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Periodic Acid - Schiff (PAS) Reaction in Fish Liver Exposed to Fungicide Contamination: A Possible Histochemical Biomarker

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Abstract. The present work aimed to study the negative effects of fungicide contamination on the liver of *Hypophthalmichthys nobilis* (Richardson, 1845) by applying the Periodic acid – Schiff reaction (PAS). The fish were treated with increasing and real applicable pesticide concentrations in agriculturepreprared according to the guidelines of the producer for a total acute period of 96 hours. Overall, we found that the intensity of the PAS staining increased proportionally with the increasing of the tested fungicide. Based on the obtained results, we consider that the PAS-staining could be successfully applied as a biomarker in toxicological research. In addition, as fungicide studies are in general less compared to the other pesticide groups, we also consider that these results could be used in future risk assessment and monitoring programs, as well as better agricultural activities.

Key words: PAS, fungicide, *Hypophthalmichthys nobilis*, biomarkers, water contamination.

Introduction

Indiscriminate and wide spread use of pesticides in the modern agricultural practices leads to a series of environmental problems due to their toxicity, long term persistence and their adverse effects on living organisms (De Forest *et al.*, 2007). Among pesticides, fungicides' effects are probably less studied compared to herbicides and insecticides and among fungicides, propiconazole is mostly studied (Li *et al.*, 2010; Chu *et al.*, 2016; Tabassum *et al.*, 2016; Tu *et al.*, 2016; Hano *et al.*, 2017; Fan *et al.*, 2018; Cao *et al.*, 2019) According to Păunescu *et al.* (2018) and Choudhury (2018) fungicides have multiple purposes in agriculture, industry and households including: seed protection during germination, storage and transport; prevention from mildew, etc. However, with their wide range of uses, fungicides can have side effects in human poisoning. The most severe "epidemic" of pesticide poisoning has been caused by the unconscious consumption of grain seeds treated with organic mercury or hexachlorobenzene. Most currently used fungicides do not cause severe poisoning because they generally have low toxicity in mammals, and are also poorly absorbed and applied in the form of suspensions which are rapidly absorbed by the plants. In addition, these biocides are able to reach surface and ground waters, mainly due to water runoff

and soil leaching, as well as by spray-drift during applications (MARQUES *et al.*, 2016; MISHRA & SINGH, 2018).

Although chemical analyses allow the qualitative and quantitative measurement of fungicides, quantifying all the pollutants in the water is not feasible as stated by SAMANTA *et al.* (2018). Furthermore, chemical analyses alone are limited to assess the synergistic/antagonistic effects of mixtures of pollutants in real field conditions (KERAMBRUN *et al.*, 2011). Therefore, an alternative monitoring technique involving biomarkers (physiological, biochemical, and cellular or molecular responses) has been widely accepted for assessment of environmental quality in freshwater systems (VAN DER OOST *et al.*, 1996; RENDÓN-VON OSTEN *et al.*, 2005). Furthermore, in recent years biomarkers are recognized as tools for the assessment of impacts of pollution on the aquatic environment, and they are already incorporated in various environmental monitoring and risk assessment programs (VIARENGO *et al.*, 2007).

In the field of aquatic toxicology, fish are important sentinels of environmental quality and ecological safety (LIN *et al.*, 2014; MARIGOUDAR *et al.*, 2018). When fish are exposed to contaminants, they can cause different lesions ranging from alterations in single cells to the organism (MISHA & VERMA, 2016). Thus, histopathology and histochemistry are considered as one of the best methods for assessing the short and long-term hazardous effects of fungicides (MISHRA & SINGH, 2018). Moreover, fish liver is among the most studied organs in toxicological research because it is responsible for processing and storage of nutrients, synthesis of enzymes, and metabolism of xenobiotics due to which it usually represents one of the most frequently altered organs (WOLF & WOLFE, 2005). Furthermore, ČOŽ-RAKOVAC *et al.* (2008) and MARCHAND *et al.* (2009) stated that because of liver important functions, multiple hepatic lesions are to be expected in wild fish populations. According to ALBAÑIL SÁNCHEZ *et al.* (2019) and NYESTE *et al.* (2019) the liver concentrates a large portion of pollutants which can eventually damage the organ. CHIVITTZ *et al.* (2016) and HALUZOVÁ *et al.* (2011) also stated that the liver plays a central role in detoxification and biotransformation of contaminants. Therefore, the hepatic alterations may indicate that this organ is overloaded, and at the same time it can be an alert for a possible failure of the liver which would impair the detoxification capacity of fish.

The present research aims to find the possible negative effects in terms of aquatic toxicology of a widely applied, but less studied fungicide in Bulgaria on Bighead Carp, *Hypophthalmichthys nobilis* (Ricahrdson, 1845), a non-target and important for aquaculture Cyprinid fish, by applying histochemical methods.

Materials and Methods

Chemicals. All the chemicals of analytical grade were purchased from Merck (Germany). The fungicide was purchased under the trade name Verita® WG (Bayer CropScience, Germany) from an agricultural supply shop in Plovdiv, Bulgaria. As previously described (see GEORGIEVA *et al.*, 2013 and STOYANOVA *et al.*, 2015) it is a systemic and contact fungicide, effective against plant diseases, caused by fungi of the class Oomycetes. The active substances in this particular fungicide are fosetyl-Al and fenamidone (Table 1 and Table 2). Fosetyl-Al (Table 3) is a member of the phosphonates, which constitute a relatively new class of systemic fungicides (COHEN & COFFEY, 1986). Fenamidone belongs to the chemical group of imidazolinone and isopropanol, respectively (Pest Management Regulatory Agency, 2003). Verita® WG contains 667 g/kg of the active substance fosetyl-Al (Aluminium tris-O-ethyl phosphonate) and 44 g/kg of fenamidone (1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one), respectively.

Experimental fish. The Bighead Carp is native to eastern China, eastern Siberia and extreme North Korea (Fig. 1). It occurs in

rivers of eastern Siberia (mouths of the Tumannaya and Razdolnaya rivers of the Primorsky District, Russia, south of the Amur (Heilongjiang) River, along the China, Russia, and North Korea borders), southward in rivers of the North China Plain including the Yellow (Huanghe) River and Yangtze (Changjiang) Rivers and southern China including the Pearl (Zhujiang) River. The native range of Bighead Carp has been reported to be 47° to 24°N (HSEIH, 1973; MOZSÁR *et al.*, 2017). Nevertheless, CHEN *et al.* (1998) reported a range of 47° (Amur River Basin, where it is an introduced species), to approximately 21°N (Hainan Island), another introduction. The actual native range of this species may never be determined accurately because this species has been widely introduced in eastern Asia (KOLAR *et al.*, 2005).

The Bighead Carp is deep-bodied, spindle-shaped, moderately compressed, with a smooth keel between the anal and pelvic fins that does not extend anterior of the base of the pelvic fins (Fig. 1). Head and mouth of the Bighead Carp are disproportionately large. The premaxillary and protruding mandible form rigid bony lips. Coloration of the body is dark gray above and cream-colored below with dark gray to black irregular blotches on the back and sides. This color pattern develops when the fish is about 2 months old. The blotched or mottled pattern is often lost in turbid water. Scales are small, cycloid, lateral line complete, strongly convex ventrally, continuing posteriorly along middle of caudal peduncle, with about 98 to 100 scales. Scale rows above lateral line 26-28, and scale rows below lateral line 16-19. Dorsal and anal fins are without spines. The number of dorsal fin rays is typically 8, anal fin 12-14, pelvic fin rays 8-9, pectoral fin rays 17-19, which extend posteriorly beyond the origin of the pelvic fins. Pharyngeal teeth are in a single row, four on each arch. They have a spoon-like shape with the grinding surface shallowly concave. The grinding surfaces of the pharyngeal teeth of the Bighead Carp differ from those of the Silver Carp, which have fine striations (YOKOTE, 1956) that are visible with magnification. Gill rakers are long and slender, rays closely set, with many membranous septa. The intestine is long and highly convoluted. CREMER & SMITHERMAN (1980) reported intestinal length to be 2.4-4.5 times total length (mean of 3.3 times total length). Large individuals may reach a weight of 40 kg (KOLAR *et al.*, 2005).

Table 1. Chemical information on Fosetyl-aluminum.

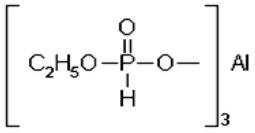
Common name (ISO)	*Fosetyl-aluminum*
Chemical nomenclature (IUPAC)	Aluminium tris-O-ethyl phosphonate Ehtyl hydrogen phosphonate, Aluminium salt, Phosphonic acid monoethyl ester
Minimum purity	960 g/kg
Chemical formula	$C_6H_{18}AlO_9P_3$
Molecular weight	354.14
Structural formula	

Table 2. Chemical information on Fenamidone.

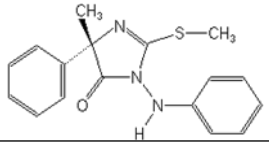
Common name (ISO)	*Fenamidone*
Chemical nomenclature (IUPAC)	(*S*)-1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one, 4H-Imidazol-4-one, 3,5-dihydro-5-methyl-2-(methylthio)-5-phenyl-3-(phenylamino)-,(S)-(9CI)
Minimum purity	975g/kg
Chemical formula	$C_{17}H_{17}N_3OS$
Molecular weight	311
Structural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

Table 3. Toxicity classification of different fungicides according to CHOUDHURY (2018).

Chemical Name	Trade Name	Toxicity Classification	96 hr LC_{50} (mg/L): Rainbow Trout
Benomyl	Benlate	High	0.2
Captan	Agrox, Captan, Captec	Extreme-moderate	0.06
Carboxin	Vitavax	High-moderate	>0.1
Chlorothalonil	Bravo, Daconil, Terlanil	High	0.3
Coppersulfate	Basicop, Bluestone	High-moderate	0.14
Fenarimol	Rubigan	High	0.2
***Fosetyl -Al**	Aliette	Minimal	428
Iprodione	Rovral	Moderate	4
Mancozeb	Dithane, Fore, Manzate	High-moderate	2(48 hr)
Maneb	Maneb, Manex	High-moderate	2 (48 hr)
Metalaxyl	Ridomil	Minimal	>100
†Propiconazole	Alamo, Orbit, Banner, Tilt	High-moderate	0.9
Thiram	Thiram, Spotrete	High-moderate	0.1
Ziram	Ziram	Moderate	5 (5 hr): Goldfish

***Fosetyl –Al –** (one of the) tested fungicide in the present study;
†Propiconazole – one of the most studied fungicides in aquatic toxicology (see LI *et al.*, 2010; CHU *et al.*, 2016; TABASSUM *et al.*, 2016; TU *et al.*, 2016; HANO *et al.*, 2017; FAN *et al.*, 2018; CAO *et al.*, 2019).

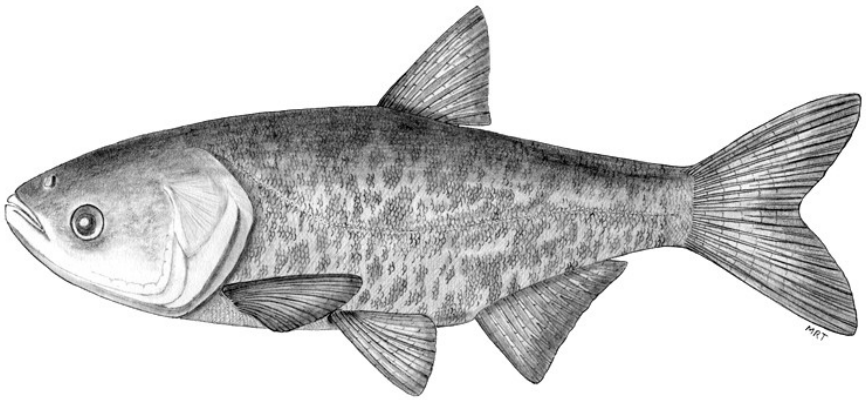


Fig. 1. Bighead Carp. Other common names frequently applied to Bighead Carp include Bighead and Bigheaded Carp (KOLAR *et al.*, 2005).

Fifty healthy Bighead Carps were purchased from the Institute of Fisheries and Aquaculture, located in Plovdiv, Bulgaria where fish are reared under strict and controlled condition. They were of a similar size-group (mean total length 18.65 cm ± 1.33; mean body mass 53.02 g ± 6.3) with no external pathological abnormalities. The fish were transported on the same day to the laboratory of Department of Ecology, Plovdiv University, Bulgaria. After transportation, the fish were placed in glass tanks with 100 L chlorine-free tap water (by evaporation) to acclimatize for a week. After acclimatization they were divided into four groups (n=15) and not fed prior or during the experiment. Fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (ROSSELAND *et al.*, 2003). All experiments were 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 EC (2010).

Experimental exposure

For the purposes of the study three groups of fish were exposed to the fungicide at 30, 38 and 50 mg/L, representing 50, 40, 30 times dilution of the stock solution, as explained by the manufacturer in the instructions for agricultural use. These concentrations are also considered as actually applicable in the agricultural practice. The fourth fish group served as control and the fish were kept in a tank with no added fungicide. All the tanks had a permanent aeration with air pumps and the water was kept oxygen saturated. For the entire duration of the experiment, the fish were maintained under a natural light/dark cycle (12:12). They were not fed prior or during the exposure. No fish mortality was observed. Basic water physical characteristics such as pH, temperature, dissolved oxygen; oxygen saturation, were measured three times per day according to a standard procedure (APHA, 2005) with a combined field-meter (WTW, Germany).

Histochemical technique

Histochemical analysis was carried out in the laboratory at the Department of Anatomy, Histology and Embryology at Medical University of Plovdiv, Bulgaria. Cryostat (Leica, Jung Frigocut 2800 N) was used to cut the samples. Multiple Bighead Carp liver sections (6 µm) of each specimen were prepared according to a standard PAS methodology (MCMANUS, 1948) as previously described (see GEORGIEVA *et al.*, 2013). Liver histochemical alterations of all specimens, including control fish livers were appraised individually and semi-quantitatively by using the grading system of BERNET (1999) which was adopted for the purposes of this study. Positive PAS-reaction was presented in purple-magenta staining. Evaluation of the histochemical changes was carried out and presented as an

average value. Each grade represents specific histochemical characteristics and is categorized as follows: (0) – negative reaction of histochemical staining; (1) – very weak positive reaction of histochemical staining; (2) – weak positive reaction of histochemical staining; (3) – moderate positive reaction of histochemical staining; (4) – strong positive reaction of histochemical staining in the hepatocytes.

Statistical analysis

The statistical analysis was performed using the program Graph Pad Prism 7 for Windows (GraphPad Software, San Diego, CA, USA). The raw data on basic water physical properties and histochemical scores were tested for normal distribution with the D'Agostino-Pearson normality test. The differences between the variables were tested using Student's T-test with 95% confidence interval. The results were reported as average.

Results and Discussion

Overall, in the treated with fungicide groups, we found a slightly positive reaction of histochemical staining (Table 4, Fig. 2). The degree of intensity of the PAS reaction increased as the concentration of fungicide increased. At the lowest concentration of 30 mg/L, we found an increase in the intensity of the PAS reaction. This fact refers to an increase in the accumulated glycogen in the hepatocytes of the fish group treated with 30 mg/L fungicide. On the semi-quantitative scale, we determined the degree as moderately positive with intense pink-violet staining in the hepatocytes. In the fish group treated with 38 mg/L fungicide, similarly to the 30 mg/L, we also observed pink-violet staining in the hepatocytes. At the highest concentration of 50 mg/L fungicide, we found a strong positive histochemical reaction with intensive dark violet staining. The strong reaction was also expressed in the presence of diffusely dispersed glycogen in the cytoplasm of hepatocytes, as well as concentration of conglomerates. In addition, when analyzing the cryostat sections of the liver, we find, in addition to increasing the intensity of the PAS-positive reaction, proportional to the increase in the fungicide concentration and the presence of conglomerates of accumulated glycogen in the cytoplasm. Such glycogen accumulations in the hepatocytes of control.

Table 4. Intensity of PAS-reaction in liver of Bighead Carp after fungicide exposure.

Concentration of the fungicide	Control group	30 mg/L	38 mg/L	50 mg/L
Intensity of PAS-reaction staining	1*	3	3	4*

* – statistically significant difference ($p<0.05$).

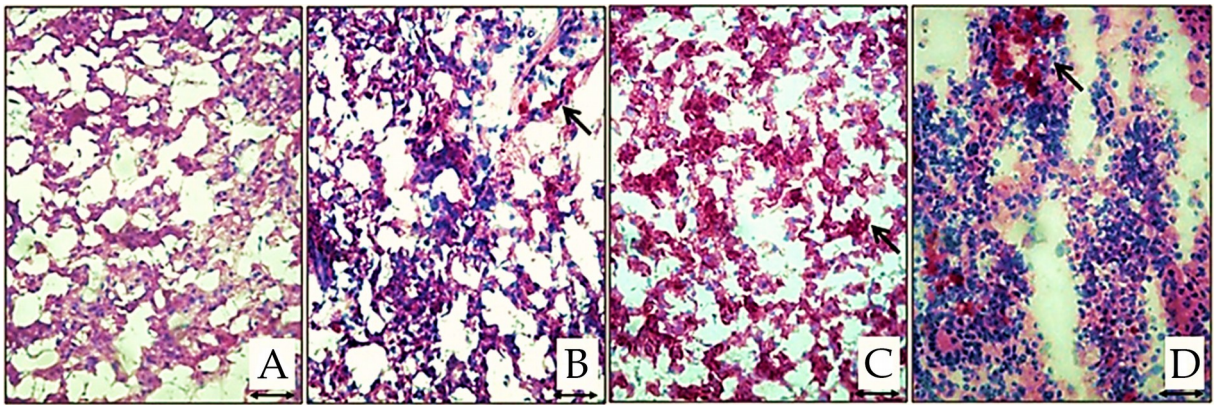


Fig. 2. Intensity of PAS-reaction in liver of Bighead Carp after fungicide exposure: A – control, x400; B – 30 mg/L, x400; C – 38 mg/L, x400; D –50 mg/L, x400; (conglomerates of accumulated glycogen are marked with arrows).

The excessive application of fungicides in plant protection can cause water contamination and thus, histochemical changes such as the amount of glycogen in the liver of fish. These changes however, can serve as a reliable biomarker for hepatotoxicity. Thus, the histochemical methods can be applied as cellular tools for the accumulation of glycogen, or lipids and proteins under the action of various toxicants.

Our results of PAS response intensity indicated glycogen accumulation in the hepatocytes and we also found a tendency towards increasing of the glycogen levels after exposure to the tested fungicide. These results confirmed our previous study which concerns the histochemical effects of the same fungicide on glycogen and lipid storage in Common Carp liver (see GEORGIEVA *et al.,* 2013). Similarly to us, SHRIVASTAVA (2007) considers that the changes in the liver glycogen amount indicate changes in the carbohydrate metabolism due to the effects of different pesticides. According to RAMESH & SARAVANAN (2008) the liver stores essential carbohydrates in the fish body and it also participates in the blood glucose homeostasis by maintaining a balance between glycogenesis and glycolysis. Therefore, we consider that the alterations associated with increased amount of glycogen in the hepatocytes compared to the fish control group may be due to changes in the amount of pyruvate which on the other hand, may affect glycogenesis and glycolysis.

The results of the histochemical study showed an accelerated process of accumulation of glycogen in the hepatocyte cytoplasm under the action of all three different and increasing fungicide concentrations. In addition, the amount of glycogen in the hepatocytes of the tested fish was increased in a direct proportion to the increase in fungicide concentrations. GEORGIEVA *et al.* (2013), STOYANOVA *et al.* (2019) and YANCHEVA *et al.* (2019) found similar histochemical alterations, but also fat infiltration in Common Carp and possibly linked them to the absence of the enzyme glucose-6-phosphatase, and the inability to release glucose in the blood which leads to hypoglycemia in the fish body. Probably, the increased amounts of glucose-6-phosphate lead to increased activity of the pentose phosphate pathway, and hence higher amounts of pyruvate. However, in the present study we have not presented results on the Sudan III staining (unpublished) and therefore, we suggest a further research in this particular area.

Conclusions

In sum, we can conclude that PAS-reaction is a rapid and reliable biomarker which can be easily applied for fungicide contamination and all three different, environmentally relevant concentrations of Verita® WG (Fosetyl-aluminum + Fenamidone) caused histochemical alterations in the liver of Bighead Carp. Furthermore, based on our previous study (GEORGIEVA *et al.,* 2013) we also found that Bighead Carp is a good bioindicator for water contamination, but it is slightly more susceptible to fungicide pollution compared to Common Carp. Lastly, the present study dropped a strong hint about possible antagonistic and synergistic interactions between Fosetyl-aluminum and Fenamidone, thus we strongly recommend that future studies are carried out.

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