successfully applied as reliable biomarkers for monitoring the degree of negative effects on the organisms due to the pesticide toxicity. The results from such experiments could be applied in the legislation in order to protect the water bodies from pesticide contamination, in areas with intensive application of plant protection products used in agricultural practices.

Key words: histology, fish, gills, biomarkers, pollution, pirimiphos-methyl, 2, 4 - dichlorophenoxyacetic acid, propamocarb hydrochloride.

Introduction

Pesticides play a significant role not only in the agriculture industry, because they increase the production of food, but also improve human health as they reduce the rate of vector-borne diseases (Blindauer et al., 1999). Pesticides, such as insecticides, herbicides and fungicides have been a part of practices in agriculture throughout the world for many years. Therefore, a great number of synthetic organic pollutants are daily released into many types of wastewaters, enter into natural water channels and accumulate in the aquatic environment (Agarwal et al., 2016; Bartczak et al., 2016; Goscianska et al., 2017). Unfortunately, the indiscriminate use of these compounds for improving production may have different impacts on non-target organisms, and especially aquatic animals. The World Health Organization (WHO, 1992) reported that around 3 million cases of pesticide poisoning occur annually, which is resulting in 220.000 deaths worldwide. Some of these chemicals are mutagenic (Garaj-Vrhovac & Zeljezic, 2000; Kumar et al., 2009; Nwani et al., 2010), related to development of cancers (Leiss & Savitz, 1995) or may cause developmental deficits (Arbuckel & Sever, 1998).

According to Qian & Lin (2015) organophosphorus compounds (OPs) have been widely used due to their low persistence under natural conditions and high effectiveness for pest control. In addition, OPs have been applied not only in agricultural industry, but also suburban and urban settings (Costa, 2018). The mode of action of OPs results inhibiting the enzyme acetylcholinesterase (AChE), which is responsible for conduction of nerve impulses during the process of neurotransmission, plays a vital role in the neurons development and formation of the network in the central nervous system (Boelsterli, 2007; Paraoanu & Layer, 2008; Arun Kumar & Jawahar, 2013). Also, OPs may act as an endocrine disruptors and producers of genotoxic, immunotoxic effects and oxidative stress in aquatic animals (Yonar & Sakin, 2011; Chang et al., 2013; Lavarias et al., 2013). According to Biswas et al. (2019) pesticide residues can be detected for long periods after their application due to the decreased biodegradation in the environment. Furthermore, they could be absorbed by fish, which leads to negative influence on their health and meat properties, and this will eventually have a negative effect on humans as well (Naiel et al., 2020).

Pirimiphos-methyl is a rapid-acting organophosphorus pesticide, which is frequently used in prevention and control of beetles, snout beetles and moths during the storage of the grains (Mhadhbi & Beiras, 2012). The use of these chemicals in water bodies in many developing countries could cause reduction of fisheries (Ayoola, 2008).

Among numerous herbicides, 2,4-dichlorophenoxyacetic acid is the most popular element of plant protection products. It is often applied in agriculture practices due to its low cost and good selectivity (Liu et al., 2016). 2,4-dichlorophenoxyacetic acid was developed during World War II (Suzuki, 2017) and was introduced on the market in the 1940s (Islam et al., 2018). The herbicide has high toxicity to broadleaf plants. It is used around houses, gardens, on golf courses, ball fields, parks, in agriculture and forestry (Garabrant & Philbert, 2002). Studies have described post-2,4-D exposure effects in different organisms, such as endocrine disruption (Guerrero-Estevez & Lopez-Lopez, 2016), reproductive disorders (Pattanasupong et al., 2004), genotoxicity (Lajmanovich et al., 2015) and carcinogenic effects (Loomis et al., 2015). In humans, 2,4-D has been related to the development of Parkinson's disease (Tanner et al., 2009) and autism (Roman, 2007). Herbicides cause damage to important vital organs and reduce the survival, growth and the reproduction of aquatic organisms (Rahman et al., 2002).

Propamocarb hydrochloride [propyl 3-(dimethylamino)propylcarbamate

hydrochloride, is a systemic carbamated fungicide with protective action against phyco-mycetous diseases (*Phythium, Phytophthora spp*.) (Fernandez-Alba et al., 2001). According to Pieroh et al. (1978) propamocarb was introduced into European markets in 1978 for control of oomycetes in decorative crops and some vegetables.

The freshwater environment includes different groups of organisms like fish, amphibians, invertebrates, plants and microorganisms. Pesticides can have direct effects on them, as a result of the physiological action of the substance, and indirect effects, which include the ecological interactions between species (Preston, 2002).

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 usualy inhabits ponds, lakes and rivers, and also rarely inhabits brackish-water environments (Barus et al., 2001). The fish has excellent commercial value and may be a good model to study the responses to various environmental contaminants (Vinodhini & Narayanan, 2009). According to Xing et al. (2012) common carp is a widely distributed bottom-dwelling fish, with feeding habits, which expose them to many different types of environmental toxicants. Furthermore, they are easily captured and are often used for human consumption. All these features make common carp a very good indexical organism for the study of contamination on aquatic systems.

Fish gills are comparatively vulnerable to toxic stress due to their continual contact with contaminated water and large surface area. They are the first and the major site of uptake of waterborne toxicants and toxic impacts (Vigliano et al., 2006).

Histopathological alterations in fish gills have been frequently used as bioindicators in evaluation of the health of fish exposed to different pollutants, both in the laboratory and field studies. Therefore, histopathological changes can be used as tools for monitoring the effects of multiple contaminants, and are also a reflection of the whole ecosystem's health status. (Drishya et al., 2016; Olaniyi, 2020).

The present study aims to assess and compare for the first time the negative effects of different concentrations of pirimiphos-methyl, 2,4-dichlorophenoxyacetic acid and propamocarb hydrochloride**,** based on their LC_{50} on the gills histological architecture in the bioindicator species common carp (*Cyprinus carpio* Linnaeus, 1758), after acute (96h) laboratory exposure.

Material and Methods

Test species

Common carp selected as a bioindicator species due to its excellent commercial value and an excellent model to study the responses to various environmental contaminations (Vinodhini & Narayanan, 2009). According to Xing et al. (2012) common carp is a widely distributed bottom-dwelling fish, with feeding habits that expose them to many different types of environmental contaminants.

Test chemicals

In the present experiment, all pesticides were purchased as a commercial products used in the agricultural practice. The decreasing pesticide concentrations were prepared as a dilution of LC_{50} of each test toxicant. The applied concentrations were as follows: 10 µg/L and 60 µg/L pirimiphos-methyl; 50 µg/L and 100 µg/L 2,4-dichlorophenoxyacetic acid; 40 µg/L and 80 µg/L propamocarb hydrochloride.

Acute experimental exposure

Carp juveniles with normal morphology and no visible alterations, were provided by the Institute of Fisheries and Aquaculture (Plovdiv, Bulgaria). After transportation, fish were placed in a 100-L glass tank and they were not fed for 2 days before the experiment according to De Moura et al. (2017). After the acclimatization period (two weeks), fish were divided into eight groups

(n = 15), including a control group with no added toxicant. Acute exposure of 96 hours in static conditions was carried out according to Modesto et al. (2010). The experimental setup was performed only once and as a pilot study in order to identify the histopathological effects of different concentrations of the tested pesticides. The basic physicochemical characteristics of the water, such as conductivity, dissolved oxygen, pH, and temperature, were measured once a day with a multi-parameter portable meter (MultiLine® Multi 3510 IDS, WTW-Xylem Analytics, Weilheim, Germany) according to APHA (2005). The basic physicochemical properties of the water stayed relatively constant during the experiment, without any significant changes between the control and the tested aquaria, therefore they will not be further discussed.

Histopathological analysis

Fish dissection was held carefully following the guidelines of Directive 2010/63/EU, regarding the protection of animals used for scientific purposes. For each pesticide concentration, the gills from 15 fish were fixed in 10% neutral buffered formaldehyde, washed in tap water and dehydrated in increasing ethanol concentrations, cleared with xylene and then infiltrated with liquid paraffin with a melting point of 54–56°C. Histological sections of 5 µm were prepared by using a rotary microtome (Leica RM 2125 RTS, Leica Microsystems, Wetzlar, Germany) and then stained with hematoxylin-eosin (H&E) following the method of Gautier (2011). Gills histological sections were examined with a light microscope (Leica DM 2000 LED, Leica Microsystems, Wetzlar, Germany) connected to a microscope camera (Leica DM 2000 LED, Leica Microsystems, Wetzlar, Germany). The histopathological alterations were classified using the semi-quantitative method according to Bernet et al. (1999), which we slightly modified. The severity of each histopathological alteration in the fish gills was specified according to the five-degree (0–5) severity gradation scale proposed by Saraiva et al. (2015). Lastly, organ index values (IO) were calculated using classes based on the scoring system introduced by Zimmerli et al. (2007).

Results

The results showed normal morphology of the gill histological structure in the control group. We observed primary lamellae, which were closely spaced and arranged in rows. The secondary lamellae were observed across the filaments, and they were covered by a single-layer epithelium. In the circulatory system in each lamella, we observed two main blood vessels: an afferent one, which extends from the gill arch to the tip of the filament, and an efferent blood vessel, which returns the blood to the gill arch. According to the five-degree (0–5) severity gradation scale, the control gill sections were determined as 0, despite the fact that in some individuals, we found lamellar lifting, which occupied less than 10% of the section surface. The normal histological structure of the common carp gills is shown in Table 1 and Fig. 1 A.

After the acute exposure with pirimiphos-methyl we observed histopathological alterations in the epithelial tissue of the gills, as well as in the circulatory system. The degree of expression of each of the observed changes is presented in Table 1. The histopathological lesions were scored according to Bernet et al. (1999). The changes were observed in both, the primary and secondary lamellae.

In regard to the first group of histopathological alterations classified according to Bernet et al. (1999), we found vasodilation of the blood vessels in the filament, which was expressed in a mild degree in both tested concentrations of pirimiphos-methyl (Table 1, Fig. 1 B,F). In contrast to the observed changes in the primary lamellae, in the secondary lamellae we found increasing of the degree of expression at the 60 µg/L pirimiphos-methyl (Table 1). In addition, we also found aneurisms of the secondary lamellae only at concentration of 60 µg/L pirimiphos-methyl (Fig. 1F).

Table 1. Histopathological lesions in common carp gills after acute (96-h) exposure to pirimiphos-methyl. Severity of the histopathological alterations, according to Saraiva et al. (2015) gradation scale: 0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations.

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value – concentrations of pirimiphos-methyl (µg/L)		
				Control	10	60
Changes in the circulatory system	*Filament*	Vasodilation	$W_{GC1} = 1$	0	2	2
	Secondary lamellae	Vasodilation	$W_{GC2} = 2$	0	3	4
	Secondary lamellae	Aneurysms	$W_{GC3} = 2$	0	0	2
Index for changes in the circulatory system				**I_{GC} =0**	**I_{GC} =8**	**I_{GC} =14**
Degenerative changes	*Gill epithelium (filament)*	Necrosis	$W_{GR1} = 3$	0	0	1
	Gill epithelium (secondary lamellae)	Necrosis	$W_{GR2} = 3$	0	1	1
Index for degenerative changes				**I_{GR} = 0**	**I_{GR}= 3**	**I_{GR}=6**
	Gill epithelium (filament)	Edema	$W_{GP1} = 1$	0	1	1
		Proliferation of stratified epithelium	$W_{GP2} = 2$	0	3	2
Proliferative changes		Proliferation of glandular cells	$W_{GP3} = 1$	0	1	0
		Fusion	$W_{GP4} = 3$	0	3	3
	Gill epithelium (secondary lamellae)	Lamellar lifting	$W_{GP5} = 1$	0	1	2
		Proliferation of stratified epithelium	$W_{GP6} = 2$	0	2	2
Index for proliferative changes				**I_{GP}=0**	**I_{GP}=20**	**I_{GP}=20**
Index for organ I_G				**I_G=0**	**I_G=31**	**I_G=40**

The degenerative changes in the filament were expressed in a very mild degree of necrosis of the epithelial tissue only at the highest concentration. Likewise, necrotic lesions were also observed in the secondary lamellae of the gills in a very mild degree (Table 1; Fig. 1D). The proliferative changes were detected in both, the filament and secondary lamellae. Edema was expressed in a very mild degree at both concentrations. Moreover, the proliferation of the stratified epithelium of the filament was observed in moderate degree at 10 µg/L pirimiphos-methyl and in mild degree at 60 µg/L pirimiphos-methyl. The proliferation of glandular cell in the filament was found only at the highest insecticide concentration in a very mild degree of expression. As a more severe proliferative change - fusion was detected at both tested concentrations in a moderate degree (Fig. 1 C). In regard to the proliferative alterations, we observed also lamellar lifting and proliferation of the stratified epithelium of the gill tissue. Lamellar lifting was expressed in a very mild degree at the lowest concentration and in a mild degree at 60 µg/L pirimiphos-

methyl. Furthermore, proliferation of the stratified epithelium of the secondary lamellae was expressed in a mild degree (Table 1). Comparing the indices of histopathological changes in the circulatory system (I_{GC}), for the lower pirimiphos-methyl concentration of 10 µg/L I_{GC} was 8, while at the concentration of 60 µg/L pirimiphos-methyl I_{GC} was found to be 14. This indicates that the higher concentration of pirimiphos-methyl has a greater effect on the degree of expression of the changes in the circulatory system, which proves that it has a more severe effect on the structure of the organ too (Table 1). Moreover, we observed vasodilatation mainly at the secondary lamellae. Likewise, to the changes in the circulatory system, the indices of degenerative changes (I_{GR}) showed a tendency to increase the degree of expression. According to Table 1, at the lowest tested concentration I_{GR} was 3, while at the highest concentration we observed I_{GR} 6. The indices for proliferative changes (I_{GP}) after pirimiphos-methyl were 20 for both tested concentrations, but we found different degrees of expression of the proliferative lesions (Table 1). Based on the obtained results and the scheme proposed by Zimmerli et al. (2007) we calculated that gill index (I_G) falls into the Class IV (index 31-40) for both concentrations of pirimiphos-methyl. Furthermore, we observed more severe degenerative alterations and changes in the circulatory system at the concentration of 60 µg/L pirimiphos-methyl.

The observed histopathological alterations under the action of 2,4-dichlorophenoxyacetic acid were found to be more severe than those observed in pirimiphos-methyl exposure (Table 2).

After 2,4-dichlorophenoxyacetic acid treatment, alterations of sinus vasodilation were detected in the filament in a mild degree at the concentration of 50 µg/L and in a moderate degree at the concentration of 100 µg/L. In addition to the changes observed in the primary lamellae, similar degree of vasodilation of secondary lamellae was found at both experimental concentrations. The observed degrees in both, filament and secondary lamellae, were increased with the increasing concentration of the herbicide (Table 2, Fig. 2 B,E).

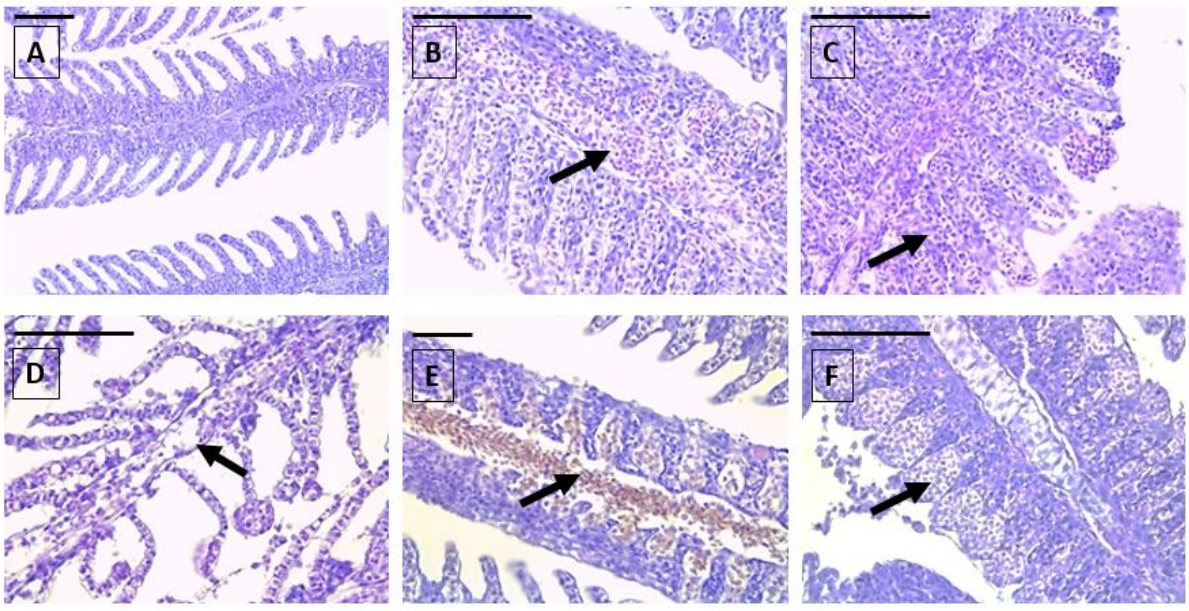

Fig. 1. Histopathological alterations in carp gills after acute exposure with pirimiphos-methyl exposure (H&E): A - control group, x200; B – vasodilatation in the filament at 10 µg/L pirimiphos-methyl, x 400; C – fusion of the secondary lamellae at 10 µg/L pirimiphos-methyl, x 400; D – degenerative changes (necrosis) at 60 µg/L pirimiphos-methyl, x400; E – vasodilatation in the filament at 60 µg/L pirimiphos-methyl, x200; F – aneurisms of the secondary lamellae 60 µg/L pirimiphos-methyl, x400.

Table 2. Histopathological lesions in the gills of common carp after 96-h exposure to 2,4-dichlorophenoxyacetic acid. Severity of the histopathological alterations, according to Saraiva et al. (2015) gradation scale: 0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations.

Reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value – concentrations of 2,4-Dichlorophenoxyacetic acid (µg/L)		
				Control	50	100
Changes in the circulatory system	*Filament*	Vasodilation	$W_{GC1} = 1$	0	2	3
	Secondary lamellae	Vasodilation	$W_{GC2} = 2$	0	2	3
	Secondary lamellae	Aneurysms	$W_{GC3} = 2$	0	0	0
Index for changes in the circulatory system				**I_{GC} =0**	**I_{GC} =6**	**I_{GC} =9**
Degenerative changes	*Gill epithelium (filament)*	Necrosis	$W_{GR1} = 3$	0	1	0
	Gill epithelium (secondary lamellae)	Necrosis	$W_{GR2} = 3$	0	0	1
Index for degenerative changes				**I_{GR} = 0**	**I_{GR}=3**	**I_{GR}=3**
Proliferative changes	*Gill epithelium (filament)*	Edema	$W_{GP1} = 1$	0	2	2
		Proliferation of stratified epithelium	$W_{GP2} = 2$	0	3	4
		Proliferation of glandular cells	$W_{GP3} = 1$	0	1	0
		Fusion	$W_{GP4} = 3$	0	3	3
	Gill epithelium (secondary lamellae)	Lamellar lifting	$W_{GP5} = 1$	0	2	3
		Proliferation of stratified epithelium	$W_{GP6} = 2$	0	2	2
Index for proliferative changes				**I_{GP}=0**	**I_{GP}=26**	**I_{GP}=30**
Index for organ I_G				**I_G=0**	**I_G=35**	**I_G=41**

The degenerative changes in the histological structure of the gills were expressed in a very mild degree of necrosis of the epithelial tissue. In the filament, necrosis was found only at the concentration of 50 µg/L, while at the concentration of 100 µg/L necrosis was found only in the secondary lamellae (Table 2, Fig. 2C). The proliferative changes were detected in both, the filament and secondary lamellae of the carp gills. Edema was expressed in a mild degree at the both concentrations. Furthermore, the proliferation of the stratified epithelium of the filament was observed in a moderate to severe degree and showed an increase with increasing concentrations of the pesticide. The proliferation of glandular cell in the filament showed very mild degree of expression, with a decreased degree at the increased concentration of the herbicide. Fusion was detected at both concentrations in a moderate degree (Fig. 2D). The proliferative alterations found in the secondary lamellae were expressed in lamellar lifting and proliferation of the stratified epithelium of the tissue. Lamellar lifting was observed in a mild to moderate extend. Meanwhile, proliferation of the stratified epithelium of the secondary lamellae was expressed in a mild degree of expression at both tested concentrations (Table 2, Fig. 2F). Comparing the indices of histopathological changes in the circulatory system (I_{GC}), for the lower concentration I_{GC} was 6, while for the higher concentration I_{GC} was 9. This indicates that the higher concentration of 2,4-dichlorophenoxyacetic

acid has a greater effect on the degree of expression of the changes in the circulatory system of the organ, which proves that it has a more severe effect on the structure of the organ as well (Table 2). Concerning the indices for degenerative changes (I_{GR}), the calculated values were similar (I_{GR} 3) at both tested concentrations (Table 2). Unlike the indices of degenerative changes, those for proliferative changes (I_{GP}) after the 2,4-dichlorophenoxyacetic acid exposure increased to the increasing pesticide concentration. For the concentration of 50 µg/L exposure I_{GP} was 35 and for the 100 µg/L tested concentration I_{GP} was 41 (Table 2). According to the scheme proposed by Zimmerli et al. (2007) and our results after 2, 4-dichlorophenoxyacetic acid exposure, the calculated gill index falls into the Class IV (index 31-40) for the lower concentration of the pesticide, which means the structure of the tissue is with pronounced histopathological alterations. The higher tested concentration falls into the Class V (index > 41), which showed that the structure of the tissue is affected by severe histopathological alterations. The indices of both experimental concentrations of the tested herbicide confirm the toxicity of 2,4-dichlorophenoxyacetic acid.

After the exposure with propamocarb hydrochloride we observed histopathological alterations in the epithelial tissue of the gills and in the circulatory system. The degree of expression of each of the histopathological changes is presented in Table 3. Moreover, the histopathological lesions were scored according to Bernet et al. (1999) in three groups – lesions in the circulatory system of the organ, degenerative and proliferative changes (Table 3). The changes were observed in both, the primary and secondary lamellae of the fish similarly to the other two tested pesticides.

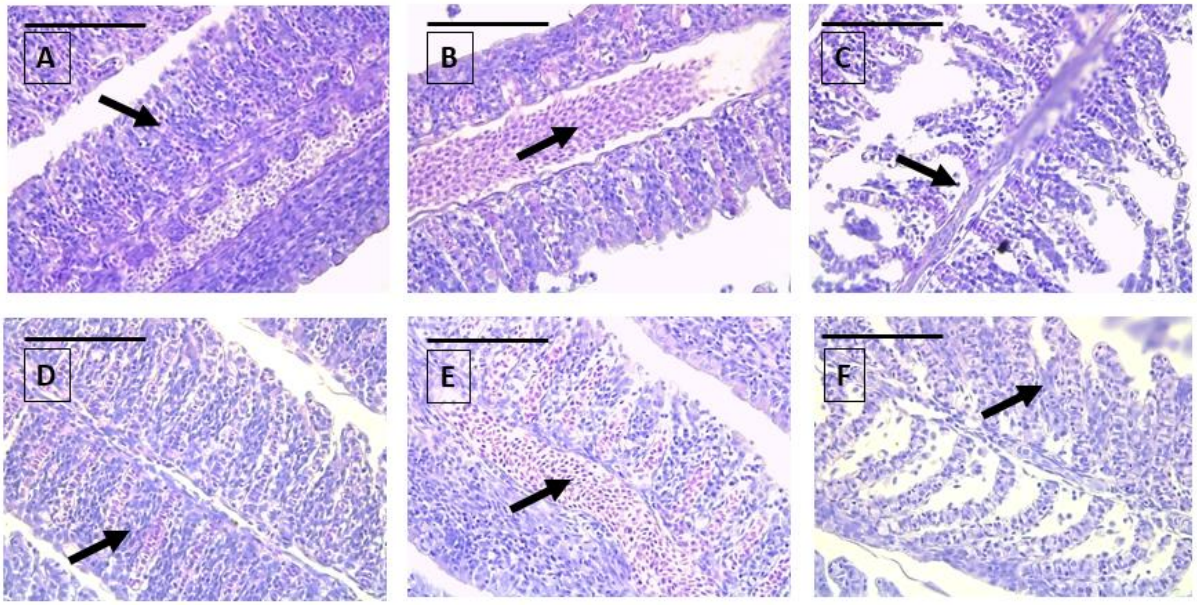

Fig. 2. Histopathological lesions in common carp gills after 2,4-Dichlorophenoxyacetic exposure (H&E), x400: A – proliferation of stratified epithelium in the filament after exposure with 50 µg/L 2,4-Dichlorophenoxyacetic acid; B – vasodilation of the blood vessel in the filament after exposure with 50 µg/L 2,4-Dichlorophenoxyacetic acid; C – degenerative changes of the stratified epithelium in the filament after exposure with 50 µg/L 2,4-dichlorophenoxyacetic acid; D – fusion of the secondary lamellae after exposure with 100 µg/L 2,4-dichlorophenoxyacetic acid; E - vasodilation of the blood vessel in the filament after exposure with 100 µg/L 2,4-dichlorophenoxyacetic acid; F - proliferation of stratified epithelium in the filament after exposure with 100 µg/L 2,4-dichlorophenoxyacetic acid.

Table 3. Histopathological lesions in the gills of common carp after 96-h exposure to propamocarb hydrochloride. Severity of the histopathological alterations, according to Saraiva et al. (2015) gradation scale: 0 – none; 1 – very mild alterations; 2 – mild alterations; 3 – moderate/pronounced alterations; 4 – severe alterations; 5 – very severe alterations.

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

In regard to the alterations in the circulatory system, we found vasodilation of the blood vessels in the filament and in the secondary lamellae expressed in a mild to moderate degree in both concentrations of propamocarb hydrochloride (Table 3, Fig. 3A, D). We found aneurisms only at the lower fungicide concentration (Table 3, Fig. 3C). Concerning the degenerative changes in the filament and in the secondary lamellae, no changes of the epithelial tissue were observed in both tested concentration of the fungicide (Table 1). The proliferative changes were showed in both filament and secondary lamellae of the gills. Edema was observed in a moderate degree in the lower concentration, but in a mild in the higher concentration. We found the similar tendency in the observed proliferation of the stratified epithelium of the filament (Table 3, Fig. 3F). The proliferation of glandular cells in the filament was found only in the lower concentration. As more pronounced proliferative change, fusion was shown at both concentrations in a severe degree (Fig. 3B). Also, the lamellar lifting showed similar degree of expression in both concentrations (Fig. 3E). Proliferation of the stratified epithelium of the secondary lamellae was expressed in a severe degree at concentration of 40 µg/L and in moderate degree at concentration of 80 µg/L (Table 3). The indices of histopathological changes in the circulatory system (I_{GC}) for the concentration of 40 µg/L I_{GC} was 8, while at the concentration of 80 µg/L I_{GC} was 6. In addition to the changes in the circulatory system, the

indices of proliferative changes (I_{GP}) showed a tendency to decrease the degree of expression similarly to the circulatory alterations. According to Table 3, at the lower tested concentration I_{GP} was 36, while at the higher concentration we observed I_{GP} 30. The indices for degenerative changes (I_{GR}) after propamocarb hydrochloride exposure were scored as 0 for both fungicide concentrations due to the fact that we found no necrosis in the fish gills after the acute treatment (Table 3). Based on the obtained results and the scale proposed by Zimmerli et al. (2007) we calculated that gill index (I_G) falls into the Class V (index > 41) for the concentration of 40 µg/L propamocarb hydrochloride, which is an indicator of the severe histopathological alterations in the gill tissue caused by the negative effects of propamocarb hydrochloride. The concentration of 80 µg/L falls into the Class IV (index 31-40), which showed pronounced histopathological alterations. Generally, we found a tendency towards decreasing the degree of expression with the increasing concentration of the tested fungicide propamocarb hydrochloride (Table 3) from which can be assumed that the fish treated with lower concentration of the pesticide showed a higher degree of adaptive mechanisms.

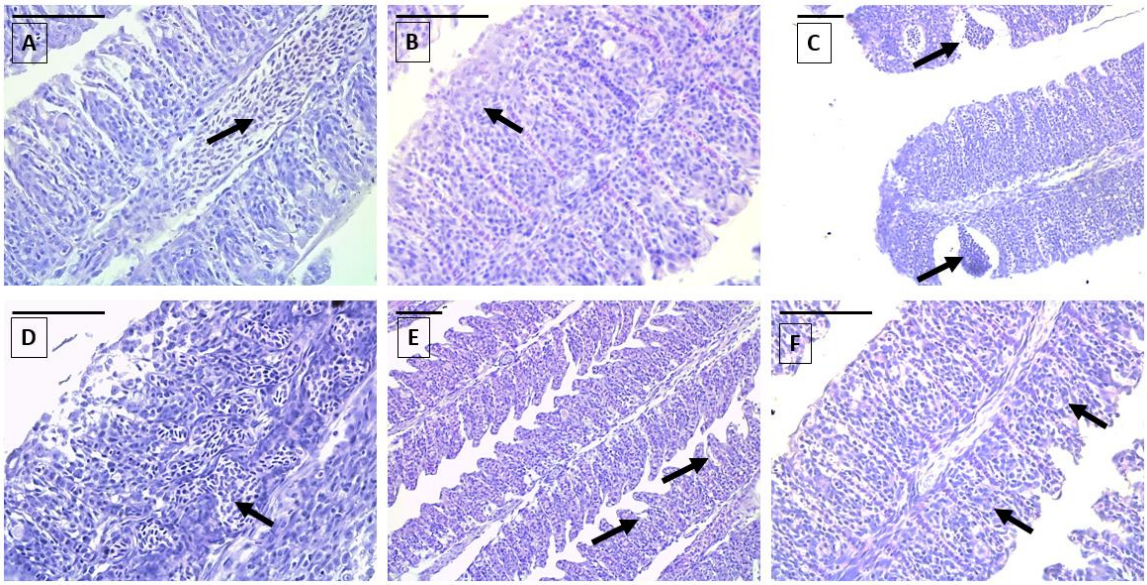

Fig. 3. Histopathological lesions in common carp gills after propamocarb hydrochloride exposure (H&E): A – vasodilatation of the blood vessel in the filament after exposure with 40 µg/L propamocarb hydrochloride, x400; B – fusion in the secondary lamellae after exposure with 40 µg/L propamocarb hydrochloride, x400; C – aneurisms in the secondary lamellae after exposure with 40 µg/L propamocarb hydrochloride, x200; D – vasodilatation of the secondary lamellae after exposure with 80 µg/L propamocarb hydrochloride, x400; E – lamellar lifting of the secondary lamellae after exposure with 80 µg/L propamocarb hydrochloride, x200; F - proliferation of stratified epithelium in the filament after exposure with 80 µg/L propamocarb hydrochloride, x400.

Discussion

Fish gills play an important role in exchange of gas and ionic regulation. Pesticides, and especially organophosphates, have a severe effect on the structure and function of the gills. According to Menezes-Faria et al. (2007) gills are firstly affected from entered pesticides into fish, and in response to

the changes in the environment, they may produce adaptive strategies to preserve the physiological function. The histopathological alterations can be used as efficient indicators of toxicity in fish. The high variety of different chemical substances in water is an additional factor influencing their toxicity in aquatic organisms.

In addition, the alterations in the gill architecture are adaptive, necessary to reduce the rate of absorption of toxicants. Epithelia hypertrophy increases the water-blood distance and as a result reducing the rate of absorption of xenobiotics (Agamy, 2013). However, epithelia hypertrophy decreases the respiratory surface area thus, reducing the effectiveness of gas exchange and leading to osmoregulatory dysfunction (Sakuragui et al., 2003).

The obtained results from the present study show a high degree of proliferative changes in the gill epithelium due to the negative effects of the tested pesticides. These alterations serve as an indicator of the occurrence of compensatory-adaptive mechanisms in the organism of the studied individuals under the influence of the applied pesticide concentrations and short-term exposure. Although the experimental concentrations are lower than the LC_{50} their application in the plant protection product leads to an increase in the toxicity of the pesticides. As a result of the toxicant effects, protective mechanisms were activated in the organism and specifically in the gills, which are in direct contact with the contaminated water. An increase in the thickness of the epithelial layer, often leading to fusion of the secondary lamellae, increases the distance between the organism and the toxicant. This statement is also confirmed by the lower expression of degenerative changes in the gill epithelium, which we observed. The highest degree of proliferation was found after the exposure to the tested fungicide, where the indices ranged between 30-36. On the other hand, the lowest degree of proliferative changes was observed after pirimiphos-methyl exposure. In the case of the insecticide exposure, in parallel to the lower values of proliferative changes, the highest values of necrotic changes in the gill epithelium were also found. We can assume that with chronic exposure, the proliferative and degenerative processes could most likely equalize or even lead to a preferential increase in necrobiosis and necrosis.

Unlike the insecticide and herbicide exposures, during the exposure to the tested fungicide necrotic changes were not observed. Moreover, a higher degree of proliferative changes was observed at the lower concentration. In parallel, the degree of changes in the circulatory system was also higher at this concentration. The observed changes could be due to oxidative stress occurring in the organism at a concentration of 40 µg/L, which leads to a higher need for oxygen delivery, which is an indicator for vasodilation and aneurysms. The higher need for oxygen is a result of intense mitoses, which reach a severe and very severe degree of expression with changes, such as proliferation of the epithelium at the filament and fusion of the secondary lamellae. At concentration of 80 µg/L, these values decreased and we could possibly assume that during the chronic exposure, the degree of proliferative changes and those in the circulatory system may equalize, which could also lead to the appearance of degenerative changes in the epithelial cells due to disruption of their metabolism.

Our results on gill histology after pirimiphos-methyl treatment are similar to those reported by Xing et al. (2012), who studied the effect of chlorpyrifos and found varied degrees of epithelial hypertrophy, telangiectasis, oedema with epithelial separation from basement membranes, general necrosis, and epithelial desquamation. Similar changes were observed by Al-Mamoori et al. (2014), who found partial lamellar deformation,

abnormal lamellae, partial terminal attachment of the lamellae, marginal dilation, hyperplasia of epithelial cells, marked gill deformation, marked lamellar aneurysm, marked lamellar fusion with epithelial cells hyperplasia, and diffuse mass of the gill lamella after treatment with chlorfos.

We agree with Devi & Mishra (2013) who observed signs of epithelial lesions when fish were exposed to sublethal concentration of chlorpyrifos, which was time dependent even treated with low concentration. Our results are also in agreement with those obtained by Kunjamma et al. (2008) in regards to short-term exposure of pesticides (hyperemia, clubbing and edema).

Our results on gill histology are similar to those reported by Makinde et al. (2015) who stated there was an evidence of lifting of epithelia layer, vacuolization, inter-lamella hyperplasia and mild desquamation of the epithelia lining. The alterations in the gill architecture the authors observed in all fish exposed to the acute concentrations of 2, 4-D amine except the control group. The degenerative lesions (vacuolization, epithelia lifting and desquamation of the epithelia lining) and proliferative response (hypertrophy) in their study were severe in the fish exposed to the highest concentration of 2,4 D - amine.

We agree with Vigario & Saboia-Morais (2014) who found signs of high gill vascularization, epithelial hyperplasia, lamellar fusion and changes in glycoconjugate granules of mucous cells after exposure to 2,4 – D herbicide. As explained by the authors, we agree that lamellar fusion is a defense mechanism that decreases the surface of gas exchange, which is vulnerable to the action of the herbicide. However, such defense responses to pollutant agents are usually irreversible toxic effects (Ortiz et al., 2003).

Furthermore, we agree with Rocha et al. (2015) who found histopathological changes, such as regressive, vascular and progressive disorders, and no neoplastic disorders after a herbicide treatment.

Our result on gill histology after propamocarb hydrochloride treatment are similar to the result reported by Boran et al. (2012) who observed hypertrophy and necrosis of epithelium, separation of epithelium from lamellae (epithelial lifting), lamellar fusion, hyperplasia of lamella and the space under the epithelium filled with eosinophilic material after a captan exposure. In addition, Choudhury (2018) found epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, edema, general necrosis and degeneration of primary and secondary gill lamellae after fungicide treatment.

Conclusions

In sum, we can conclude that both concentrations of each pesticide (pirimiphos-methyl, 2,4 - dichlorophenoxyacetic acid and propamocarb hydrochloride), which were lower than LC_{50} negatively affected the histological structure of common carp gills and also activated compensatory-adaptive mechanisms, resulting in pathological proliferative changes. We consider that these histopathological lesions affect the gills by disrupting their functions. There was a tendency towards enhancing the morphological alterations and their degree of expression was proportional to the increasing pesticide concentrations. Overall, based on our results, the test insecticide showed higher toxicity with more severe irreversible necrotic changes in the common carp gills compared to the herbicide and fungicide exposure.

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