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Toxicological impact of a neonicotinoid insecticide and an organophosphorus fungicide on bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) gills: a comparative study

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Abstract. The main aim of the present study was to compare the toxicological effects of a fosetyl-Al and fenamidone based fungicide and a thiamethoxam based insecticide on the gill histological structure of bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) in a short-term laboratory conditions (96 hours). We used of the insecticide 6.6 mg L^{-1}, 10 mg L^{-1} and 20 mg L^{-1} representing 30, 20, 10 times dilution, and of the fungicide – 30 mg L^{-1}, 38 mg L^{-1} and 50 mg L^{-1} representing 50, 40, 30 times dilution, respectively. These concentrations were considered as real applicable pesticide concentrations in plant protection practices. Overall, we found pronounced alterations in the gill histological structure such as proliferative and regressive, as well as changes in the circulatory system in the fish treated with both pesticides. The histopathological alterations indicated the negative effects of the applied chemicals on non-target aquatic species such as bighead carp. In addition, we found that, in terms of the histological lesions and tested fish species, the fungicide had more severe effects compared to the insecticide.

Key words: pesticides, gills, histology, bighead carp.

Introduction

Pesticides have been widely applied to protect agricultural crops since the 1940s, and, since then, their use has increased steadily (Grung et al. 2015). To maintain the water at an adequate quality and to preserve natural ecosystems and biodiversity, it is necessary to sustainably use, protect and manage the water resources. In the countries of the European Union (EU), national water agencies, which follow EU policy and the requirements of the EU Water Framework and its Daughter Directives (Directive 2000/60/EC, Directive 2008/105/EC, Directive 2008/56/EC, European community's environmental objectives 272/2009, European community's technical report 2010), implement regular water monitoring with the aim to control and prevent pollution (Milačič et al. 2017, Rashid et al. 2018). The worldwide usage of pesticides and high diverseness of all these substances make them one of the most important contaminants of aquatic habitats (Khoshnood 2017). According to FAO (2003), when pesticides are not used according to appropriate agricultural practices, they initially enter into the environment via point sources, but they are further dispersed via diffusion processes. As stated by Tsaboula et al. (2019) and according to EU (EC Technical guidance on priority substances and pesticides, 2012) point sources are considered as any spills of concentrated or diluted pesticides during transport, storage, filling, spraying, cleaning, and management of residual spray and maintenance of spray equipment. Different ways of motility for these compounds have been described, for instance, wind can carry out the pesticides from one field to another, runoff (from excessive watering system or rain) can lead them to different water bodies, even undergrounds water reservoirs; and by all these ways pesticides can affect the non-target organisms (Khoshnood 2017). Neonicotinoid insecticides nowadays present one of the most sold pesticides worldwide. These chemical compounds are applied as seed coating, leafspray and soil drenches when used in crops (Bonmatin et al. 2015), and they act on the central nervous system of insects, interfering with neural transmission (Gibbons et al. 2015, Iturburu et al. 2018). According to the European Food Safety Authority report (2006), fosetyl-Al belongs to the chemical class of phosphonates, but has a structure and mode of action that are different from those of most other phosphorus compounds used as pesticides. It can only be used as a fungicide. It was first commercialized as an agricultural systemic fungicide in the 1970s in France. It also acts indirectly by stimulating the natural defense mechanisms of the plant. In short-term non-target organ toxicity tests, it causes histological changes in the kidneys and interferes with the calcium-phosphorus metabolism. At higher doses, it causes urinary hyperplasia. Genix et al. (2003) described the mechanism of action of the imidazoline compound fenamidone. According to the authors, it belongs to the group of quinone external inhibitors (QoI), a name derived from their mode of action. The report of the European Food Safety Authority (2006) does not provide a combined risk assessment of these two ingredients of the tested fungicide. The majority of toxicity data of the fungicide come from studies carried out with its main ingredient, which is fosetyl-Al.

Khoshnood (2016) stated that a large range of biological techniques, such as molecular, biochemical, morphological, physiological, or at the level of community and population are used to monitor the impact of various pollutants or various concentrations of a single pollutant. Therefore, the histopathological alterations are widely applied as biomarkers for the effects of different pollutants on fish health. In addition, fish tissues and organs were repeatedly used as biomarkers for contamination due to their specific characteristics. According to Khoshnood (2016), among these organs, the gills are one of the most common tissues in environmental contamination studies due to their wide surface area, which is in a permanent contact with the surrounding water, high activity in ion transport, high amount of blood, etc. Other authors add that the inclusion of histopathologic pa-

rameters in toxicity tests can reveal structural and specific toxic effects on organs at sublethal level. Therefore, histopathology has been used in toxicity assessments aiming to identify tissue damage in aquatic animals exposed to contamination (Dutra et al. 2017, Soares et al. 2019).

Fish are among the group of aquatic organisms, which represent the largest and most diverse group of vertebrates (Antal et al. 2013, Czédli et al. 2014). Likewise, they are virtually present in all environments and many species have been found to be susceptible to environmental pollutants (Van Der Oost et al. 2003). Yancheva et al. (2015, 2016) describe in reviews why in ecotoxicology fish have become the major vertebrate model. In addition, fish as bioindicators are sentinel organisms that respond to changes at various structural levels, from cellular, physiological, biochemical, genetic and histological factors to variations in patterns of behavior, which may affect the population structure of the species as a response to stressors present in the environment (Rodrigues et al. 2010, Velusamy et al. 2014, Lima et al. 2018).

Bighead carp (*Hypophthalmichthys nobilis*) is a freshwater species, which has the advantages of fast growth, it is strongly resistant to diseases, and has also a good meat quality and rich nutrition. As stated by Afzal et al. (2008) and Lin et al. (2018), the studies on bighead carp locally and overseas mainly focuses on the preservation of fish growth and propagation, ecological regulation, digestion and absorption of algae. According to our preliminary studies (see Stoyanova et al. 2015; Stoyanova et al. 2016; Yancheva et al. 2016; Yancheva et al. 2016; Stoyanova et al. 2018) common carp and bighead carp are usually reared together in aquaculture, in addition bighead carp is more sensitive to organic contaminants. Therefore we consider that it could be also used as bioindicator. Even though the toxic effects of pesticides on fish are well documented, the data on lesions in the gill histological structure of bighead carp are relatively scarce. Furthermore, as stated by Albañil Sánchez et al. (2019), the water ecosystems are usually contaminated with a combination of different pollutants. Thus, it is important to study the mixtures and not just the effects of a single compound, which does not appear isolated in the environment. Similarly to Albañil Sánchez et al. (2019), who have chosen not to perform tests with a single active substance, we aimed to investigate the effects of commercially and extensively applicable plant protection products on bighead carp. Therefore, we compared the effects of two pesticides – a fungicide and an insecticide on the gill histological structure of bighead carp in a short-term laboratory conditions (96 hours).

Materials and methods

Test pesticides

Fungicide. A fosetyl-Al (Aluminium tris-O-ethyl phosphonate) and fenamidone (1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one) based fungicide in the form of dispersible granules (WG) was used (Table 1 and 2). According to European Food Safety Authority (2006) fosetyl-Al is a phosphonate compound, but its structure and mode of action differ from the most of the other organophosphorus compounds used as pesticides. Moreover, fosetyl-Al is used for control of various plant pathogenic phycomycetes and ascomycetes. This active substance is used for preventing crops and also for inhibiting fungal spore germination and penetration of pathogens into plants. Fosetyl-Al also acts indirectly by stimulating the plant's natural defense mechanisms. Fenamidone belongs to the Quinone outside Inhibitors (QoI) group (Genix et al. 2003) and provides an excellent control of *Oomycetes* diseases (Leake 2003).

Table 1. Chemical information on Fosetyl-aluminium.

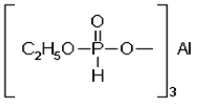
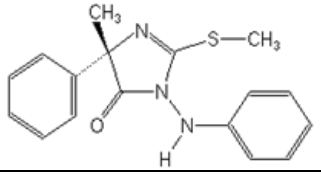
Common name (ISO)	*Fosetyl-aluminium*
Chemical nomenclature (IUPAC)	Aluminium tris-O-ethyl phosphonate Ehtyl hydrogen phosphonate, Aluminium salt, Phosphonic acid monoethyl ester
Minimum purity	960 g/kg
Chemical formula	$C_6H_{18}AlO_9P_3$
Molecular weight	354.14
Structural formula	

Table 2. Chemical information on Fenamidone.

Common name (ISO)	*Fenamidone*
Chemical nomenclature (IUPAC)	(*S*)-1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one, 4H-Imidazol-4-one, 3,5-dihydro-5-methyl-2-(methylthio)-5-phenyl-3-(phenylamino)-,(S)-(9CI)
Minimum purity	975g/kg
Chemical formula	$C_{17}H_{17}N_3OS$
Molecular weight	311
Structural formula	

*Fosetyl –Al – (one of the) tested fungicide in the present study.

Insecticide. A thiamethoxam ((E,Z)-3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-N-nitroamine) based insecticide was used. Thiamethoxam is the second-generation neonicotinoid insecticide that is used for the control of several commercially important insect pests on a variety of crops (Tang et al. 2017). Despite thiamethoxam represents a low risk for certain non-target organisms (e.g. mammals), the low soil absorption, high leaching capability, high solubility in water, and resistance to biological treatment make thiamethoxam a potential contaminant of surface and underground waters, which represents an important risk for fish health (Jeschke et al. 2011, Zhang et al. 2012, Baldissera et al. 2018).

Experimental exposure

Bighead carps were obtained from the Institute of Fisheries and Aquaculture, Plovdiv, Bulgaria where fish are reared under controlled conditions. They were weighed (g; total weight including viscera) and the total length (cm) were recorded (53.02 g ± 6.3; 18.65 cm ± 1.33). After transportation the fish were moved in glass aquaria with 100 L chlorine free tap water (by evaporation) to acclimatize for a week. The individuals were divided into three test groups for each tested pesticide (n=15), including the control (in total 7 groups). Thereafter, the fish were treated with different soluble concentrations of the tested pesticides for 96 hours (acute, short-term and static experiment). The tested concentrations of the pesticides were prepared by a dilution of the stock solution according to the instructions of the manufacturer. We used 6.6 mg L^{-1}, 10 mg L^{-1} and 20 mg L^{-1} insecticide (thiamethoxam) representing 30, 20, 10 times dilution, and 30 mg L^{-1}, 38 mg L^{-1} and 50 mg L^{-1} fungicide (fenamidone and fosetyl-Al), representing 50, 40, 30 times dilution of the stock solution, respectively (Table 3). In our preliminary experiments (un-

Table 3. Applied concentrations of the tested pesticides.

Pesticides	Stock solution	Dilution	Concentration pesticide	Concentration active substances
Fungicide	150 g/100 L	30 times	50 mg L^{-1}	33.35 mg L^{-1} fosetyl-Al, 2.2 mg L^{-1} fenamidone
		40 times	38 mg L^{-1}	25.08 mg L^{-1} fosetyl-Al, 1.65 mg L^{-1} fenamidone
		50 times	30 mg L^{-1}	20.01 mg L^{-1} fosetyl-Al, 1.32 mg L^{-1} fenamidone
Insecticide	20 g/100 L	10 times	20 mg L^{-1}	5 mg L^{-1}
		20 times	10 mg L^{-1}	2.5 mg L^{-1}
		30 times	6.6 mg L^{-1}	1.65 mg L^{-1}

published results) 10 and 20 fungicide dilutions resulted in a lethal effect of the tested fish groups. The basic physical characteristics of the water such as: pH, temperature, oxygen level and conductivity were followed strictly during the exposure according to a standard procedure (APHA 2005) with a combined field-meter (WTW, Germany). The experiment, which was performed in triplicates, was conducted in accordance with the national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes according to Directive 2010/63/EU.

Histological techniques

The fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (Rosseland et al. 2003). The fish were sacrificed by severing the spinal cord anterior to the dorsal fin (procedure approved by the Ethics Committee, Faculty of Biology, Plovdiv University, 2014). The histological analysis was prepared according to a standard procedure for light microscopy analysis (Gautier 2011). For each fish, the gills were sampled and preserved in 10% neutrally buffered formalin for 24 hours. Then they were dehydrated in graded ethanol series, embedded in paraffin and sectioned (5 µm thick) on a rotary microtome (Leica RM 2125 RTS) according to Humason (1962). The sections were stained with hematoxylin–eosin (H&E) and observed with a light microscope (Leica DM 500, Germany). The histological alterations were presented according to the semi-quantitative system proposed by Bernet et al. (1999). They were also classified into three reaction patterns – circulatory, regressive (degenerative) and progressive (proliferative) changes. Each reaction pattern includes several histopathological alterations, which concern either functional units of the organ or the entire organ (see Bernet et al. 1999). Secondly, a six degree (0–6) severity gradation scale (SGS), which represents the severity of each alteration, was defined according to Saraiva et al. (2015). Each grade represented a specific gill histopathological alteration and was categorized as follows: (0) – no histological alterations, which represented normal gill histological structure; (1) – mild histological alterations; (2) – moderate histological alterations; (3) – pronounced histological alterations; (4) – severe histological alterations and (5) – very severe histological alterations. The relevance of a lesion depends on its pathological importance, i.e. how it affects the organ function and the ability of the fish to survive. Therefore, the pathological extent of each lesion was defined with an importance factor according to Bernet et al. (1999) as follows: (1) – minimal pathological importance, the lesion is easily reversible as toxicant exposure ends; (2) – moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized and (3) – marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. The final value for each alteration results from the multiplication of the score value with the importance factor. Summing up these final values for one reaction pattern or organ gives the index for the respective reaction pattern i.e. index for circulatory disturbance (IC), index for regressive changes (IR), index for progressive changes (IP) for the gills or organ index (OI). Organ index values were used to classify the severity of histological response using classes based in the scoring scheme proposed by Zimmerli et al. (2007): Class I (index ≤ 10) – normal gill structure with slight histological alterations; Class II (index 11–20) – normal gill structure with moderate histological alterations; Class III (index 21–30) – moderate alterations of normal gills; Class IV (index 31–40) – pronounced histological alterations of the gills; Class V (index N 40) – severe histological alterations of the gills. The sum of the multiplied score values and importance factors of all diagnosed changes for each specimen resulting in different indices were statistically analyzed. The results were presented as average. Besides the indices calculated by extent (score value) and pathological importance (importance factor) of lesions, we calculated the prevalence of histological changes. The prevalence of each lesion was presented as the percentage occurrence within each specimen and we proposed the following classification: (10-30%) – alterations, which occurred rarely; (30-50%) – alterations, which occurred frequently; (50-70%) – alterations, which occurred more frequently and (>70%) – alterations, which prevailed in the gill histological structure.

Statistics

For the statistical processing of the data, the software package Graph Pad Prism 7 for Windows (USA) was used. The histological index results were presented as average values per group. The numbers of fish affected by specific histological lesions were presented as percentage prevalence. T-test was applied to see if there is a significant difference between the histological alterations in the exposed groups and the control, as well as between the different percentage prevalence.

Results

No external abnormalities were identified on any of the bighead carps. The gills of the control group were characterized with normal morphology including primary and secondary lamellae. The secondary lamellae were placed close together, arranged in rows and covered by gill epithelium. Based on the proposed scale, we determined the gill histological structure of control common carp as relatively normal (0) (Table 4, Figure 1). However, the macroscopic examination of the bighead carp gills from the tested groups showed alterations, which differed in severity, prevalence and type. The observed changes in the pesticides treated fish are presented in Table 4, 5, 6, 7, as well as in Figure 1, and 2.

Gills histological alterations after insecticide exposure

The histological alterations in bighead carp gills after the insecticide exposure are presented in Table 4 and 5.

At the lowest tested insecticide concentration, we found lamellar lifting in a severe degree of expression. In addition, the proliferative changes concerning the squamous epithelium and the glandular cells, as well as edema at the base of the secondary lamellae, were found in a moderate degree. In

Table 4. Histological lesions in bighead carp gills induced by the applied insecticide (96 hours).

Gills reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value				Index Control group	Index 6.6 mg L^{-1}	Index 10 mg L^{-1}	Index 20 mg L^{-1}
				Control	6.6 mg L^{-1}	10 mg L^{-1}	20 mg L^{-1}				
Circulatory disturbances	Blood vessels of secondary lamellae	Vasodilatation along the length of blood vessel	W_{GC1}=1	**0***	2	**0***	2	I_{GC}=0	I_{GC}=2	I_{GC}=1	I_{GC}=2
		Vasodilatation at the basal part of the blood vessel	W_{GC2}=1	0	0	0	0				
		Vasodilatation at the apical part of the blood vessel	W_{GC3}=1	0	0	1	0				
		Aneurysms	W_{GC4}=3	0	0	0	0				
	Blood vessels of primary lamellae	Vasodilatation	W_{GC5}=2	0	0	0	**2***				
Regressive changes	Epithelium	Degeneration (necrosis)	W_{GR1}=3	0	2	3	3	I_{GR}=0	I_{GR}=6	I_{GR}=9	I_{GR}=9
Progressive changes	Epithelium (secondary lamellae)	Lamellar lifting	W_{GP1}=1	0	3	3	4	I_{GP}=0	I_{GP}=13	I_{GP}=14	I_{GP}=**19***
		Proliferation	W_{GP2}=2	0	0	0	0				
	Epithelium (primary lamellae)	Edema	W_{GP3}=1	0	2	2	3				
		Proliferation of stratified epithelium	W_{GP4}=1	0	2	3	3				
		Proliferation of glandular cells	W_{GP5}=1	0	2	2	2				
		Proliferation of cartilaginous tissue	W_{GP6}=1	0	1	1	1				
		Fusion	W_{GP7}=3	0	1	1	2				

Bold* – statistically significant than the other concentrations ($P < 0.05$)

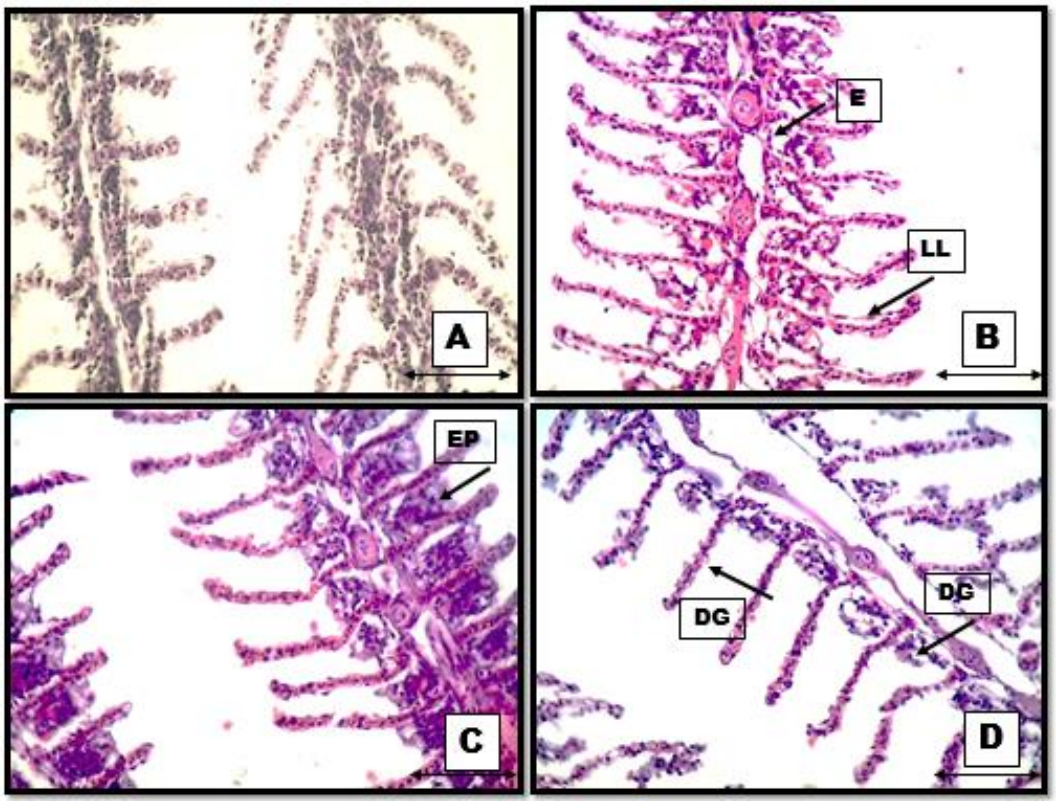


Figure 1. Histopathological lesions in bighead carp gills after the insecticide exposure, H&E: A – normal histological structure of bighead carp gills, x 200; B – edema (E) and lamellar lifting (LL) at 6.6 mg L^{-1} insecticide, x 400; C – epithelial proliferation (EP) at 10 mg L^{-1} insecticide, x 400; D – epithelial degeneration (DG) at 20 mg L^{-1} insecticide, x 400.

Table 5. The percentage prevalence of histological lesions in bighead carp gills after the insecticide exposure (96 hours).

Histological alterations		Prevalence, %			
		Control	6.6 mg L^{-1}	10 mg L^{-1}	20 mg l^{-1}
Circulatory disturbances	Vasodilatation along the length of blood vessel in the secondary lamellae	0	56	0	49
	Vasodilatation at the basal part of the blood vessel in the secondary lamellae	0	0	0	0
	Vasodilatation at the apical part of the blood vessel in the secondary lamellae	0	0	23	0
	Vasodilatation of central venous sinus	0	0	0	53
	Aneurysms	0	0	0	0
Regressive changes	Degeneration (necrosis)	0	36	68	73
Progressive changes	Lamellar lifting	0	65	67	85
	Proliferation of epithelium covering secondary lamellae	0	0	0	0
	Edema	0	43	51	71
	Proliferation of stratified epithelium	0	35	48	56
	Proliferation of glandular cells	0	39	41	53
	Proliferation of cartilaginous tissue	0	17	21	26
	Fusion	0	14	19	37
Average, %		0	23.5	26	**38.7***

Bold* – statistically significant the other concentrations ($p<0.05$)

Table 6. Histological lesions in bighead carp gills induced by the applied fungicide (96 hours).

Gills reaction pattern	Functional unit of the tissue	Alteration	Importance factor	Score value				Index Control group	Index 6.6 mg L^{-1}	Index 10 mg L^{-1}	Index 20 mg L^{-1}
				Control	30 mg L^{-1}	38 mg L^{-1}	50 mg L^{-1}				
Circulatory disturbances	Blood vessels of secondary lamellae	Vasodilatation along the length of blood vessel	W_{GC1}=1	0	2	2	2	I_{GC}=0	I_{GC}=**8***	I_{GC}=13	I_{GC}=13
		Vasodilatation at the basal part of the blood vessel	W_{GC2}=1	**0***	0	2	2				
		Vasodilatation at the apical part of the blood vessel	W_{GC3}=1	0	0	0	0				
		Aneurysms	W_{GC4}=3	0	0	1	1				
	Blood vessels of primary lamellae	Vasodilatation	W_{GC5}=2	0	3	3	3				
Regressive changes	Epithelium	Degeneration (necrosis)	W_{GR1}=3	0	3	3	4	I_{GR}=0	I_{GR}=9	I_{GR}=9	I_{GR}=**12***
Progressive changes	Epithelium (secondary lamellae)	Lamellar lifting	W_{GP1}=1	0	3	3	4	I_{GP}=0	I_{GP}=13	I_{GP}=14	I_{GP}=17
		Proliferation	W_{GP2}=2	0	0	0	0				
	Epithelium (primary lamellae)	Edema	W_{GP3}=1	0	2	3	3				
		Proliferation of stratified epithelium	W_{GP4}=1	0	2	2	2				
		Proliferation of glandular cells	W_{GP5}=1	0	0	0	1				
		Proliferation of cartilaginous tissue	W_{GP6}=1	0	3	3	4				
		Fusion	W_{GP7}=3	0	1	1	1				

Bold* – statistically significant than the other concentrations ($P < 0.05$)

Table 7. The percentage prevalence of histological lesions in bighead carp gills after the fungicide exposure (96 hours).

Histological alterations		Prevalence, %			
		Control	6.6 mg L^{-1}	10 mg L^{-1}	20 mg L^{-1}
Circulatory disturbances	Vasodilatation along the length of blood vessel in the secondary lamellae	0	37	41	46
	Vasodilatation at the basal part of the blood vessel in the secondary lamellae	0	0	39	43
	Vasodilatation at the apical part of the blood vessel in the secondary lamellae	0	0	0	0
	Vasodilatation of central venous sinus	0	51	57	65
	Aneurysms	0	0	17	23
Regressive changes	Degeneration (necrosis)	0	43	49	71
Progressive changes	Lamellar lifting	0	53	58	76
	Proliferation of epithelium covering secondary lamellae	0	0	0	0
	Edema	0	45	56	61
	Proliferation of stratified epithelium	0	33	41	47
	Proliferation of glandular cells	0	0	0	25
	Proliferation of cartilaginous tissue	0	53	67	83
	Fusion	0	13	21	27
Average, %		0	**25.2***	34.3	43.6

Bold* – statistically significant the other concentrations ($P < 0.05$)

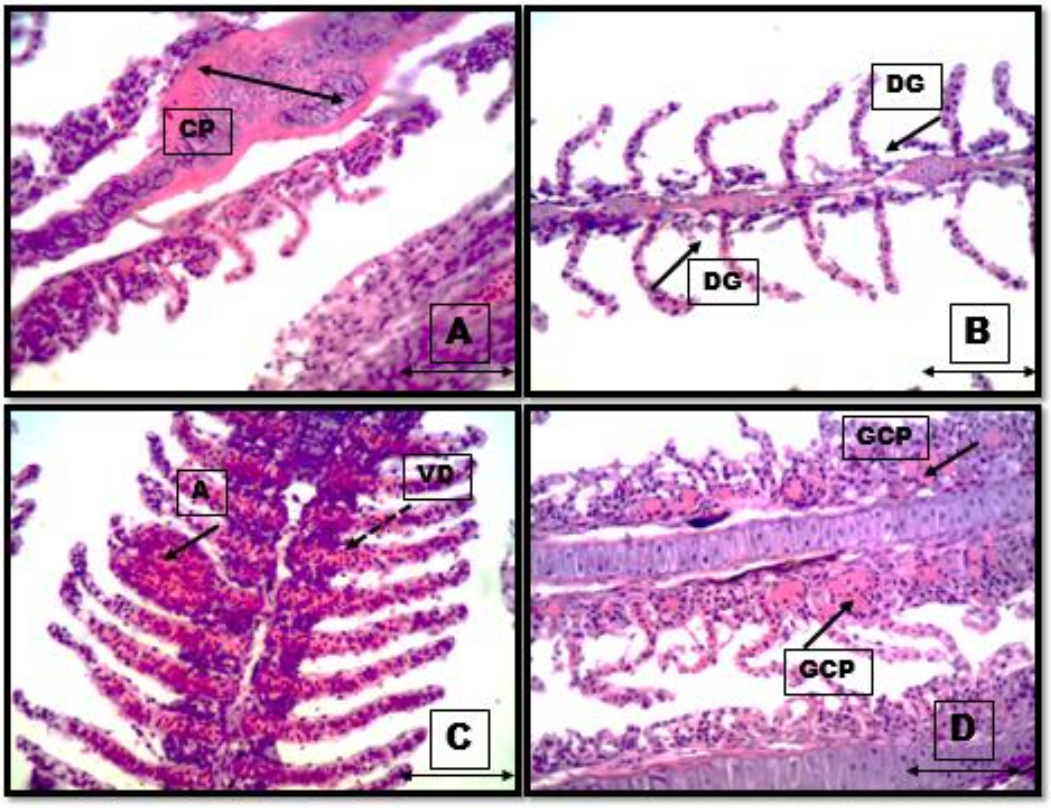


Figure 2. Histopathological lesions in bighead carp gills after the fungicide exposure (96 hours), H&E: A – proliferation of cartilaginous tissue (CP) at 30 mg L^{-1} fungicide, x 400; B – epithelial degeneration (DG) at 38 mg L^{-1} fungicide, x 400; C – aneurysms (A) and secondary lamellae vasodilatation (VD) at 50 mg L^{-1} fungicide, x 400; D –glandular cells proliferation (GCP) at 50 mg L^{-1} fungicide, x 400.

regard to the proliferation of squamous epithelium, it was expressed in hyperplasia reaching up to half the length of the secondary lamellae. On the other hand, the degenerative changes, which we observed in the gills histological structure, were also presented in a moderate degree of expression. Therefore, we found an equalization of the necrosis and proliferation processes. We observed an equalization of the degree of expression (score values) of the proliferative and degenerative lesions (score value = 2) which is due to an equalization of the expression of the epithelial proliferation

(score value = 2), glandular cells proliferation (score value = 2) and edema (score value = 2) at the lowest insecticide concentration. In addition, we determined the score value of the lamellar lifting as 3, while the fusion was expressed as 1, which complements our thesis of the equalization of the necrosis and proliferation processes (average for both alterations 2). Fusion was found to be in separate sections of the gill structure affecting several neighboring lamellae, and therefore on the proposed scale, we defined its degree as mild. In addition to the proliferative lesions, we observed cartilaginous tissue proliferation, which was found in a moderate degree. It mainly affected the chondroblasts. Histological alterations related to the circulatory system, such as vasodilatation, were found only along the length of the blood vessels in the secondary lamellae, with a moderate degree of expression. Vasodilatation of the main sinus and aneurysms, as a more severe degree of changes in the circulatory system, were not observed (Table 4, Figure 1). At the higher insecticide concentration, similarly to the previous concentration, we found proliferative and degenerative changes in the bighead carp gills. We observed edema and proliferation of glandular cells in the squamous epithelium in a moderate degree. Fusion, found in a mild degree of expression, was present in single areas with several fused secondary lamellae. In the circulatory system, we found vasodilatation at the apical part of the blood vessels in the secondary lamellae. Moderate cartilaginous tissue proliferation was also observed. Similarly to the previous concentration, aneurysms and vasodilatation in the main venous sinus were not observed (Table 4, Figure 1). At the highest insecticide concentration, we found a lamellar lifting in a very severe degree of expression. At a severe degree, we also observed proliferative and degenerative alterations in the gills. This indicated that even at the highest concentration there was an equalization of the process of necrosis and proliferative processes. In addition, in a severe degree we also found edema located at the base of the secondary lamellae. In regard to the proliferative lesions affecting the glandular cells in the squamous epithelium, as well as fusion, these histological lesions occurred in a moderate degree of expression. At this concentration, fusion was found in larger areas of the analyzed gill lamellae, compared to the previous two tested concentrations. The histological changes in the circulatory system were in a moderate degree and consisted of a vasodilatation along the length of the blood vessels in the secondary lamellae, as well as in the main sinus. At the highest concentration, similarly to the previous one of 10 mg L^{-1} insecticide, we found cartilaginous tissue proliferation, which was also observed in a moderate degree. Aneurysms were also not detected at the highest applied insecticide concentration (Table 4, Figure 1).

The gills in the group treated with the highest insecticide concentration were most affected in terms of percentage prevalence of histological lesions – 38.7%. According to the proposed scale, the histopathological alterations occurred frequently in the individuals. In addition, the other two insecticide concentrations showed histopathological lesions, which occurred rarely in the gills structure (Table 5).

Gills histological alterations under fungicide exposure

The histological alterations in bighead carp gills after the fungicide exposure were presented in Table 6 and 7.

At the lowest fungicide concentration, we found lamellar lifting, degenerative changes, as well as proliferative alterations in the cartilaginous tissue, expressed in a severe degree. On the other hand, we observed proliferation, related to the epithelial tissue covering the filament and edema in a moderate degree. Proliferation of the glandular cells was not detected. Fusion was observed in single areas between several of the secondary lamellae. The circulatory system lesions we found, were presented as vasodilatation along the length of the blood vessels in the secondary lamellae, and in the main sinus with a moderate degree of expression. Aneurysms affecting the blood vessels were not detected at the lowest fungicide concentration (Figure 2). At the higher concentration of 38 mg L^{-1}, we found lamellar lifting, edema, epithelial degeneration and cartilaginous tissue hyperplasia in a severe degree. In addition, the proliferative alterations in the gills were in a moderate and mild degree of expression. Changes in the blood vessels, similarly to the previous concentration of 30 mg L^{-1}, were observed along the secondary lamellae and in the main sinus in a moderate degree. In contrast to the lowest concentration, aneurysms were detected in single blood vessels and based on the proposed scale, we determined the extent of this lesion as mild (Table 4, Figure 2). At the highest fungicide concentration, we observed lamellar lifting, degenerative changes, as well as proliferation of cartilaginous tissue at a severe degree of expression. The proliferative changes of epithelial and glandular cells were determined in a moderate degree of expression. Fusion was found in single sections of the gill histological structure. Similarly to the previous two tested fungicide concentrations, the degree of expression was mild. In addition, similarly to the previous concentration of 30 mg L^{-1}, the histopathological alterations in the circulatory system were expressed in a similar degree of expression. They were also localized at the length and the basal part of the secondary lamellae, and along the main sinus. Aneurysms affecting single blood vessels in the secondary lamellae were localized at the apical part of the blood vessels, and were expressed in a mild degree (Table 5, Figure 2).

The percentage prevalence of histopathological alterations at the lowest fungicide concentration was 25.2%. We found an increasing tendency of the percentage prevalence with increasing the applied concentration. At the highest fungicide concentration we observed 43.6% percentage prevalence of the histopathological lesions in the bighead carp gills (see Table 7). According to Table 5 and 7, we found a higher percentage prevalence of gill alterations in the case of fungicide exposure compared to insecticide exposure ($p<0.05$).

Discussion

We agree with Montes et al. (2010), Lima et al. (2018), Ullah et al. (2018, 2019) and Soares et al. (2019) that the histopathological examination is an useful and powerful biomarker for evaluating the toxic effects of chemical pollutants and other environmental stressors. Moreover, histopathology alterations in fish could serve as an important biomarker in toxicological studies. Therefore, they could be applicable to differ-

ent tissues of the fish, such as gills. Since, the gills are involved in a number of extremely important functions such as respiration, excretion, acid-base balance, and osmoregulation, we support the statement of Ullah et al. (2019) that gills morphology is a key indicator in ecotoxicological and environmental monitoring studies.

Based on the obtained results, the histopathological alterations in the gill structure, such as proliferative alterations, were mainly expressed in a severe and very severe degree. In addition, the degree of severity of the observed histopathological lesions increased with increasing the concentrations of the applied pesticides. Our findings are in agreement with Marigoudar et al. (2018) who also found cellular lesions such as accumulation of mucous, epithelial lifting, epithelial fusion of secondary lamellae and degeneration of cells in gill filaments after exposure to sublethal concentrations of chlorpyrifos herbicide.

The proliferation of the squamous epithelium in the gills of experimental individuals treated with the fungicide remained at the same degree of expression at all three test concentrations. On the other hand, the proliferative changes, which we observed during the insecticide exposure, were presented mainly in a severe degree as well. In addition, at the insecticide concentrations, we found an increasing tendency in the degree of expression. On the other hand, we observed a mild fusion in all three test concentrations. Therefore, we suggest that the applied fungicide concentrations could activate cell division processes, but at the higher concentrations of the pesticides and/or longer exposure may be required to produce fusion. These lesions were also found earlier in the studies of Velmurugan et al. (2009) and Marigoudar et al. (2018), according to whom epithelial lifting is caused due to the thickening of lamellae and fusion of secondary lamellae, and therefore resulted from lamellar hyperplasia.

Along with the proliferative changes in the gill epithelium, we also observed proliferation of the cartilaginous tissue, mainly in a moderate and severe degree of expression in both exposures. An increasing tendency was observed due to the fungicide toxicity. Similarly to us, Xing et al. (2012) also found severe histopathological alterations in the gills of common carp (*Cyprinus carpio* Linnaeus, 1785), such as epithelial proliferation, edema with epithelial separation from the basement membranes, necrosis and epithelial desquamation, but under atrazine and chlorpyrifos exposures.

Furthermore, we found that the degree of degeneration and proliferation processes was equalized at the three concentrations of the tested insecticide. This, in turn, showed an equalization of the intensity of necrosis and cell division processes. In addition, we observed severe and very severe degenerative changes due to the fungicide toxicity. In this regard, in all fungicide concentrations the degenerative changes of the gill histological structure dominated over the proliferative ones.

The gill lesions associated with the lamellar aneurysms were also observed from Deepasree & Rajendran Nair (2016) in *Channa punctatus* (Bloch) exposed to the toxic effect of pesticides. We agree with the authors who stated that these lesions may be due to the disturbances in the blood flow. Therefore, concerning the observed changes in the circulatory system in our study, we found that the test fungicide showed a more severe toxic effect on the gill structure. On the other hand, significant changes were observed mainly at the highest insecticide concentration, but the lower concentration showed more often a lack of this histological alteration.

Our results confirmed those of Schwaiger et al. (1997) that the indexes of histopathological changes reveal the distribution frequency of alterations and the state of functioning or impairment of the tested organs. Overall, progressive (proliferative) changes were the most prevalent stress reaction pattern in the bighead gills during the insecticide exposure with lamellar lifting, edema and epithelial proliferation being the most frequent alterations. In regard to the insecticide effect on bighead carp gills, the regressive (degenerative) changes were recorded at a lesser prevalence than progressive changes and they were mainly located in the filaments. On the other hand, all tested fungicide concentration affect the circulatory system of the studied organ to a greater extent compared to the epithelial tissue. The comparison between the tested groups showed that all concentrations of the applied pesticides increased the gill indices at 96 hours, in relation to the control ($P < 0.05$). According to Table 4 and 6, the I_{GC} fungicide was significantly higher than the I_{GC} insecticide ($P < 0.05$). It means that the gills were lesser affected by the insecticide compared to the fungicide. Regarding the I_{GR} and I_{GP}, a significant difference between the test pesticides, was established only at the highest applied concentration ($P < 0.05$). The comparisons between the treatments showed that all fungicide concentrations increased the gill indices mainly at the highest concentrations for progressive and regressive changes compared to the insecticide exposure. On the other hand, the circulatory disturbances were most affected in the fish exposed to all fungicide concentrations.

Overall, the observed gill histological changes were nonspecific, but we were able to compare their degree of expression after the exposure to both tested pesticides. Summarizing the degree of expression on the scale of Bernet et al. (1999) we also monitored whether the proliferative changes were involved in compensatory adaptive processes or in degenerative changes and such in the blood system associated with irreversible necrotic changes, respectively. A higher degree of degeneration also indicated higher toxicity of the tested pesticide, which, in our case, was the fungicide. Along with that, the determined threshold was when the organ was functionally affected, but the changes were still reversible; above this threshold, irreversible necrotic changes occurred.

Conclusion

In sum, we can conclude that the tested fungicide showed more severe negative effects on gill histological structure of bighead carp compared to the tested insecticide. The tested fungicide showed a higher degree of negative impact on the occurrence of degenerative changes, associated with necrotic processes, as well as changes in the circulatory system, including vasodilatation and aneurysms. In contrast, the tested insecticide also caused degenerative changes, but to a lesser degree. Moreover, the insecticide toxicity is found to be connected more strongly with the proliferative alterations, indi-

cating a different degree of expression. Hence, proliferation of the epithelial tissue indicates the activation of compensatory adaptive mechanisms in the studied organ. On the other hand, the high degree of degenerative changes induced by the fungicide, affected the gill histological structure by thinning of the filaments and secondary lamellae, which could also affect the faster penetration of the toxicant through the gills. In addition, we found the highest degree of aneurysms after fungicide exposure, which is an indicator of the blood flow and a higher number of red blood cells, filling the vessels to compensate for the organ structural disturbances. Lastly, we consider that these results could be carefully taken into account in monitoring and risk assessment programs and when updating the legislation in the field of water conservation, as the studied pesticides are not yet considered as priority substances in surface waters according to EU legislation.

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