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Histochemical alterations in liver of Common Carp *Cyprinus carpio* (Linnaeus, 1785) after glyphosate exposure: Preliminary study

VESELA YANCHEVA[1], ELENKA GEORGIEVA[2]*, ILIANA VELCHEVA[1], PEPA, ATANASOVA[3], STELA STOYANOVA[2]

[1] *Department of Ecology and Environmental Conservation, Faculty of Biology, Plovdiv University, 24, Tzar Assen Str., BG-4000 Plovdiv, Bulgaria*
[2]* *Department of Developmental Biology, Faculty of Biology, Plovdiv University, 24, Tzar Assen Str., BG-4000 Plovdiv, Bulgaria, e_tomova@abv.bg*
[3]*Department of Anatomy, Histology and Embryology, Medical University of Plovdiv,15A, Vasil Aprilov Str., BG-4000 Plovdiv, Bulgaria*

Abstract. The present study was designed to provide some preliminary data on the toxic effects of 96 h exposure to glyphosate on the liver of Common Carp (*Cyprinus carpio* L.) under *ex situ* conditions. For this purpose we used Sudan III staining which could be suggested as fast and low-cost histochemical biomarker for pesticide contamination effects.

Key words: fish, pesticides, liver, histochemistry.

Introduction

Aquatic environments are inevitably the ultimate receptors for many different contaminants, including pesticides. Throughout the world, 40% of the applied pesticides are herbicides, 33% are insecticides, while 10% are fungicides and 17% are classified as others (Glinski *et al.* 2018). Glyphosate (N-phosphonomethyl glycine) is a post-emergent and broad-spectrum herbicide that belongs to the glycine group, and it is the most widely used non-selective herbicide to control plant growth worldwide because it has a powerful herbicidal action and interferes with 5-enolpyruvyl-shikimate-3-phosphate synthase, an important enzyme for the synthesis of essential aromatic amino acids in plants (Sergiev *et al.* 2006). It is currently the best-selling herbicide in the world, used in agricultural and non-agricultural areas and the use of glyphosate for crop production is world-wide spread, both in industrialized and developing countries (Benbrook 2016). According to WHO (2005) the acute toxicity of glyphosate to animals is considered low. However, given its dramatic increase in usage over the last 20 years, there have been growing concerns regarding chronic low-dose exposure to glyphosate (Myers *et al.* 2016). Moreover, the major problem with the continuous and uncontrolled use of this herbicide is its effect on non-target organisms. Thus, at present glyphosate is receiving increased attention as potential toxicant to aquatic organisms and ecosystems (Van Bruggen *et al.* 2018). In fish, it has been shown that glyphosate-based herbicides cause biochemical alterations and morphological lesions in tissues such as gills and liver (Stoyanova *et al.* 2015). Use of sentinel organisms for environmental quality monitoring by biological tools provide a sensitive and reliable approach to estimate the possible negative effects of pollutants. Fish, among them, are

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recognized as an excellent experimental model for toxicological studies because of their importance as protein source. Furthermore, fish are concerned as a proper indicator for the assessment of contamination in aquatic ecosystems, as they receive the toxicants both directly through water and indirectly through food, thereby resulting in bioaccumulation in their tissues and biomagnification in the food web.

Based on the literature above we find that it is essential to obtain information on the toxic effects of glyphosate on non-target organisms such as fish. Therefore, the objective of the present work is to study the toxic effects of glyphosate (commercial product NASA 360) on the liver of Common Carp, *Cyprinus carpio* (Linnaeus, 1785) which is an important species for aquaculture and aquatic toxicology by applying histochemical methods. We also aimed to see if Sudan III staining could be proposed as a sensitive, rapid and low-cost biomarker for the negative effects of pesticides.

Material and Methods

The fish were purchased from the Institute of Aquaculture and Fisheries, Plovdiv, Bulgaria where they are reared in strictly controlled conditions. The Common Carps were of the same size-age group without external pathological changes. Their average weight and length were as follows: 47.8 g ± 15.2 and 16.3 cm ± 2.7. After transportation, they were placed into 100 L glass tanks with dechlorinated water to acclimate for a week. During the acclimation period a constant temperature was maintained within 23°C ± 1.5. A twelve-hour light period was provided for the fish. Thereafter, the Common Carps were randomly divided (n=15) in each glass tank and treated with glyphosate once for 96 h (short-term exposure). The concentrations of glyphosate (NASA 360) were determined by dilution of a stock solution prepared according to the manufacturer's guidelines for recommended crop-specific quantities as previously described (see Stoyanova *et al.* (2015)). The basic physical parameters of water (pH, oxygen level, temperature and conductivity) were monitored three times a day with a combined field meter (WTW, Germany). The fish were dissected and the requirements of Directive 2010/63/EU on the protection of animals used for scientific purposes were met. The liver was immediately frozen at -25°C for further histochemical analysis. The histochemical study was conducted on a freezing microtome (Leica, Jung Frigocut 2800 N) and cryosections of 6 µm were prepared from the liver. They were then stained for lipid determination by Sudan III staining (Sigma, USA) as described by Daddi (1896). By this colouring method, the lipids in the hepatocytes are stained in orange and the nuclei of the cells in pale blue. In addition, the histochemical changes in the liver were evaluated according to a standardized assessment tool by using a modified version of the protocol described by Bernet *et al.* (1999). The Sudan III staining evaluation was scored as follows: (0) - Negative histochemical staining reaction; (1) - Very weak positive histochemical reaction with discreet yellow coloration; (2) - Slightly positive histochemical reaction with yellow-orange staining; (3) - Moderately positive reaction of histochemical staining with intense yellow-orange staining; (4) - Highly positive histochemical reaction in hepatocytes with intense orange coloration.

The statistical analysis was performed using Graph Pad Prism 7 for Windows. The raw data on basic physical properties and histochemical scores were distributed normally and analyzed using Graph Pad Prism 7 for Windows (USA). The differences between the variables were tested using Student's t-test at significance level of 95% ($p<0.05$). The results were reported as mean±SD.

Results and Discussion

The values of water properties were constant during the experiment, without significant differences ($p<0.05$) and they are not presented in the manuscript. Thus, we

consider that the histochemical alterations that we observed in the liver of Common Carp are not due to changes in the abiotic factors.

The results of the histochemical alterations are presented on Fig. 1 and the average scores in Table 1, respectively. Overall, from the obtained results on histochemical alterations in the liver of Common Carp after glyphosate exposure changes we observed a tendency towards an increase of the lipid content in the hepatocytes along with the increase in the concentration of glyphosate. The scores were evaluated as (1) - Very weak positive histochemical reaction with discreet yellow coloration; (2) - Slightly positive histochemical reaction with yellow-orange staining; (3) - Moderately positive reaction of histochemical staining with intense yellow-orange staining for 20, 40 and 72 mg/L concentrations, respectively.

Table 1. Average results for Sudan III staining intensity in liver of Common Carp after 96 h exposure to glyphosate (n=10 for each concentrations). † - statistically significant than the others ($p<0.05$).

Common Carp liver	**Nasa 360/glyphosate concentration, mg/L**			
	control	20	40	72
Sudan III staining intensity score	0	1	2	3†

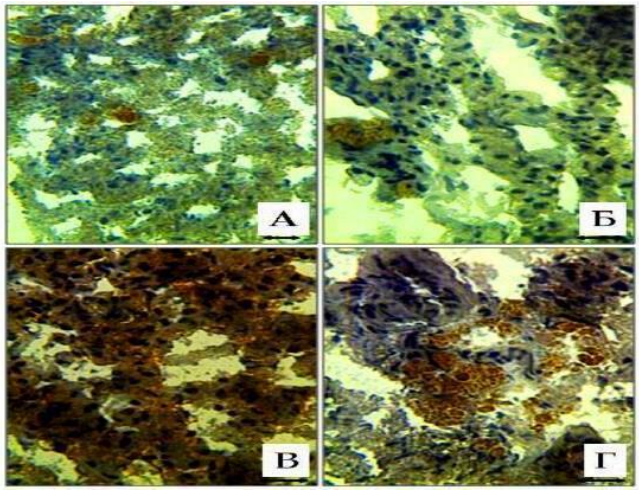

Fig. 1. Sudan III staining intensity in liver of Common Carp after 96 h exposure to glyphosate: А – control, x200; Б – 20 mg/L glyphosate, x400; В – 40 mg/L glyphosate, x400; Г – 72 mg/L glyphosate, x400.

The negative effects of pesticides, along with changes in glycogen levels in the liver, may cause other degenerative changes such as fat degeneration, expressed as accumulation of lipid droplets in hepatocytes which may affect lipid metabolism negatively.

The large amount of lipid droplets accumulated in the cytoplasm of hepatocytes is due to fatty tissue degeneration in the liver cells which is confirmed in our previous study (see Stoyanova *et al.* 2015). This is probably due to increased amounts of pyruvate in the liver, and hence through the pyruvate dehydrogenase complex of increased amounts of Acetyl-CoA which is used for the synthesis of fatty acids and cholesterol. The increased fatty acid synthesis leads to increased triglyceride synthesis and hyperlipidemia associated with fat infiltration in hepatocytes. Along our findings, other authors similarly detected changes in the lipid content of hepatocytes after pesticide exposure. Gultekin *et al.* (2000) observed fat degeneration in the liver due to lipid metabolism disorders after insecticidal exposure.

Similarly to us, Ayoola (2008) found fatty degeneration in the African catfish, *Clarias gariepinus* (Burchell, 1822) hepatocytes under the action of glyphosate for 96 h. We agree with the authors that variations associated with changes in the amount of lipids (or glycogen) in the liver of exposed animals generally may be due to changes in the glycolysis processes which in turn depend on the toxicant concentrations or its toxic character, as well as exposure period.

In sum, we can conclude that glyphosate has a negative effect on the liver of Common Carp and Sudan III staining could be recommended as a sensitive, rapid and low-cost biomarker for the effects of pesticide contamination on freshwater fish.

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