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A N I N T E R N A T I O N A L J O U R N A L

Histological Alterations in Common Carp (*Cyprinus carpio* Linnaeus, 1758) Gills as Potential Biomarkers for Fungicide Contamination

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ABSTRACT

The present study aimed to investigate the histological alterations in common carp gills caused by a fosetyl-Al and fenamidone based fungicide tested in laboratory conditions at 30, 38 and 50 mg/L concentration. In general, all the tested concentrations activated compensatory-adaptive mechanisms, which caused pathological changes in the fish gills. Results showed different histological alterations in the gill structure, which included lamellar lifting, edema, proliferation of the glandular cells and epithelium, covering the gill filament, fusion and degenerative alterations. Blood circulatory system showed vasodilatation of the secondary lamellae and aneurysms. Overall, there was enhancement of the gill histological changes, which was dose-dependent, i.e., proportional to the increasing fungicide concentrations. Thus, based on the results, it was concluded that the histological alterations in common carp gills could be applied as possible biomarkers in risk assessment and monitoring programs for pesticide contamination of aquatic ecosystems.

Key words: histology, fish, gills, pesticides, contamination, biomarkers

INTRODUCTION

Extensive use of pesticides for the effective control of plant diseases has become crucial for agriculture in the last decades (Panigrahi et al. 2014). Pesticides, however, are the class of compounds that despite of their benefits may produce a range of toxic effects that pose potential hazards to the environment. Therefore, increasing agricultural production has resulted in increasing number of aquatic systems being impacted by the contaminants present in wastewater releases (Figueiredo-Fernandes et al. 2007). It is well known that the pesticide application could represent a contaminant source for the aquatic environment and thus, affect aquatic fauna and in particular, fish (Pathan et al. 2010; Botaro et al. 2011; Thomas et al. 2012; Wang et al. 2013; Agbohessi et al. 2014; Karthigayani et al. 2014; Barnhoorn et al. 2015). Factors to be considered in this regard are pesticide persistence in the environment and their potential for bioaccumulation and biomagnification in the food chain (Muthukumarave et al. 2013; Rathnamma and Nagaraju 2014).

Fish are often used as indicators of pesticide water contamination due to the fact that they respond to its low concentrations with physiological and

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behavioral changes (Ayas et al. 2007; Banaee et al. 2011; Murthy et al. 2013). Thus, monitoring sentinel fish species is widely used to assess the degree of toxicant accumulation and their effects on health status (De la Torre et al. 2005). In teleost fish, the gills, liver, kidney and muscles are the tissues, which are the most commonly, used in eco-toxicological and pathological studies (Sauer and Watabe 1989). Fish gills are multifunctional organs involved in ion transport, gas exchange, acid–base regulation and waste excretion (Dang et al. 2001). They are comparatively more vulnerable to pollutant stress because of their permanent contact with contaminated water and large surface area. Fish gills are not only the major site of uptake of waterborne toxicants, but they are also the first and most important site of toxic impact. Their condition has a direct relation with the water quality and any deterioration in their structure and function has adverse impact on the biology and survival of the fish. Therefore, gills can be applied as an effective indicator not only for the fish health, but also for the ecosystem health and the level of water pollution (Perry and Laurent 1991; Tkacheva et al. 2004; Rosseland et al. 2007; Heier et al. 2009; Ba-Omar et al. 2011; Singh 2014).

Biomarkers have been proposed as sensitive tools for the early detection of environmental exposure to pollutants and their negative effects on aquatic organisms (van Der Oost et al. 2003; De la Torre et al. 2005). They also serve as links between the environmental contamination and its effects, providing unique information on the ecosystem health status (Au 2004; Maria et al. 2009). Histological alterations in various tissues and organs, including gills are directly related to effects of many contaminants and could serve as important biomarkers to assess the pesticides toxicity (Thophon et al. 2003). Due to being exposed to the pollutants, major structural damages may occur in the target fish organs, histological structure may change and physiological stress may occur. This stress also causes some changes in the metabological functions. In addition, changes in the functions are initiated with changes in the tissue and cellular level before significant changes could be identified in fish behaviour or external appearance (Van Dyk et al. 2008). Even though, histological alterations are non-specific biomarkers, they have been examined for decades in various fish tissues and organs in order to assess the effects of different chemical contaminants (heavy metals, pesticides, POPs, etc.) both in the field and laboratory studies (Hinton and Lauren 1990; Manera et al. 2000; Au 2004; Ramírez-Duarte et al. 2004; Figueiredo-Fernandes et al. 2007; Fontaínhas-Fernandes et al. 2008; Mohamed 2009).

Common carp (*Cyprinus carpio* L.) is a wide-spread fish species, which is preferred in aquaculture and is also very popular in sports fishing due to its fast growth rate, hardiness and prolific breeding in confined water. Carp has also been proposed as a test organism in many toxicological assays because it is relatively insensitive and as a consequence, survives and accumulates contaminants even at heavily polluted sites (Snyder et al. 2004; Brumbaugh et al. 2005; Reynaud and Deschaux 2005; De Boeck et al. 2007; Oruc and Usta 2007; Reynders et al. 2008). Although there are several studies on pesticide effects on fish gills, the effects of fosetyl-Al and fenamidone-based fungicides on fish, including common carp is relatively limited. Due to its high economic importance and scarcity of data on gill histological alterations caused by this chemical, the objectives of the present study were:

1) to investigate the effects of a fosetyl-Al and fenamidone based fungicide on common carp gills;

2) to study the degree of expression of each histological alteration; and

3) to propose the histological alterations as possible biomarkers for pesticide pollution.

MATERIAL AND METHODS

Test animals

Forty healthy common carps were purchased from the Institute of Fisheries and Aquaculture, located in the city of Plovdiv, Bulgaria. They were of a similar size-group (mean length 16.3 cm ± 2.7; mean body mass 47.8 g ± 5.2) with no external pathological abnormalities. After transportation, the fish were placed in glass tanks with 100 L chlorine-free tap water (by evaporation) to acclimatize for a week. They were divided into four groups (n=10) and not fed prior or during the experiment.

Chemicals and experimental setup

The test pesticide in this experiment was "Verita WG", a systemic and contact fungicide, effective against plant diseases, caused by fungi of the class Oomycetes. The active substances in this particular pesticide are fosetyl-Al and fenamidone. Fosetyl-Al is a member of the phosphonates, which constitute a relatively new class of systemic fungicides (Cohen and 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. The test chemical was provided by Bayer CropScience, Germany. 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. The fourth fish group served as control and the fish were kept in a tank with no added fungicide.

All the tanks were equipped with air pumps for permanent aeration and water was kept oxygen saturated. The fish were kept under a natural light/dark cycle (12:12 h). Physico-chemical characteristics of the water such as pH, temperature, dissolved oxygen, oxygen saturation and conductivity were measured once per day according to a standard procedure (APHA, 2005) with a combined field meter (WTW, Germany). They were as follows: pH – 8.2 ± 0.1; temperature – 24.5ºC ± 0.12; dissolved oxygen – 7.26 mg/L ± 0.2; oxygen saturation – 90.25% ± 3.3 and conductivity – 320 S/m. The study was performed in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Histopathological analysis

Fish dissection was performed according to the procedures given in the EMERGE Protocol (Rosseland et al. 2003). Samples were placed in vials with 10% neutral buffered formaldehyde solution (pH 7.0). They were rinsed in tap water, dehydrated in a graded series of ethanol concentrations, cleared in xylene, embedded in paraffin wax with melting point of 54–56ºC, sectioned to a thickness of 5–7 µm using a semi-automated rotary microtome (Leica RM 2245) and mounted on sterilized glass slides. Sections were then deparaffinised, stained with hematoxylin and eosin (H&E) for histological examinations and prepared for light microscopy analysis (Romeis 1989).

Semi–quantitative scoring

Ten paraffin sections were produced from the gills of each specimen. Each section was taken from a different location from the paraffin block, instead of in series. The main purpose was to follow the histological changes in a maximum part of the sample. Histological alterations in the gill structure were analyzed by observing the whole gill surface. Degree of expression of the histological changes in each of them was studied, including corresponding changes in the gill surface in relation to the normal histological structure. Histological alterations in the gill epithelium were determined semi–quantitatively by using the grading system of Peebua et al. (2006), which was slightly modified. The system provided an objective assessment of the fish gill histological integrity in the histological analyses and clearly distinguished between the normal gill structure and gill pathology. Each grade represented specific histological characteristics and was categorized as follows: (-) – no histological alterations, which represented normal histological structure; (+/-) – mild histological alterations; (+) – moderate histological alterations; (++) – severe histological alterations; (+++) – very severe histological alterations in the gill surface morphology.

RESULTS AND DISCUSSION

Gills of most teleost are typically composed of four pairs of gill arches, which are supported by a bone skeleton. Filaments come from the gills arches, supported by cartilage (primary lamellae), from which the secondary lamellae exit. The secondary lamellae are constituted by a simple epithelium, where gas exchanges occur (Laurent and Perry 1995). No histological changes were observed in the control fish gills. Gill structure of the control carps are shown in Figure. 1. With regards to the proposed grading system, the control common carp histological characteristics were evaluated as relatively normal (-). Histological results indicated that the different test fungicide concentrations caused morphological alterations in the gill epithelium and the blood circulatory system of the common carp gills. Lamellar epithelium lifting; edema; proliferation of the stratified epithelium and glandular cells; fusion and degeneration in the gill epithelium were found. In addition, vasodilatation in the blood circulatory system and different aneurisms, which in fact represented the most severe disturbances in the blood vessels, was observed (Table 1).

Table 1 - Histological alterations in the common carp gills caused by the test fosetyl-Al and fenamidone based fungicide.

Histological alteration	Control	30 mg/L	38 mg/L	50 mg/L
Lamellar lifting	+/-	+	++	+++
Edema	-	+	++	++
Proliferation of stratified epithelium	-	+	+	+
Proliferation of glandular cells	-	-	+	+
Fusion	-	-	+/-	+/-
Degeneration of gill epithelium	-	-	+	+++
Vasodilatation of secondary lamellae:				
Along the length of blood vessel	-	-	-	-
At basal part of the blood vessel	-	+/-	+/-	+
At apical part of the blood vessel	-	-	-	-
Vasodilatation of central venous sinus	-	+/-	+	+
Aneurysms	-	-	+	++

(-) – no histological alterations, which represent normal histological structure; (+/-) – mild histological alterations; (+) – moderate histological alterations; (++) – severe histological alterations; (+++) – very severe histological alterations in the gill surface morphology.

Lamellar epithelium lifting at 30 mg/L fungicide was observed in a moderate degree of expression (Table 1, Fig. 1). Edema and proliferation of stratified epithelium were presented also in a moderate degree of expression (Fig. 1). In contrast, at the lowest fungicide concentration, proliferation of the glandular cells, fusion and degenerative changes were not observed. Furthermore, relatively slight changes in the blood circulatory system were also found at 30 mg/L. They were presented in vasodilatation, which was detected at the basal part of the blood vessels of the secondary lamellae (Table 1). In addition, vasodilatation of the central venous in a mild degree of expression was observed, but not aneurysms.

Lamellar epithelium lifting and edema were presented in a severe degree of expression in all the specimens treated with 38 mg/L of the fungicide. Proliferative changes in the stratified gill epithelium and glandular cells as well as degenerative changes were observed in a severe degree. However, fusion as the most serious form of proliferation of the filamentous stratified epithelium was in a mild degree (Fig. 1). While morphological changes in the blood circulatory system included vasodilatation at the basal part of the secondary lamellae, which was in a moderate degree of expression, these changes also presented vasodilatation of the central vein and aneurysms in the secondary lamellae in a moderate degree of expression (Table 1).

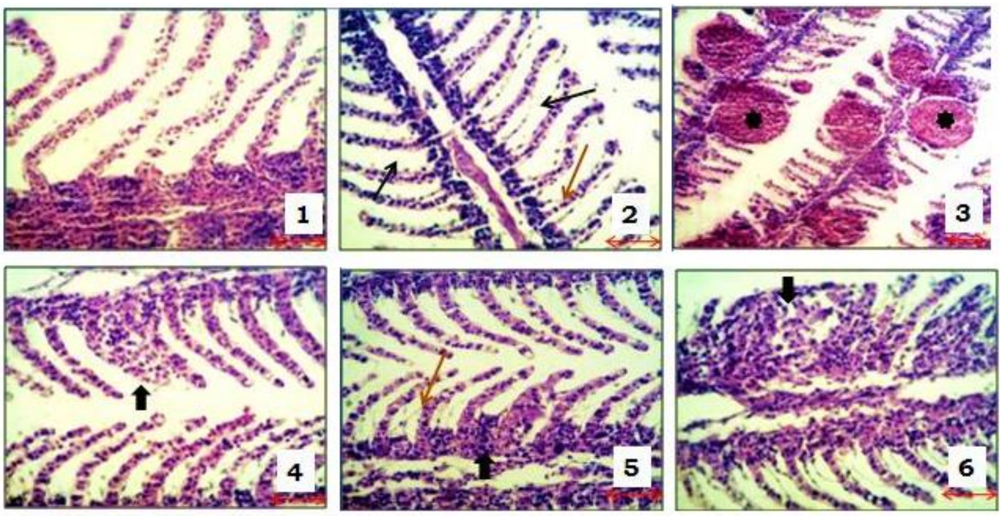

Figure 1 - Histological alterations in common carp gills, caused by the test fosetyl-Al and fenamidone based fungicide, (H&E): 1 - normal histological structure, x400; 2 - lamellar lifting (black arrow) and edema (brown arrow), x400; 3 - aneurysms in the secondary lamellae, x200; 4 - fusion between two secondary lamellae, x400; 5 - proliferation of filamentous epithelium and edema (black arrow), x400; 6 - fusion, x400.

At the highest chemical concentration of 50 mg/L, alterations in the gill histological structure were more pronounced than the lower concentrations. Lamellar lifting and degenerative changes were detected in a very severe degree. Edema was expressed also in a severe degree, but proliferative changes in the epithelial and glandular cells were in a mild degree of expression (Fig. 1). Similar to the fungicide concentration of 38 mg/L, fusion was presented in a mild degree. Vasodilatation affected mainly the basal part of the blood vessel in the secondary lamellae and the central venous sinus, which was moderate. The most severe changes in the blood circulatory system - aneurysms, were more pronounced compared to the other tested fungicide concentrations (Table 1, Fig. 1) as reported earlier by (Georgieva et al. 2014), the present study showed proliferative and degenerative changes in the fish gill structure. However, in the present study a fungicide was used at different concentrations, but not an insecticide.

In general, enhanced proliferation increases the distance between the external environment and the circulatory system and degenerative changes contribute substantially towards hindering the gill functions, and therefore, the whole body health. The degenerative changes in this study were much more pronounced compared with the ones caused by thiamethoxam in our previous one (see Georgieva et al. 2014). These suggested the presence of intensive processes of epithelial cells differentiation, as well as cell death, resulting from the high pesticide toxicity. The histological changes in the gill structure could be a reaction of the fish organism to toxicant intake or an adaptive response in order to prevent the entry of the toxicants through the gill surface. Hence, they could serve as an actual defense mechanism, which resulted in the increase of the distance between the external environment and the blood, and also could be a barrier to the entrance of different contaminants (Mohamed 2009). According to Gaber et al. (2014), the histological changes found in the fish gill structure in their study were also an adaptive response to the pesticide effects. However, the histological changes observed in this study, which occurred under the action of the test fungicide, particularly the proliferative changes, not only limited the toxicants penetration, but also reduced the normal transportation of oxygen to the red blood cells in the blood vessels. Therefore, the general physiological state of the organism would be undoubtedly impacted.

Changes in the circulatory system such as vasodilation and aneurysms indicate increased blood vessels pressure. Such histological alterations in fish gills have also been reported in other studies such as Campagna et al. (2007); Peebua et al. (2008); Ramírez-Duarte et al. (2008); Vigário and Sabóia-Morais (2014) under the influence of different pesticides. The present study found aneurysms, which actually were not observed in the common carp gills when it was exposed to thiamethoxam. This result indicated that the test fungicide was more toxic to the fish compared to the insecticide as reported previously (Georgieva et al. 2014).

The study not only focused on the histological alterations in the gill structure but also provided the degree of expression of each histological change, which was caused by the fungicide in order to establish more precisely the degree of its toxic effects. In general, the degree of expression of the gill morphological changes was in a dose-dependent manner. Barbieri and Alves Ferreira (2011) also reported the same tendency, which led to a decrease in the ability to maintain the homeostasis.

In conclusion, all fosetyl-Al and fenamidone-based fungicide concentrations in the present study caused changes in the fish gill morphological structure, which were mostly associated with the proliferative and degenerative alterations, as well as alterations in the blood circulatory system. There was also a tendency towards enhancing the morphological alterations and their degree of expression was proportional to the increasing concentrations of the fungicide. These histological changes could be successfully applied as tissue biomarkers in both, the field assessment and monitoring programs on pesticide contaminated aquatic ecosystems. Therefore, further investigations in this particular field should be carried out to better understand the negative impact of different pesticides (fungicides, insecticides or herbicides) on fish gill morphology and their protective mechanisms.

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