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Biochemical and Histopathological Markers as Indicators of Genuine Toxicity Caused by Intraperitoneal Application of Tilt 250 Fungicides of Fish Barbus Peloponnesius

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Abstract
In this paper are presented the obtained results from the research activity of the enzyme so-called *Ethoxyresorufin-O-deethylase* in the liver of the fish *Barbus peloponnesius* (Valenciennes, 1842), after intraperitoneal application of certain doses of Tilt 250 fungicide (0.18mg/l, 0.36mg/l and 1.08 mg/l). Also in this paper are presented the results of histopathological analysis of liver, kidney and gonads after the intraperitoneal application of certain dose Tilt 250 fungicide (2µg/l), during the 1,2,3,7 and 14 days. From the obtained results it was concluded that the application of the doses of 0.18mg/l, 0.36mg/l and 1.08mg/l to 250 tilt fungicides have caused the induction of EROD enzyme, whereas the dosage application of 2µg/l toxicity has caused changes in the liver, kidney and gonads.
Keywords: pesticides, fish, biomarker, EROD, lesions.

Introduction

The use of pesticides in the world has increased dramatically over the last two decades due to changes that have occurred in agricultural practices. The environmental pollution caused by pesticides, especially in aquatic ecosystems has become a serious problem for flora and fauna and for the humanity in general.
Water pollution by pesticides, whether directly or indirectly, can cause fish kills and reduce their populations as a result of reducing the reproduction process, and if their concentration level increases in fish tissues, then could also be with toxic negative consequences for the human body itself (Fig. 1).

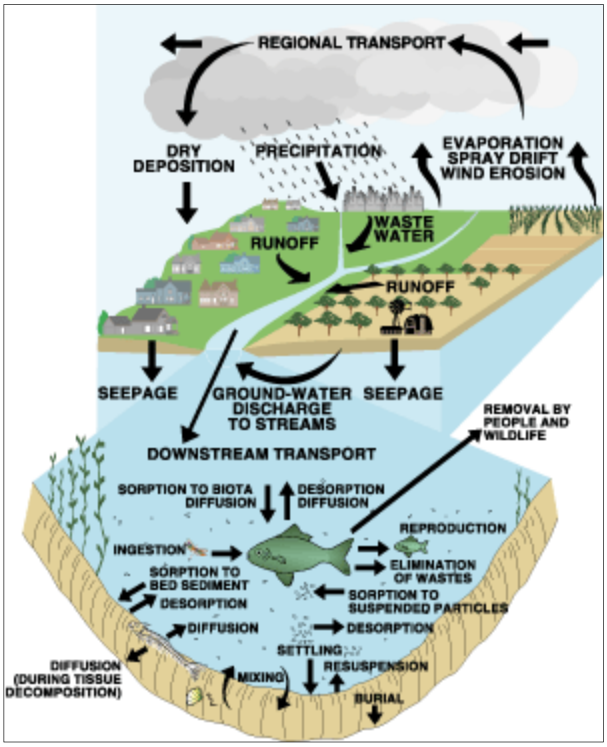


Figure 1. Pesticide movement in the hydrologic cycle including pesticide movement to and from sediment and aquatic biota within the stream. Modified from Majewski and Capel (1995). Source: http://water.usgs.gov/nawqa/pnsp/pubs/fs09200/

The remaining quantities of pesticides and their metabolites that are found in drinking water and various foods are very alarming today and they present serious threat to the human health as a result of their exposure.
The contamination of surface waters by pesticides is well documented worldwide and it constitutes an essential issue at the local, regional, national and global level (Cerejeira et al, 2003; Spalding et al., 2003). Pisces as strong indicators of exposure to pollution and as the bio indicators have a very important role in the monitoring of the pollution of aquatic biotopes since they react with high sensitivity towards changes caused in aquatic environments. The biochemical fish markers present responses that are initiated by the presence of a specific group of pollutants, which possess the same mechanism of toxic activity in the aquatic ecosystem organism. From previous studies, it is proved that the presence of Cytochrome P450 as a complex enzyme in fish, presents a very suitable detector used then to monitoring aquatic premises; (Payne et al., 1987).
The Cytochrome P450 was discovered for the first time in 1967 by Klingenberg in the trout (*Salmo gairdneri*) and since that time this protein is intensively researched (Kvasniãková 1995; Anzenbacherová and Anzenbacher 1999, Lewis 2001). From previous studies it is proven that this enzyme is not a single entity, but it includes a large number of isoenzymes, that up to today have been isolated more than 1000 of this kind (Stoilov et al., 2001, Lewis 2001).
Cytochrome P450 is classified as hemoglobin protein type b (Heme of this type have also hemoglobin, mioglobin and some peroxidases) bound to membranes of the endoplasmic reticulum non granules. Except for the endoplasmic non granules reticulum membranes, Cytochrome P450 is related to mitochondrial membranes, whereas in bacteria is found in the cytoplasm in the digested form. The name of this pigment derives from the fact that as the CO complex absorbs the light at 450 nm wavelength, that's why it is called like that. An Inactive form of Cytochrome P450 has maximum absorption of 420 nm which is similar to other hemoglobin proteins (Kvasniãková 1995; Schenkman and Jansson 1998; Anzenbacherová and Anzenbacher 1999). The Cytochrome P450 activity depends on the presence of NADPH-Cytochrome P450 reductase and phospholipids of the membrane fraction.
All these components make up the system of non-specific monooxygenase (Kvasniãková 1995). In this system are included the enzymes EROD and B(a)PMO. EROD is a very good indicator of changes in the environment and in aquatic ecosystem, and also it represents one of the enzymes that were first discovered by Stegeman 1992. These enzymes represent indicators activity of all deposited chemicals which cannot be detected by analytical methods. The biomarker presents histopathological changes of tissues and cells which are caused as a result of exposure to different fish pollutants (Hinton and Lauren, 1990, Myers and Fournie, 2002). Most of the lesions in tissues and organs were proven in the laboratory, to the fish exposed by pollutants. Macrophages' aggregates (MA) are reliable indicators of exposure of fish towards contaminated sediment and low level of dissolved oxygen in water (Myers and Fournie 2002) and in the large number are found in fish which live in contaminated waters (Fournie et al., 2001). The accumulations of macrophages in fish were found in the spleen, kidney, liver, and sometimes even in testicles. They differ in the number, size, pigment, and concentrate on the destroyed tissue (Wolk 1992).

The purpose of the study

The purpose of this study was the investigation of EROD enzyme activity and of histopathological possible changes to fish *Barbus peloponnesius* which belongs to the family of *Cyprinidae* caused by the intraperitoneal application of Tilt 250 fungicide. Also, the ultimate goal of this research is to establish the possibility of application of biochemical and histopathological markers in monitoring the level of toxicity to aquatic organisms in general; and in particular, to the fish and aquatic environment pollution.

Material and methods

For the realization of this research are involved two groups of fish: control and experimental fish, with the average length of 20.8cm and average body weight of 81.5g. Such fish were held in aquaria glass of 80l capacity, with dimensions of 80cm x 40cm x 35 cm and equipped with pumps for continual ventilatio (Shark RS-610) (Photo. 1).

Photo 1. *Barbus peloponnesius* fish stuffed in the aquarium which is used for the realization of this research.

The de-chlorinated water from the special de-chlorination filter, (So-Safe CTSLW 10D) is partially replaced by aquariums every day and every week completely, in order to reduce or eliminate the whole nitrates and avoid the risk of low alkalinity of water. Water temperature in the aquariums was 16^{o}C, the amount of dissolved oxygen in water 6mg/l and pH value of 7.4. After acclimatization (after 14 days) to the experimental group of fish that we have used for the analysis of EROD enzyme activity, we have operate with Tilt 250 fungicide (contains 250g/l propiconazole [(+/-)-1-(2-/2,4-dichlorphenyl/-4-propyl-1,3-dioxolan-2-ylmethyl)-1H-1,2,4 triazole]) by injecting intraperitoneal the fixed dose in their body, (0.18mg/l, 0.36mg/l and 1.08mg/l).

To the fish that were used for the analysis of the possible histopathological changes it is applied the dose of 2µg/l, during the 1,2,3,7 and 14 days. Tilt 250 fungicide containing 250g/l propiconazole [(+/-)-1-(2-/2,4-dichlorphenyl/-4-propyl-1,3-dioxolan-2-ylmethyl)-1H-1,2,4Triazole]).

Before the desectation was done, firstly we determined the weight (g) and length (mm) of fish by acting with a needle down to the operculum we have killed them by causing interruptions in the spinal cord. Their disectation was done with scissors by opening the abdominal cavity and by isolating the target organs. After the isolation of the liver, kidney and gonads, researchers had defined their body weight through digital scales (AND SV-200).

During the isolation of the liver (hepatopancreas), researchers have been careful not to injure the gallbladder because it possesses inhibitor that inhibits the activity of enzymes. The average weight of liver was 0.20g, for kidney it was 0.25g and for gonads it was 0.50g.

To determine the activity of the EROD enzyme we have used 200mg of liver. This amount is homogenized with puffer for homogenization (pH 7.4) in homogenizer (Polytron-PT) and it is centrifugated (10,000g; 20min; 4^{0}C), whereas the supernatant gained was re-centrifugated (100,000g; 1h; 4^{0}C) in ultracentrifuges (Sanyo, Harrier Refrigerated 18/80).

The EROD enzyme activity was measured with spectrofluorometers (SHIMADZU RF-1501), according to the method applied by Burke and Mayer (1974), for excitation/emission wavelengths setting 510nm/585nm. The microsomal protein concentration was measured with spectrophotometers (GENESYS 10uv) applying the method of Lowry et al. (1951) with the reading of adsorption at 710nm, and by using the Folins's phenol reagent.

The fragments of isolated organs for histopathological analysis were fixed in formalin 9% for 24h. After their dehydration, washing and paraffination, then they were cut in very tiny parts, with a thickness of 5µm, with the help of microtome (LEICA SM 2000R).

The staining was done according to the method of Hematoxylin and Eosin and protocol Harris, while the microscopic examination of lesions is done through the optical microscope, with digital cameras and software (NIKON Eclipse 80i) that is based on histomorphological criteria, described previously by other authors (Myers et al., 1993, 1994; Hinton et al., 1992; Hinton, 1993; Moore and Mayers, 1994; Sonia et al. 2007).

The statistical analysis

The obtained results are expressed as average values, with standard deviation and with the number of fish involved in the research. For the statistical elaboration of the obtained values researchers have used a software package IBM SPSS Statistics 20 (© IBM, Armonk, NY, USA). As the most distinctive and significant value alongside with the control is taken that value for which it was $P < 0.05$.

Results

The obtained results of the EROD enzyme activities, in the liver of fish *Barbus peloponnesius*, after 48 hours

exposure to the certain doses Tilt 250 fungicide are shown in Chart 1.

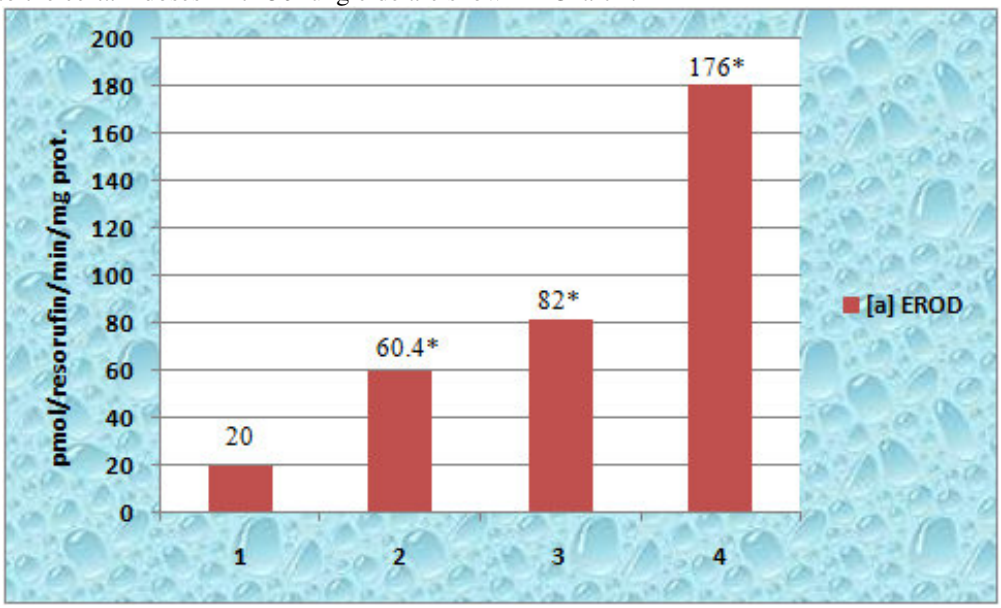
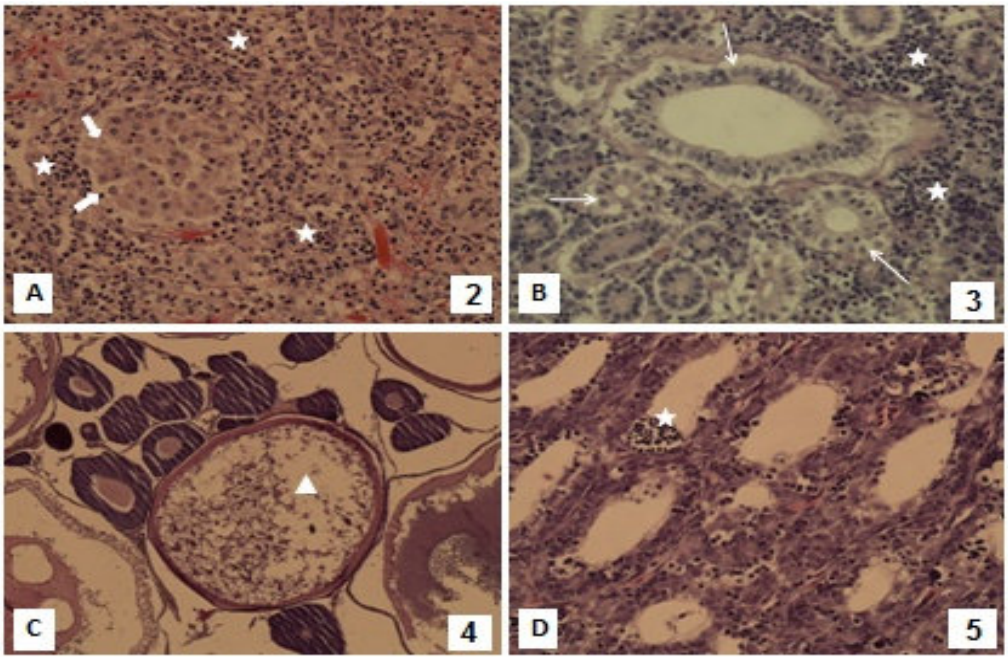


Chart 1. EROD enzyme activity (pmol/resorufin/min/mg prot.) in the liver of fish *Barbus peloponnesius* (n=5) after 48h exposure to Tilt 250 fungicide. 1 = control fish; 2-4 = fish exposed to Tilt 250 fungicide (2 = 0.108 mg/l; 3 = 0.36 mg/l; 4 = 1.08 mg / l). Results that are presented with the star (*) indicate a significant statistical difference in balance with control ($p < 0.05$) and n=5 the number of fish involved in the study.

From the graphical view of the results obtained from the analysis of the activity of the EROD enzyme, in the experimental fish liver, it is clear that during the intraperitoneal action with 0.108mg/l 250 Tilt fungicide, the enzymatic EROD activity was 60.4±10.60 pmol/resorufin/min/mg prot., or 2 times greater than that of control fish, during the action with 0.36mg/l of enzyme activity it was 82± 29.73 pmol/resorufin/min/mg prot., or 4 times greater than control fish, while in action with 1.08mg/l of enzyme activity was 176±42 pmol/resorufin/min/mg prot., or 9 times greater than that of control fish. From the results obtained it is obvious that the doses applied of Tilt 250 fungicides with experimental fish have caused expressive inducibility of the enzyme EROD compared with the control fish.

Toxicophatic lesions to liver, kidneys and gonads determined by optical microscopy are presented in pictures 2, 3, 4 and 5.



Pictures 2, 3, 4 and 5. The Toxicophatic lesions in the liver, kidney and gonads of fish *Barbus peloponnesius*, caused by intraperitoneal application of dose by 2μg/l, Tilt 250 fungicide between the 1,2,3, 7 and 14 days. A-lesion in liver (magnification 400X, H & E), B-lesions in kidney (magnification 400X, H&E), C-lesion in ovarium (magnification 200X, H&E), D-lesion in testicle (magnification 200X, H&E). Stars

show aggregates of macrophages in the liver, kidney, ovary and testicle; thick arrows show necrosis of hepatocytes; the tiny arrows indicate the necrosis of renal epithelial cell channels; and the triangle indicates the destructiveness of the follicle).

By microscopic analysis of histopathological preparations, stained with hematoxylin and eosin (H&E), it was concluded that the applied dose of 250 Tilt fungicides for a certain period of time has caused lesions in the liver, kidney and gonads.

Discussion

The carp fish of the family (*Cyprinidae*) are often used as a model for investigating the activity of EROD enzyme (Hongyan et al. 2002).

EROD enzyme is safe and exceptional biomarker of body exposure to toxic substances (Achazi, 1998: Addison and Payne 1986; Burgeot et al., 1994; Forli et al. 1986; Galgani et al. 1996; Van der Oost et al. 1996, 2003).

Even though so far there are known many factors that affect the EROD enzyme (internal factors: gender of the body, age, reproductive status, etc., as well as external factors: annual periods, the presence of inducer and inhibitors in the environment), and yet there isn't found any satisfactory model which will help the researchers in the precise interpretation of results, derived from measurements of the EROD enzyme activity.

The power or EROD enzyme is different among various types of fish. Besides the level of expressiveness of the genetic level, the activity of EROD enzyme can also be affected by other factors, such as: physiological factors, anatomical, histological and other features of the organism.

It is known that the presence of the fats in the liver can affect negatively in EROD enzyme activity.

The histopathological biomarkers are closely related to other stress biomarkers because at first many pollutants must undergo metabolism in order then to be able to provoke changes in the attacked body. For example, the acting mechanism of some ksenobionts may initiate the formation of special enzymes that can cause changes in metabolism, such changes that are followed by intoxication and death in cellular level which are manifested as necrosis and that represents the histopathological biomarker in the cellular level (Hinton et al. 1992; Велкова-Јорданоска 2005).

In our case, the application of certain doses of 250 Tilt fungicides has initiated strain on EROD enzyme activity in the liver of the fish *Barbus poleponnesius*, these findings match with findings of other authors (Siroka et al., 2005, Babin et al. 2005 Dong et al., 2009, Sanchez et al., 2009, Haluzova et al. 2011).

Also, the application of certain dose of such toxicity for a certain period of time has caused histopathological changes in the liver, kidney and gonads that are followed by bleeding, hemolysis, and aggregation of macrophage infiltration, cell necrosis and destructiveness of follicles, these changes match with changes that were obtained earlier by other authors (Staicu et al., 2007, Deka et al. 2012).

Conclusion

The application of certain doses of Tilt 250 fungicide has caused a significant increase in EROD enzyme activity in the liver of the fish *Barbus poleponnesius*, for several times compared with control fish. These results prove that Tilt 250 fungicide has caused the inducibility of this biochemical biomarker. Also the application of certain dose of this fungicide in a certain period of time has caused toxipothatic changes in liver, kidney and gonads. From the analyses done by the optical microscope in the organs that were analyzed was concluded the necrosis of hepatocytes and renal epithelial cell channels, the presence of hemosiderin, hemorragia, hemolysis, and accumulation of macrophage infiltration and degeneration of follicles. From the obtained results from the enzyme and Toxicophatic analysis in vitro conditions, we can conclude that the *Barbus peloponnesius* fish can serve as a genuine Bio indicator of aquatic biotopes pollution from pesticides and from other potential pollutants. Therefore, based on these findings, the researchers recommend to the relevant institutions which produce pesticides and fungicides that prior to bringing to the market certain preparations; preliminarily they should license them as eco-preparations, which can then be used in agriculture, crop protection, without endangering the aquatic world. Based on the data from previous research and this research, the researchers can conclude that the uncontrolled use of pesticides which are unpatented in ecological aspect may cause lethal consequences for fish in particular but also for other organisms in general.

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