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# *Histochemical Alterations in Bighead Carp (Hypophthalmichthys nobilis Richardson, 1845) Liver Under Two Pesticides Exposure: 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 glyphosate based herbicide on the liver lipid accumulation in bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) in a short-term laboratory conditions (96 hours). A histochemical method with Sudan III staining was applied. We used 30 mg/L, 38 mg/L and 50 mg/L concentrations fungicide, representing 50, 40, 30 times dilution and 20 mg/L, 40 mg/L and 72 mg/L representing 70, 40, 20 times dilution of the fungicide, respectively. These concentrations were considered as real applicable pesticide concentrations in plant protection practices. Overall, we established a different degree of lipid accumulation in the fish liver. In terms to the histochemical alterations, we found that the fungicide had a more sever effect compared to the herbicide.

**Key words:** histochemistry, liver, fosetyl-Al, fenamidone, glyphosate, fish.

## Introduction

Pesticide residues in water present a major concern as they pose a serious threat to aquatic ecosystems. According to the authors, pesticides have been directly linked to causing fish mortality worldwide. Moreover, the increase in concentration of pesticides due to its persistent and non-biodegradable nature in the tissues of organisms at each successive level of food chain is known as biomagnification. Due to this phenomenon, organisms at the higher levels of food chain experience greater harm as compared to those at lower levels (GILL & GARG, 2014). In addition, in terms of their persistent, semi-volatile character, as well as their high stability and lipophilic characteristics, which contribute to their bioaccumulation in the adipose tissues of animals and their biomagnification through the food chain (EQANI *et al.*, 2013; PAULINO *et al.*, 2014; VIEIRA *et al.*, 2019), these compounds can promote toxic effects in chronically exposed organisms, such as fish, even at low concentrations (STANLEY & PREETAH, 2016).

In several scientific studies, the authors proposed to better assess the aquatic ecosystems contamination with pesticides, the application of biomarkers, which assess

the health of organisms (DE LA TORRE *et al.*, 2005; MDEGELA *et al.*, 2006). In addition, the assessment of polluted aquatic ecosystems is related with monitoring their status. The assessment includes identifying the factors that have a negative impact on the ecosystems and measures to limit the pesticide pollution. In this regard, an overall model of the adverse effects of chemicals and their mixtures in contaminated aquatic ecosystems can be developed. On the other hand, stress in the organism caused by the effects of toxicants triggers a series of biological reactions, which can serve as biomarkers of contamination. At the established levels of the reference response, biomarkers assess the rapidly developing stress of the organism. Biological response at a higher hierarchical level is a measure of a late reaction that reflects the state of the entire ecosystem. MINIER *et al.* (2006) added that biomarkers are important tools since they present a specific information concerning the biological effects of a particular pollutant. Furthermore, they can be applied in biomonitoring programs, as well as to clarify the negative effects on the organism and the concentration of the toxicant in the health risk assessment.

Changes in fish organism in general allow the determination of water toxicity and the potential hazard associated with anthropogenic substances in aquatic ecosystems. According to MONTENEGRO RAYO (2004) and BRÖNMARK & HANSSON (2005) fish have a direct impact on the function and structure of aquatic ecosystems, including dietary dynamics, zooplankton composition, etc. This impact can be traced mostly in freshwater basins, where they are the largest consumers of the lower trophic levels. As stated by BERNET *et al.* (1999) criteria for the reliability of histological studies refers to the actual biological significance of the analyzed histological alterations. Moreover, this defines the importance of observed histological changes in different tissues, expressed to varying degrees in different organs.

The liver is the target organ of chemical intoxication because of the large blood flow. According to STENTIFORD *et al.* (2003) various liver pathological changes may serve as reliable biomarkers for toxic effects of various organic pollutants, including pesticides.

In fish species, lipids and proteins are the main organic constituents, and play many important roles in the fish physiology, which includes growth, reproduction and migration (TOCHER, 2003). According to OLIVARES-RUBIO & VEGA-LÓPEZ (2016) contaminants such as polyaromatic hydrocarbons (PAHs), polychlorinated hydrocarbons (PCBs), pesticides, and pharmaceutical products are the most investigated. Furthermore, these contaminants are hydrophobic, and due to their physicochemical properties are able to accumulate in the lipids of aquatic organism in a dose-dependent manner (KAINZ & FISK, 2009).

According to the Environmental Protection Agency (2010), the tested fungicide is a systemic and a contact pesticide. It contains the active ingredients fosetyl-Al and fenamidone, and belongs to the third category of toxicity. The report of the European Food Safety Authority (2006) does not provide a combined risk assessment of the two ingredients of the tested fungicide. The toxicity data of the fungicide are based on those of the studies carried out with its main ingredient, an ingredient of fosetyl-Al. According to the Environmental Protection Agency (2010), the tested herbicide is a total and systemic herbicide with the active substance glyphosate and belongs to the third category of toxicity. Glyphosate is an aminophosphorus analogue of the natural amino acid glycine. Although, the acute toxicity of glyphosate to animals is considered low (WHO, 2009), the glyphosate-based formulations are generally more toxic for many aquatic species

(VELASQUES *et al.*, 2016; MELO *et al.*, 2017; ALBAÑIL SÁNCHEZ *et al.*, 2019).

Bighead carp (*H. nobilis*) is a freshwater species, which has the advantages of fast growth, it is strongly resistant to diseases, and has also good meat quality and rich nutrition. However, it has not been that widely studied in terms of the effects of different pollutants compared to other Cyprinids, such as common carp.

Therefore, in the present study we aimed to propose bighead carp as a model for ecotoxicological research. Based on the excessive use of pesticides and their negative impact on the aquatic environment, we tried to determine the effects of real applicable pesticide concentrations in plant protection practices on bighead carp, which is also important freshwater species in aquaculture. Therefore, we applied a histochemical approach to assess the negative effects of the applied pesticides.

## Material and Methods

*Test chemicals*

We used 20 mg/L, 40 mg/L and 72 mg/L herbicide (glyphosate (N-(phosphonomethyl)-glycine)) representing 70, 40, 20 times dilution, and 30 mg/L, 38 mg/L and 50 mg/L fungicide (fenamidone (1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one) and fosetyl-Al (Aluminium tris-O-ethyl phosphonate), representing 50, 40, 30 times dilution of the stock solution, respectively. The selected dilutions are based on preliminary tests, with dilutions selected where no mortality was found in the individuals. In addition, we aimed to compare the effects of two widely applicable commercial products on the histological structure of bighead carp. We used a stock solution of commercial products actually applicable in agricultural practices. The stock solution was prepared according to the instructions of the company producer. The purpose of the present was to establish the toxicity of the selected products to their applicable concentrations.

*Experimental set up*

After transportation, the fish were divided in glass aquaria (100 L) with chlorine free tap water during the acclimatization period for 7 days. The fish were not fed 48 hours prior to the experiment. They were divided into three tested groups (n=15) for each pesticide, including a control group (chlorine free tap water without addition of toxicants) and were treated in static conditions for 96 hours with different concentrations of the tested pesticides. The experimental set up followed MODESTO & MARTINEZ (2010); SANTOS & MARTINEZ (2012) and ALBAÑIL SÁNCHEZ *et al.* (2019), thus the tested insecticide was added only at the beginning of the experiment and the water was not renewed. 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 (EU, 2010).

*Histochemical study*

Fish dissection was carried out according to the international standard procedures given by ROSSELAND *et al.* (2003). Histochemical analysis was performed in the laboratory at Medical University of Plovdiv, Bulgaria. Leica Cryostat (Germany) was used to cut the liver samples. Multiple carp sections of 6 µm were prepared according to a standard methodology. They were stained with Sudan III using the method of Daddy (1896) and described by PEARSE (1972). According to the applied method, the lipid droplets in carp liver were presented in yellow staining. Liver histochemical changes of all test specimens, including the control group were appraised individually and semi-quantitatively by using the scale of MISHRA & MOHANTY (2008). Positive Sudan III staining was presented with fat droplets in

the hepatocytes cytoplasm. Evaluation of the histochemical lesions was presented as an average value. Each grade represents specific histochemical characteristics and they were categorized as follows: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (+++) – strong positive reaction of Sudan III staining in the carp liver.

**Results and Discussion**

In the control group we found normal histological structure of bighead carp liver in accordance to ROCHA *et al.* (1994). In addition, we found a pale yellow staining in the hepatocytes, which we identified as a very weak positive reaction of the histochemical staining. The obtained results from the conducted histochemical study of bighead carp liver are presented in Table 1 and 2.

**Table 1.** Histochemical changes in bighead carp liver due to the fungicide exposure. Legend: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (+++) – strong positive reaction of Sudan III staining in the carp liver.

| Fungicide concentration | Control group | 30 mg/L | 38 mg/L | 50 mg/L |
|---|---|---|---|---|
| Intensity of yellow Sudan III staining | ± | ± | + | ++ |

**Table 2**. Histochemical changes in bighead carp liver due to the herbicide exposure. Legend: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (+++) – strong positive reaction of Sudan III staining in the carp liver.

| Herbicide concentration | Control group | 20 mg/L | 40 mg/L | 72 mg/L |
|---|---|---|---|---|
| Intensity of yellow Sudan III staining | ± | ± | + | + |

At the lowest fungicide concentration (30 mg/L), we found that the intensity of the applied histochemical staining was with the same degree of expression as the control. At the higher fungicide concentrations we found an increase in the intensity of the Sudan III staining. Thereby, in the tested group, exposed to 38 mg/L, we observed a weak positive reaction, expressed in a yellow-orange staining of the cytoplasm of hepatocytes, which indicated accumulation of lipid droplets in the liver cells. At the highest concentration of 50 mg/L fungicide, we found a moderate positive histochemical reaction, which was in expressed in an intensive yellow-orange staining, indicating the accumulation of a larger amount of lipids in the cytoplasm of hepatocytes (Table 1, Fig. 1).

At the lowest herbicide concentration, we observed a very weak positive reaction expressed in pale yellow. At the higher concentrations of 40 mg/L and 72 mg/L, we found a slight increase in the intensity of the histochemical staining, which showed an increase in the amount of lipid droplets in the cytoplasm of hepatocytes. The degree of the positive Sudan III staining, expressed in a yellow-orange staining in the cytoplasm, was in similar intensity at the higher two concentrations (Table 2, Fig. 2).

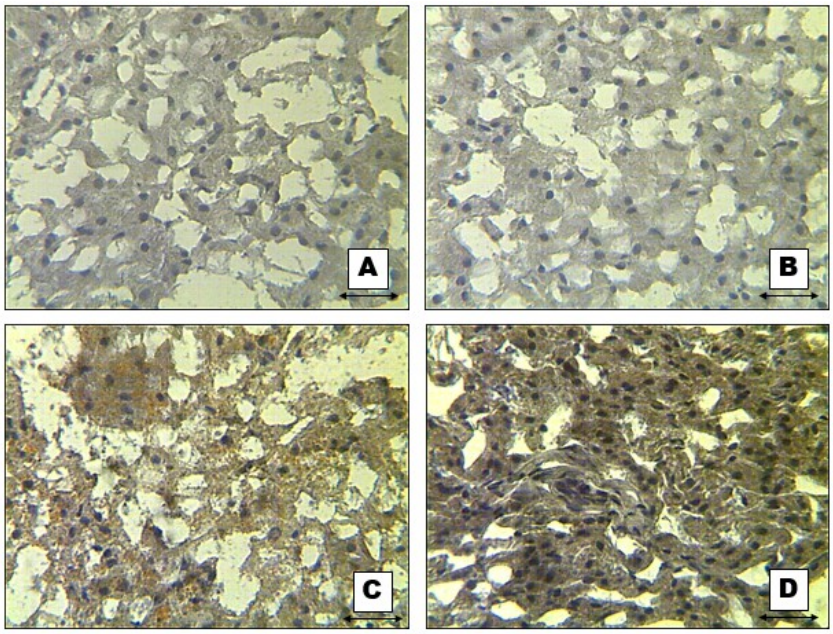


**Fig. 1.** Intensity of Sudan III staining in bighead carp liver after the fungicide exposure, x400:
A – control group fish; B – Intensity of the histochemical staining at 30 mg/L;
C – Intensity of the histochemical staining at 38 mg/L;
D – Intensity of the histochemical staining at 50 mg/L fungicide.

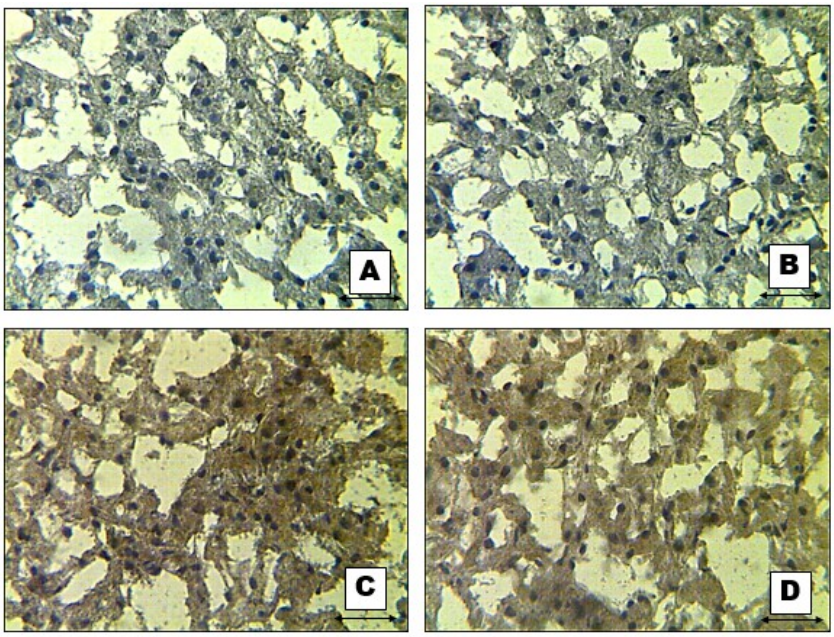


**Fig. 2.** Intensity of Sudan III staining in bighead carp liver after the herbicide exposure, x400: A – control group fish; B – Intensity of the histochemical staining at 20 mg/L;
C – Intensity of the histochemical staining at 40 mg/L;
D – Intensity of the histochemical staining at 72 mg/L herbicide.

Since, the liver is the first-line of defense against potentially harmful xenobiotics, and it is therefore not surprising that it is also the target organ that is most commonly affected by industrial chemicals (WAHLANG *et al.*, 2013), we established the degree of expression of lipid accumulation in the liver under two widely applicable pesticides in the agricultural practice. As stated by RAMESH & SARAVANAN (2008) the applied histochemical method is an indicator of the accumulation of lipids in cells and allows detection of changes, which occurs at the cellular level under the action of various toxicants, including pesticides. In addition, the liver stores essential carbohydrates in the organism and also participates in the blood glucose homeostasis by maintaining a balance between the processes of glycogenesis and glycolysis.

FABBRINI *et al.* (2010) added that lipid accumulation in the hepatocytes represents a complex interaction, which includes a balance between triglyceride synthesis (lipogenesis), hydrolysis (lipolysis), and transport. Moreover, XU *et al.* (2012) found lipid accumulation in goldfish liver due to organophosphate pesticide trichlorfon toxicity. The authors stated that hepatic triglycerides cannot then be transported from the liver and are accumulated in the liver which is associated with changes in apolipoprotein quantity. Fatty degeneration in fish liver was also observed by AL-OTAIBI *et al.* (2019) under diazinon toxicity. WANG *et al.* (2019) found lipid metabolism disorders in adult zebrafish due to chlorpyrifos exposure.

Based on the obtained results of the changes in lipid content, we found a tendency towards increasing the lipid inclusions in the cytoplasm, along with an increase in the concentration of the applied toxicants. These results are also confirmed by established fat degeneration by the histological analysis in our previous studies (see STOYANOVA *et al.*, 2014; YANCHEVA *et al.*, 2016). Moreover, the tested fungicide caused more severe degree of fatty degeneration in the hepatocytes. The large amount of lipid droplets, which were accumulated in the hepatocytes is probably a result of the occurrence of fatty degeneration in the liver cells, which was also found after histological examination of the liver (see YANCHEVA *et al.*, 2016). On one hand, the occurred lipid accumulation in the tested organ is probably due to the increased amount of pyruvate in the liver, and hence by the pyruvate dehydrogenase complex lead to increased amount of Acetyl-CoA, which is used for the synthesis of fatty acids and cholesterol. On the other hand, increased fatty acid synthesis leads to increased triglyceride synthesis and to hyperlipidemia associated with fatty infiltration in hepatocytes. In regard to the fat infiltration in liver cells, these changes can be associated with the absence of the enzyme glucose-6-phosphatase and the inability to release glucose in the blood, which in turn leads to hypoglycemia. Probably, the increased amounts of glucose-6-phosphate lead to increased activity of the pentose phosphate pathway, and hence higher amounts of pyruvate.

Based on our previous study (YANCHEVA *et al.*, 2016), we found increased levels of LDH and ALAT due to the applied fungicide toxicity. Moreover, the changes in the specific enzymatic activity probable lead to a stimulation of the process of gluconeogenesis and accumulation of the glycogen in the liver of the experimental fish species. Along with the obtained results in the present study concerning the lipid accumulation in liver, the increased pyruvate and acetyl-CoA in the liver and lipogenesis pathway lead to accumulation of fatty acids in hepatocytes and thereafter could lead to hyperlipidemia. Thus, the observed changes in the bighead carp liver could serve as protective mechanisms against pesticide toxicity.

In addition, STOYANOVA *et al.* (2014) and YANCHEVA *et al.* (2016) studied changes in the specific enzymatic activity in the liver and changes in the glycogen content under the tested in the present study herbicide.

These changes could be linked to the process of lipid accumulation in the hepatocytes, but it may differ from the one, which we supposed occurred under the fungicide exposure. We suggest, similarly to POSTIC *et al.* (2004) that decreased levels in the glycogen storage lead to an increase in the process of glycolysis and thereafter, leads to the process of *de novo* lipogenesis and lipid accumulation in hepatocytes.

**Conclusion**

Overall, we investigated an increase of the intensity of Sudan III staining with accumulation of lipid droplets in the fish liver, which correlates with the increasing concentration of the tested pesticides. Moreover, we could associate this process with changes in the processes of gluconeogenesis and glycolysis. In addition, we found that that the fungicide had a more sever effect compared to the herbicide expressed in more pronounced fatty degeneration. Therefore, this could be considered a series of compensatory mechanisms in the fish liver metabolism in response to the toxic effects of pesticides and the stress they induce. Further investigations in this particular area need to be carried out to better understand the metabolic changes in the liver under the influence of organic contaminants. We consider that these results could be carefully taken into account in monitoring and risk assessment programs, since the tested pesticides are not yet considered as priority substances in surface waters according to EU legislation.

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