Chapter

The Morphophysiological, Histological, and Biochemical Response of Some Nontarget Organisms to the Stress Induced by the Pesticides in the Environment

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Abstract

Ferns, amphibians, and fish are groups of nontarget organisms affected by many types of pesticides that end up in the environment. This chapter aims to approach the following themes: the influence of different pesticides on the spore germination process and on the differentiation of their gametophyte; aspects regarding the impact of some pesticides on breathing in fish (physiology and histopathology at the branchial level), as well as a series of effects at the hematological and biochemical levels; and changes of some hematological, biochemical, and structural parameters in amphibians. Species that are not directly targeted by the action of the pesticide in the environment, ferns can be used in their gametophyte stage, young or mature sporophyte in different biotests to evaluate the risk associated with these substances. The biochemical, hemathological, and histopathological changes recorded in both fish and amphibians can be considered biomarkers of pesticide pollution.

Keywords: fern, fish, amphibians, pesticides

1. Introduction

Contamination of the natural environment, due to natural factors and human action, has been subject of numerous studies. In many ecosystems, human activity has led to changes of natural biogeochemical cycles, resulting in the accumulation of some substances.

Chemical contaminants are found everywhere in nature, and ecologists are the ones who assess their impact on natural communities of organisms. Among these, pesticides are the most common type of aquatic ecosystem contaminant.

Considering the physical and chemical properties (vapor expansion, volatility, evaporation capacity from water—codistillation) that transform pesticides, their bioaccumulative stability and capacity as well as the publication of ecotoxicological data on the action of different pesticides have become indispensable to continually monitor changes in the environment.

Different research on morphology, physiology, and biochemistry of gametophyte and sporophyte of fern species has shown that pesticides affect these processes, depending on species sensitivity, concentration, and exposure time.

Fish assimilates pesticides through gills or contaminated food. Gills are the main channel of pesticide penetration, which is why any disease at this level will have a great influence on the adaptive changes in the fish body. The effects of pesticides on fish are numerous and varied: they cause mortality both directly and indirectly, by starvation (destroying the organisms they feed on), affects hatching, growth rate, can lead to malformations, affects reproduction rate, modifies enzyme activity, and cause histopathological changes in organs, genetic effects, etc. Although under experimental conditions fish can survive pesticides in different concentrations, under natural conditions they are more vulnerable to disease, predators, no longer competitive, and no longer dealing with stress caused by changing seasonal temperature, reproduction season or temporary starvation.

Amphibians are organisms that populate aquatic ecosystems, being involved in aquatic trophic chains both by eating food and being food for predators. Over the last 20 years, scientists have reported the global decline of amphibian populations. Therefore, in 1996, the International Union for Conservation of Nature registered 156 species of amphibians in the Red List, and recent data show that 1856 species (about 32.5% of all amphibian species) are currently registered.

2. Influence of pesticides on spore germination and gametophyte differentiation in ferns

2.1 Gametophyte of ferns

Ferns and lycophytes (pteridophytes) represent 4% of Terra's vascular plants [1], numbering about 11,000 species in 40 families and 300 genres [2]. Different species of pteridophytes are used as medicinal, food, horticultural, and agricultural plants [1] and in the last decade as organisms in acute [3] and chronic phytotoxicity tests [4].

The life cycle of pteridophytes is characterized by an alternation of generations between a well-developed sporophyte and a reduced gametophyte, independent of the sporophyte [1]. The gametophytic generation of ferns begins with the formation of spores (meiospores), from which, a gametophyte or multilcellular prothallium is differentiated by germination. The gametophyte of leptosporangiate ferns is above ground, photosynthetic, short lived, and heart shaped [1]. From the spore to the mature gametophyte stage with gamentagia, the differentiation of the gametophyte involves the passage through the prothallium filament and spatula-shaped prothallium lamella [5].

The spores germinate on the ground, the first division resulting in a smaller base cell (the initial cell of the rhizoid) and a larger apical cell (the initial cell of the prothallium). The initial cell of the rhizoid elongates and forms a hyaline rhizoid having no chloroplasts. The initial cell of the prothallium will form a multicellular prothallium filament, consisting of a variable number of cells with numerous chloroplasts. One or more rhizoids are formed on the cells at the base of the prothallium filament. Lamella formation (prothallium plate) is initiated by the longitudinal division of the end cell of the prothallium filament. One of the daughter cells divides through a slanted wall resulting in an apical (initial) wedge-shaped cell. The fore side of the prothallium then extends to a width of 3–4 cells and becomes spatula shaped. When the apex of the prothallium is heart shaped, the initial cell is replaced by a flat multicellular meristem. The activity of the lateral zones of this multicellular meristem helps the formation of the prothallium wings. The cells

behind the apical meristem divide through a plan parallel to the underlayer, leading to the formation of a multi-layered medial crest. On the underside of the prothallium, there are antheridia, archegonia, and rhizoids. Prothallium differentiates different types of prothallium trichomes in some species.

The processes of spore germination and differentiation of gametophyte in ferns are influenced by a series of endogenous and exogenous factors, with environmental pesticides in the latter category.

2.2 Changes in the fern gametophyte caused by pesticides

Over the time, many researchers have highlighted the advantages of using spores and gametophytes of ferns in their experimental research [6] and ecotoxicology in recent years [3, 7]. The most advantages include: (1) the formation of a large number of spores/individual, (2) spores can be preserved in the laboratory and can thus be available throughout a year (3) are small and light, requiring reduced storage space, (4) spore germination and gametophyte differentiation may be obtained and monitored *in vitro* in small recipients, in simple laboratory conditions, and (5) conclusions are relevant for higher plants.

The spore germination process can be completely inhibited or delayed by pesticides in the environment. The glyphosate herbicide in concentrations of 0.48–19.20 mg/l significantly inhibited the macrospores germination process of *Regnellidium diphyllum* aquatic fern. From a total of 1050 megaspores used in the experiment, only 744 germinated [8].

A fungicide containing 50% metal copper applied in a concentration of 0.1–0.3 g/100 ml Knop solution significantly inhibited the spore germination process in *Athyrium filix-femina* and *Polypodium vulgare* ferns. The highest concentration of fungicide in the first species inhibited germination completely [9]. After 50 days of exposure to the fungicide, gametophyte differentiation was delayed compared to the control variant, which was in young cordate prothallium stage. Except for the lowest concentration of fungicide applied to *P. vulgare* in which the gametophyte was in the stage of prothallium lamella, at all the others, the stage of differentiation was prothallium filament. In addition, the filament cells had necroses, and the rhizoids were unelongated. Also *P. vulgare* species had more sinuous and more intensely colored cell walls at the level of rhizoids, [9] compared to the control variant.

A bifenthrin-based insecticide applied in a concentration of 0.01–0.04 ml/100 ml of Knop solution to the two above-mentioned species resulted in a decrease in spore germination rates, and at the highest concentration in both species, the gametophyte was in the form of a three-dimensional cell mass, whereas the gametophyte in the control variant was in cordate prothallium stage [9].

Asplenium scolopendrium spores which were grown *in vitro* on 20% copper metal fungicide containing culture medium showed a decrease in the germination percentage by up to 37.66% compared to the control variant. The species proved more sensitive than *Athyrium filix-femina* when the highest fungicide concentration was applied [10]. Gametophyte differentiation of both species was affected, so after 3 weeks of exposure, the gametophyte was in the stage of prothallium lamella formation for *A. scolopendrium* control variant, respectively, in the stage of young cordate prothallium for *A. filix-femina* control variant. The gametophyte was in the form of germinated spores when the highest concentration of fungicide (0.7%) was applied. Necrosis of prothallic chlorocytes, inhibition of rhizoid elongation, and even the absence of rhizoids have been observed in variants exposed to fungicide.

Acetamiprid-based insecticides (20%) dramatically affect spore germination in *Asplenium scolopendrium* and *Athyrium filix-femina*, the process being reduced by 63.34–100% in the first species, respectively, 41.34–100% in the second. In *A.*

scolopendrium, in the variant exposed to 0.02% insecticide with acetamiprid, the gametophyte did not differentiate, the spores remaining ungerminated, while in *A. filix-femina*, after 6 weeks, the gametophyte was in the stage of three-dimensional cell masses and after 14 weeks in the cordate prothallium stage with archegonia [11].

The evaluation of glyphosate herbicide impact on *Blechnum appendiculatum, Macrothelypteris torresiana*, and *Thelypteris dentata* was made by applying it in concentrations of 0.33, 0.65, 2.72, and 10.89 g (active ingredient) l^{-1} at different stages of the life cycle: spore, gametophyte, and sporophyte [12]. The results indicated almost complete inhibition of the spore germination process, discoloration of the prothallic chlorocytes, as well as the chloroplastic tissues of the young sporophyte. The authors state that the herbicide has a negative impact on spore banks in the soil, resulting in a mortality of 31–50% in all green stages of their life cycle [12].

Also, the fern sporophytes are affected by pesticides. The relative growth rate, the amount of chlorophyll pigments, and the photosynthetic activity of *Azolla microphylla* aquatic fern were also significantly affected by treatment based on endosulfan, an insecticide applied in a concentration of 0–600 ppm. The authors consider the photosynthetic activity and the amount of chlorophyll pigments in order to explain how pesticides act on the photosynthetic mechanism in *Azolla* [13].

Saturn herbicide in *Azolla pinnata* caused the decrease of nitrogen, phosphorus, and potassium uptake in concentrations between 0 and 0.004 ppm applied for 5–25 days, while furadan insecticide in concentrations of 0.001 and 0.002 ppm stimulated uptake of nitrogen, phosphorus, and potassium after 20 and 25 incubation days on the culture medium [14]. Saturn also affected the fresh and dry mass of *Azolla pinnata.*

The paraquat herbicide applied in the culture medium of *Azolla microphylla*, in a concentration of 2–6 μM, resulted in overproduction of reactive oxygen species (ROS) which determined the induction of antioxidant enzyme activity: superoxide dismutase, catalase, guaiacol peroxidase, and peroxidase ascorbate, the species tolerating herbicide toxicity for 72 h [15]. In higher concentrations, this antioxidant defense mechanism is no longer working, and *Azolla* does not survive. Fragmentation and browning of fronds were also observed. In addition to the abovementioned changes, the presence of paraquat in the medium determines the decrease of both chlorophyll and protein amount [15].

The mature sporophyte of *Asplenium scolopendrium*, *Asplenium trichomanes*, *Athyrium filix-femina*, *Blechnum spicant*, *Dryopteris dilatata*, *Phegopteris connectilis*, *Polystichum* aculeatum, and *Woodsia ilvensis* species subjected *in situ* to treatment with asulam herbicide showed important damage, the maximum being recorded 1 year after application [16].

3. Influence of pesticides on histological and biochemical parameters in fish

As a result of penetration into the aquatic environment, pesticides affect a wide range of nontarget organisms such as invertebrates and fish [17]. Aquatic ecosystems are the "final destination" of pesticides used in agriculture [18]. Early life stages are the most sensitive. Many toxicity tests are focused on the study of hatching and the occurrence of deformities in hatched larvae, decreased mobility or even immobility, lack of coordination in swimming movements; for example, deltamethrin in concentrations >0.005 ppb decreases hatching rate in common carp [19]; bifenthrin in concentrations of 50–200 ppb determines the lack of coordination in swimming movements, malformations of axial development in *Danio rerio* species [20].

Acute exposure to pesticide resulted in reduced fish populations and increased mortality [21, 22]. Chronic exposure to small amounts of pesticide increased the

incidence of disease, stress, and behavioral disorders [23]. Pesticide bioaccumulation causes a major danger—bioaccumulation factor of cypermethrin in fish is 1200× [24]. Acute tests of toxicity are used to evaluate pesticide toxicity and get rapid consistent results at least within certain limits, regarding the concentration-response relationship. These tests allow the determination of LC50 values for different periods of time, NOEC values, providing a very rich database on the toxic effect of many pesticides on different fish species.

Considering the large amount of data in the specialized studies on the effects of pesticides on fish, the data in this paper refer in particular to the pesticide groups on which we have conducted our own experiments. Many authors have established concentrations or lethal doses for different pesticides and species. For example, the toxicological values for chlorpyrifos are between 1.3 μg l^{-1} (96-h LC50) for *Lepomis macrochirus* and 2600 μg l^{-1} (72-h LC50 72) for *Gambusia affinis* [25]; LC_{50} in fish is less than 30 μg bifenthrin/l water [26, 27]; for most species, 96-h LC_{50} values for bifenthrin determined by the static method are between 2 and 5 μg l^{-1}, and between 0.5 and 4 μg l^{-1} by the continuous flow method [28]; propiconazole is moderate to slightly toxic for most aquatic organisms—96-h LC50 values for *Cyprinus carpio* 18.9 mg/l, *Oncorhynchus mykiss* 19 mg/l, and 21 mg/l for *Lepomis macrochirus* [29]; 48-h LC50 was 9 mg/l in *Carassius auratus*, 2.2 mg/l in *Oncorhynchus mykiss*, and 4 mg/l in *Cyprinus carpio*; the toxicity of chlorpyrifos in fish is generally between 0.01 and 1 mg/l (96-h LC50): 0.41 mg/l in *Oncorhynchus mykiss* and 0.015 mg/l in *Salmo gairdneri* [30].

As regards their action, pesticides block a certain metabolic process; the practical way in which this is done is sometimes difficult and in many cases is unknown or only partially clarified. The use of high sensitivity physiological indices is a tendency of recent years in aquatic toxicology. The most used physiological method is to determine pesticide action on fish breathing. Specialists can determine the concentrations in which breathing disorders begin to occur by recording the frequency of breathing movements and determining the oxygen consumption of fish kept in sublethal solutions of toxic substances (in state of rest or activity). The fish alters their energy metabolism in terms of spending a greater amount of energy to alleviate toxic stress [31], which results in improved oxygen use under hypoxia and anoxia conditions [32]. Determination and monitoring of oxygen consumption in aquatic organisms can be considered a better method to assess a substance toxicity than doing acute toxicological tests, because it has good results even in low concentrations of toxic substance [33]. One of the early symptoms of fish poisoning is breathing difficulties. Therefore, decreases in oxygen consumption in the gills were found by Cebrián et al. [34], even 24 h after exposure of *Procambarus clarkii* to chlorpyrifos, in the corresponding 96-h LC50 concentration. The frequency of opercular movements initially appears to support physiological activities in the polluted environment and is followed by the decrease in the breathing rate, which may be the result of gill diseases, as seen in the rainbow trout exposed to the action of fenvalerate and cypermethrin by Bradbury et al. [35].

Hematological parameters reflect fish state faster than determining other parameters because they change extremely rapidly under modified environmental conditions. For this reason, they are widely used to describe the state of fish health. Fish-nucleated red cells can be an ideal model to understand the harmful action of pesticides in different cell compartments; mitochondria and the nucleus of fish red blood cells are sensors in the programmed cell death mechanism [36].

Svobodova et al. reported significantly lower values of erythrocyte and hemoglobin in carp after acute exposure to deltamethrin (as a result of changes in hematopoiesis process), while the number of leukocytes did not change significantly [37]. Research carried out by Sopinska and Guz on permethrin poisoned carp revealed a decrease in the number of white blood cells, granulocytes in particular [38].

Anemia resulting from carp exposure to cypermethrin was also reported by Doruncu and Girgin [39].

Jayaprakash and Shettu evaluate the changes in some hematological parameters of the freshwater fish *Channa punctatus* exposed to sublethal concentrations of deltamethrin [40]. This study revealed: decrease in the hemoglobin content, total erythrocyte count, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin concentration, and a significant increase in the total leukocyte count.

Decrease in the number of erythrocytes in carp poisoned with pyrethroid insecticides (permethrin and cypermethrin), due to dysfunction of hematopoiesis [37, 39]. Exposure to Talstar EC 10 in a concentration of 57.5 μg l^{-1} had no effect on the number of erytrocytes in common carp (*Cyprinus carpio*), with not significantly different values compared to those in the control group [41]. Atamanalp et al. [42] reported a significant increase in the number of erythrocytes in *Oncorhynchus mykiss* exposed to cypermethrin. Ahmad et al. observed significant decreases of hemoglobin in trout exposed to a sublethal dose of mancozeb, resulting in decreasing the amount of oxygen in the tissues and a decrease in the energy production of the animals [43]. Similarly, hemoglobin decreased percentually in *Heteropterus fossilis* after 30 days of exposure to deltamethrin [43].

Galloway and Handy studied the effect of organophosphorus pesticides (parathion, chlorpyrifos, malathion, and diazinon) on the immune system in invertebrates, fish, and other vertebrates [44]. Studies on fish have demonstrated an immunosuppressive effect to the action of these pesticides [45].

Physiological stress indicators, such as plasma cortisol and glucose level are commonly used to assess whether fish is stressed and whether the hypothalamo-pituitary-interrenal system works properly [46]. The intensive use of lactic acid in gill oxidation during stress caused by exposure to pesticides is mentioned in the literature [47] and is due to the increase in their energy demand [48] which is only covered by exogenous glucose. Glycogen mobilization is linked to the increase of energy demand; during pesticide-induced stress, a large amount of glycogen can be synthesized and stored in the muscles [49] which may later be in the form of glucose into blood tissues [50].

Due to the fact that fish live in a carbohydrate-deficient but protein-rich environment, they can use lipids and proteins as sources of energy more efficiently than carbohydrates [51]. Anaerobic glycolysis, recycling, and use of lactic acid preserve the energy potential of fish, thus ensuring better adaptation of fish to the polluted environment. Increases in the amount of energy (glucose) associated with lactate production have been reported in fish poisoned with pyrethroids [51].

High levels of lactic acid and strongly stimulated LDH activity in various tissues suggest the great importance of anaerobic glycolysis in adapting fish to stress caused by pyrethroid pesticides [52]. Velisek et al. examined the biochemical profile of carp (*Cyprinus carpio*) after 96 h exposure to bifenthrin in a concentration of 57.5 μg l^{-1} and found significant increases in glucose level [41]. Bálint et al. observed an increase of blood glucose in carp after exposure to deltamethrin [53].

Velisek et al. showed an increase in blood glucose in trout and prussian carp as a result of metabolic stress induced by the action of Talstar 10 EC insecticide [41]. Jee et al. recorded an increase of blood glucose level in *Sebastes schlegeli* fish due to their exposure to cypermethrin (pyrethroid insecticide); researchers also reported a decrease in cholesterol and plasma proteins [54]. Datta and Kaviraj recorded increases in plasma glucose and decrease of liver glycogen in *Clarias gariepinus* exposed to a concentration of 0.005 mg cypermethrin/l [55]. Decrease in blood glucose levels following exposure to thyram was reported in *Salmo gairdneri* species by Van Leeuwen et al. [56].

Acetylcholinesterase is a highly used biomarker in aquatic ecotoxicology research [57], sensitive to low concentrations of organophosphates. Determination of

acetylcholinesterase activity in erythrocytes is used to assess the degree of intoxication with acetylcholinesterase inhibitors. Recovery of acetylcholinesterase activity affected by exposure to organophosphorus pesticides can be carried out by dephosphorilation or AchE synthesis, which is a very slow process in fish; the disappearance of the inhibitory effect on acetylcholinesterase in *Gambusia affinis* exposed to oraganophosphorus compounds occurs within 45–60 days after exposure [58].

Costin et al. found a decrease of the specific activity of catalase, glutathione peroxidase, and glutathione reductase in prussian carps exposed to a sublethal concentration of deltamethrin (2 μg/l water), while glutathione-S-transferase activity increased [59]. Examination of the biochemical profile after 96 h of carp exposure to bifenthrin in a concentration of 57.5 μg l^{-1} resulted in significant increases in glucose, ammonia, aspartate aminotransferase, creatine kinase, and monocytes [41].

Bifenthrin inhibits the production of ATPase [60], which explains the stronger effect of bifenthrin on aquatic organisms compared to terrestrial ones (maintaining the critical concentration of ions against the concentration gradient, in the much diluted aquatic environment, requires intensive ionic transport processes, the necessary energy being supplied by ATPase; the decrease in production of this enzyme leads to the death of organisms). Li et al., following the study of propiconazole action on *Oncorhynchus mykiss*, suggested Na/K-ATPase activity in the fish brain as potential biomarkers for intoxication [61].

Fish gills are the main place of ion exchange with the environment and, at the same time, the main channel of pesticide penetration. They are in constant contact with water, and any change in its composition may affect the gills' permeability and their osmoregulatory functions. Cengiz found histopathological changes in the carp gills following acute exposure to deltamethrin in concentrations of 0.029 and 0.041 mg/l^{-1} (exfoliation, necrosis, edema, hyperplasia, fusion of secondary lamellae, etc.) [62]; similar changes caused by deltamethrin action were identified in *Gambusia affinis* by Cengiz and Unlu [63]. Costin et al. found morphological changes in the carp gills exposed to sublethal concentrations of deltamethrin (2 μg/l water), 48 h after the exposure to 2 μg deltamethrin/l water, which accentuated after 14 days of exposure (longest interval); the author reported hyperemia, fusion of secondary lamellae, epithelial layer damage, and chlorogenic cell proliferation [59].

Fusion of secondary lamellae as a result of exposure to pesticides appears to have a protective role in diminishing the affected gill surface; this response slows down the penetration of toxic and may result in fish choking [64, 65]. The toxic effect of pyrethroids on fish increases with increasing liposolubility [66]; because of their strong lipophilic character, the pyrethroids are well absorbed by the gills, even in low-pesticide waters, thus decreasing the availability of oxygen to the tissues of the internal organs [67].

Histological changes due to exposure to pesticides also occur in other organs. Melo et al. observed hepatic tissue alterations in *Rhamdia quelen* (loss of cell form, piknotic nuclei, necrosis, and increase of hepatocytes loaded with by gallbladder pigment) following the exposure of fish to organophosphates (0.01 ml/l Folidol for 4–72 h) [68]. Examination of liver tissue samples after 96 h of carp exposure to bifenthrin in concentration of 57.5 μg l^{-1} revealed hepatocyte degradation [41]. Cengiz and Unlu found histopathological changes caused by deltamethrin on the liver of *Gambusia affinis* [63]; cypermethrin causes hyperplasia and necrosis of hepatocytes in *Labeo rohita* species [69]. Ali et al. found DNA damage in erythrocytes and gill cells in *Channa punctatus* (Bloch), 5 days after exposure to chlorpyrifos in a concentration of 203 μg/l [70].

Much of the research on toxicological aspects of pesticide poisoning in fish refers to oxidative stress, causing the activity of antioxidant enzymes, proposed as biomarkers of pesticide intoxications [71–73]. It has been demonstrated that

pyrethroid metabolism generates oxygen reactive species in poisoned fish [74]. The occurrence of oxidative stress in different fish tissues as a result of exposure to deltamethrin is supposed to be the main cause of product toxicity [75]. Chlorpyrifos toxicity is also manifested by the induction of oxidative stress [76]. It seems that oxidative stress contributes to the development and severity of syndromes in acute intoxication with these insecticides [77]. Mancozeb stimulated oxidative stress, while the amount of antioxidant serum enzymes decreased significantly; Fabra et al. demonstrated that this fungicide caused significant biochemical changes in cell membranes [78].

Exposure to pesticides may bring about behavioral changes such as: hyperactivity, loss of balance, and impossibility of maintaining the normal position in trout exposed to cypermethrin [79]. Symptoms of fish exposed to pyrethroid pesticides are: swimming near water surface, hyperactivity, loss of balance, increase of branchial mucus secretion, etc. Levine et al. found a decrease of swimming intensity in *Danio rerio* due to exposure to chlorpyrifos in a concentration of only 100 mg/l [80].

Toxicity of different pesticides on fish and other aquatic organisms can be influenced by various factors such as: pH, temperature, dissolved oxygen, water hardness, etc. Mauck et al. found that bifenthrin is more toxic at low temperatures, and its toxicity is only slightly influenced by water hardness [81]. Macek et al. recorded 96-h LC50 values of 7.1, 15, and 51 μg l^{-1} in *Oncorhynchus mykiss* at temperatures of 12.7, 7.2, and 1.6°C [82].

Although the data on histochemical, biochemical, hematological, and behavioral changes in poisoned fish are quite numerous, they are little related to *Carassius gibelio, Perca fluviatilis*, and *Alburnus alburnus* species which are not commonly used in toxicological tests. In toxicology research, the emphasis is placed on biochemical, chemical, structural, and ultrastructural changes, disregarding the physiological processes which are part of the primary response of organisms to stressors.

As a contribution to the study of pesticide effect on fish, we will briefly present some of our results for these species exposed to the action of six pesticides—3 insecticides (Talstar One—in concentrations of 0.05, 0.1, 0.2 and 0.4 μl bifenthrin/l water, Reldan 40 EC—0.4, 0.8, 1.6, and 3.2 mg chlorpyrifos/l water, and Actara 25 WG—0.016, 0.032, 0.064 and 0.128 mg thiamethoxam/water) and 3 fungicides (Tilt 250 EC—1.06, 2.12, 4.24, and 3.2 mg chlorpyrifos/l water, Dithane M-45—2, 4, 8, and 16 mg mancozeb/l water, and Tiradin 70 PUS—0.007, 0.014, 0.028, and 0.56 mg thiram/l water).

The analyzed pesticides reduce energy metabolism and breathing rhythm in crucian carp, bass, and bleak [83–92]. In many of the variants, the immediate response to the toxic action is a breathing rate stimulation of variable duration, in inverse ratio to the pesticide concentration, with high decrease in oxygen consumption, followed by the "stabilization phase" to the new conditions, with small fluctuations of the mentioned indices around an average value [85–92]. The advantage of determining energy metabolism and breathing rate is the rapidity of this response to the action of the stressor (pesticide) on the one hand and their noninvasive character on the other.

The number of erythrocytes increases after exposure to Reldan 40 EC, Tilt 250 EC, and Tiradin 70 PUS [86–89, 92]; decreases of this hematologic parameter are recorded under the action of Actara 25 WG and Talstar One [84, 90, 92]. Blood glucose levels in fish increase for 2 weeks under the action of Talstar One, Actara 25 WG, and Tilt 250 EC [84–86, 90, 92]. Dithane M-45 fungicide and Reldan 40 EC insecticide have a hypoglycemic effect [83, 86, 92]. Tiradin does not change blood glucose level in crucian carp and bleak intoxicated with a concentration of 0.02 ml/l for 2 weeks [91, 92].

Behavioral changes occur in crucian carp and perch exposed to pesticides: hyperactivity in the first 24–48 h, disordered movements, frequent rising at the

surface of water in the first hours after exposure to toxic, and apathy under the action of insecticides and fungicides [85–92]. Increases in mucus production occur especially in crucian carp under the action of fungicides [83, 87–89, 91, 92].

There are different results as regards the sensitivity of the species: the prussian carp was more sensitive under the action of Reldan 40 EC and Actara 25 WG [86, 92] as well as Tiradin 70 PUS and Tilt 250 EC fungicides [87, 88, 91, 92]; the bleak was more sensitive to Talstar One and Dithane M-45 [92]. In some experimental variants, there were no significant differences in the reactivity of the species under the action of the six pesticides.

Among the physiological parameters, blood glucose values and the number of red blood cells give a clear signal of fish stress, which is why we recommend using these parameters as biomarkers for pesticide-induced toxic stress. The study of oxygen consumption and breathing rate combined with the number of erythrocytes and blood glucose as well as the main behavioral changes allow the formation of a symptomatological picture of fish poisoned with pesticides.

4. Influence of pesticides on histological and biochemical parameters in amphibians

The impact of pollutants on humans in their environment is particularly complex and explains the lack of action or late and often confusing reactions regarding the protection measures. The degree of exposure depends on the simultaneous presence of some essential factors (nature and concentration of pollutants) and modifiers that ultimately influence the absorbed quantity.

Amphibians are some of the most representative species of vertebrates in aquatic and agricultural ecosystems, because they are the natural enemies of many pests of crop plants. Due to their high sensitivity to the changes occurring in their natural environment and because their larval development occurs exclusively in the aquatic environment, amphibians have been considered bioindicators for the aquatic and agricultural ecosystems [93–96] and have been used as test specimens to study the action of different chemicals in these ecosystems.

Bridges [97] conducted numerous studies on the effects of long-term exposure to pesticides. The presence of contaminants in the environment may alter the predator-prey interaction among aquatic species by changing the predator or prey levels of activity or behavioral change of the predator. The effect of predator-prey meeting may be dependent on the fact that both species are exposed simultaneously to a contaminant/pollutant or exposure occurs only to one of the two species.

Due to the short period of exposure to nonpersistent pollutants, it is important to examine the long-term effects of short-term exposure on amphibians and the sensitivities at a certain stage in their development cycle. Any delay in metamorphosis or any decrease in size during metamorphosis may have an impact on the evolution of the amphibian populations, leading to its decline or local extinction.

Sampath et al. [98] studied the toxic effect of two pesticides (carbaryl and methyl-parathion) on the excretory system of *Rana tigrina* tadpoles and showed an increase in the rate of N-urea and NH3-N elimination in intoxicated specimens depending on the concentration of pesticide. Short-term exposure (96 h) to action in low doses of endosulfan organochlorine insecticide caused changes in the growth of *Litoria freycinetti* tadpoles either immediately or on a long term [99]. Tadpoles exposed to toxic action grew more slowly, and those who survived were more easily captured by predators in their natural environment.

Tomizawa and Casida [100, 101] demonstrated that neonicotinoid insecticides acted selectively on the nicotine-acetylcholine receptor in insects and mammals,

making them the safest insecticides (as a mode of action). They described the mechanism of selective toxicity of neonicotinoid insecticides.

Gendron et al. [102] published the conclusions of a study on the effects of the leopard frogs' exposure to a mixture of pesticides. The hypothesis shows that the exposure of *Rana pipiens* leopard frogs to agricultural pesticides can affect the dynamics of the parasitic worm infections, *Rhabdias ranae*. The pesticide treatment did not influence the growth of the parasites, the results indicating that they got matured and reproduced earlier in frogs exposed to pesticides, compared to control specimens. Such alternations in developmental cycle characteristics that increase the transmission of parasites may lead to an increase in virulence. The results contribute to further discussion on the role that anthropogenic factors might have in the gradual death of amphibians due to complications arising from the disease observed in different parts of the world.

Greulich and Pflugmacher [103] suggested a number of factors for the recent decrease in amphibian populations, one of them being exposure to pesticides. Specialists studied the absorption and effects of the pyrethroid cypermethrin insecticide in relevant concentrations from the environment for two different amphibian species, *Bombina variegata* and *Rana arvalis*, with the observation that cypermethrin absorption caused deformities, abnormal behaviour, and mortality. These changes depended on the dose of pesticide.

Some studies show that glyphosate has a harmful effect on the environment, especially on amphibians. Howe et al. [104] studied the effect of glyphosate on four North American amphibian species: *Rana clamitans*, *R. pipiens, R. sylvatica*, and *Bufo americanus*, and observed the following aspects: decrease of the anteroposterior diameter of the body, increased time for metamorphosis, tail malformations in tadpoles, and gonadal disorders. These effects may partly appear as a result of changes in the hormone level. There was a high level of transcription for ARNm segments encoding β-receptor synthesis for thyroid hormones, following exposure of individuals to solutions containing glyphosate.

Chen et al. [105] conducted studies on the effect of glyphosate on fauna in wetlands, having the zooplankton, *Simocephalus vetulus*, and *Rana pipiens* tadpoles as research subject. Significant effects of the pesticide action on the two species were determined at concentrations lower than those expected in the environment. Increased pH values (7.5) also showed the toxic effects of the pesticide on the two species under study, although the reproduction rate for *S. vetulus* improved to a pH level above 5.5 in the absence of the pesticide. The stress caused by the lack of food, associated with a pH of 5.5, decreased the survival rate of *S. vetulus.*

Relya [106] studied how four commercial compounds (diazinon, carbaryl, malathion, and glyphosate) affected the survival and larval growth rate of five species of amphibians (*Rana pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolo*). The combination of pesticides has occasionally resulted in lower survival rates and development than those determined by each pesticide, but never lower than those caused by the worse of the two. This suggests that the combination of the four pesticides had the same effect as the estimated total concentration of pesticides on the ecosystem.

The toxicity of pesticides in general and their genotoxicity on nontarget organisms in particular was of special interest to researchers. Shaolong et al. [107] studied the toxicity and genotoxicity of two pesticides (imidacloprid and RH-5849), used in China since 1992, for two species of amphibians. RH-5849 insecticide did not prove to be toxic to tadpoles even if they were kept for 96 h in a saturated solution. Two techniques were used to study the genotoxicity of the two insecticides: micronucleus test and single cell gel electrophoresis. These tests showed significant changes in the DNA of amphibian erythrocytes.

Relya [108] studied six North American species of amphibian larvae (*Rana sylvatica*, *R. pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*) to investigate the long-term effects of Roundup, many of the existing studies focusing on short-term effects (1–4 days). He also studied the effects of Roundup's association with other sources of stress, such as predators, on the tadpoles' survival rate during 16 days with and without chemical signals from predatory salamanders (*Notophthalmus viridescens*).

The values range from 0.55 to 2.52 mg of active ingredient (IA)/L, considerably lower than those used in previous studies. Stress increased by predators made Roundup two times more toxic to one of the six species (*R. sylvatica*). This finding suggests that the synergistic action of pesticides and predators may be a general phenomenon for amphibians (the range of pesticides can be very wide). Based on this research, pesticides such as Roundup certainly play a significant role in the decline of amphibian populations around the globe.

Relyea et al. [109] also studied the effect of pesticides (glyphosate and malation) on the natural environment of amphibians in the presence of zooplankton and phytoplankton (algae). Three species of amphibian larvae (*Hyla versicolor, Bufo americanus,* and *Rana pipiens*) were studied and combined with their predators (no predators with *Notophthalmus viridescens* and *Dytiscus sp*. Larvae) and pesticides (no pesticides, with malathion insecticide and Roundup herbicide). Roundup proved to have a negative effect on tadpoles, reducing their biomass by 40%, with no indirect effects on the amphibian community through predators or abundance of algae. Malathion in a concentration of 0.3 mg/l caused the number of tadpoles to decrease. This insecticide associated with the tritone in the amphibian environment does not have significant effects; the presence of beetle larvae associated with malathion has a positive effect, which determines the increase in the survival rate of the tadpoles in parallel with the decrease in the number of predators. While high concentrations of malathion can cause the death of amphibian larvae, the small concentrations may have positive effects as beetle larvae, and the predators of amphibian larvae are killed. These data lead us to the conclusion that pesticides can have direct and indirect effects on natural amphibian communities.

Toxicological investigations on amphibian larvae have mainly focused on the effects of heavy metals and pesticides on their growth, development, and behaviour, and only a few data refer to the bioaccumulation of toxins under natural conditions. Hofer et al. [110] studied the accumulation of inorganic (Pb and Cd) and organic (organochlorine and polyaromatic hydrocarbon pesticides) toxic in the body of *Rana temporaria* tadpoles at various altitudes in the Alps. They found an increase in the accumulation of Pb and Cd in the body of tadpoles in low pH waters, with a high concentration of metals. The amount of organochlorine substances was relatively low due to the age of the tadpoles (about 2 months) and the absence of lipid deposits necessary for the absorption of these substances.

Seifert and Stollberg [111] investigated the interaction of the neonicotinoid imidacloprid insecticide with the nicotine-acetylcholine receptor (nAcChR) in the frog embryonic muscle cells. The response of the muscle cell to the action of acetylcholine, nicotine, and imidacloprid was recorded as a contraction. The contractions of acetylcholine or nicotine are inhibited by α-bungarotoxin. Imidacloprid does not lead to the contraction of the muscle cell, but it can attenuate the contractions produced by acetylcholine or nicotine. They have found that imidacloprid is an antagonist of nAcChR receptor in the muscle cell and an agonist in its toxic action on insect nerve receptors.

Honda et al. [112] studied how to activate or inactivate 11 nicotinic insecticides in a two-step system of metabolic coupling and receptor binding. The authors incubated neonicotinoid insecticides with CYP3A4 and NADPH or AOX on a

cosubstrate of N-methyl-nicotinamide for metabolism. They used ketoconazole or menadione for inhibition of subsequent conversions. They also used *Drosophila* nAChR receptor and [^{3}H] imidacloprid or α4β2 and [^{3}H](−)-nicotine receptor to determine changes in the action of neonicotinoid insecticides. *Drosophila* nAChR receptor coupled with CYP3A4 system activates imidacloprid and thiamethoxam, while the other nine insecticides do not undergo any change in their toxic potential. AOX system coupled with *Drosophila* nAChR receptor strongly inactivates clothianidin, dinotefuran, imidacloprid, desmethyl-thiamethoxam, and thiamethoxam has a low inactivation effect on nitenpiram and nithiazines but has no effect on the other four insecticides studied.

Neurotoxicity of pesticides has been studied by many researchers on the sciatic nerve of *Rana ridibunda*. Zafeiridou et al. [113] studied the influence of three herbicides: acetochlor, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4-dichlorophenoxyacetic acid, on the value of the action potential of the sciatic nerve in *Rana ridibunda.* Since the action of 2,4-D is pH-dependent, the toxicity of the three chemical compounds has been studied at a pH of 3.3. It has been found that a low pH increases the toxicity of 2,4,5-T and 2,4-D pesticides and decreases acetochlor toxicity.

Casco et al. [114] demonstrated high toxicity of cypermethrin in *Bufo arenarum* larvae. They showed that low doses of cypermethrin, lower than those used in agriculture, can cause massive apoptosis in the central nervous system of the tadpoles through a neurotoxic mechanism.

In 2006, Relya [115] published an article on the effects of pesticides, pH, and stress due to predation on amphibians. It was shown that pH and stress due to predation and a single application of the insecticide (carbaryl) affected the survival and growth of *Rana catesbein* larvae and *Rana clamitans* green frogs under natural conditions. Decreasing the pH had no effect on the survival rate but caused a faster growth of tadpoles. Low concentrations of carbaryl did not affect the two species, but higher concentrations resulted in a lower survival rate and higher increases in individuals of *Rana catesbein* species. The stress caused by predation and low pH did not increase herbicide lethality due to its rapid rate of degradation under natural environmental conditions. Even if stress, pH, or predators could make carbaryl more lethal under laboratory conditions, after repeated insecticide applications, these stress factors did not interact under natural conditions even if a single carbaryl application was used.

The application of auxin herbicides of 2,4-dichlorophenoxyacetic acid (2,4-D) type in deciduous forests is mainly used in reafforested areas. The absorption of this herbicide in the target plants is reduced due to its low solubility in water. Because of this, the acid is esterified to increase its liposolubility and to diffuse into the plant-bearing vessels. But the esterification of auxin herbicides increases their absorption in other organisms, such as amphibians [116].

Numerous researches have shown that the gelatinous shell of amphibian embryos has a protective role against various environmental pollutants. Edginton et al. [117] studied the rate of absorption, metabolism, and excretion of butoxyethyl ester for 2,4-dichlorophenoxyacetic acid (2,4-D BEE) in *Xenopus laevis* embryos. They found that embryos with a gelatinous coating had a low absorption of toxic compared to embryos without the coating after 8 h from exposure. Metabolism of 2,4-D BEE lasted between 35 and 42 min, and the accumulation of radioactive residue was 35% located in the gelatinous shell and 65% in the embryo.

Vonesh and Buck [118] have shown that pesticides influence the process of laying eggs in amphibians. Four experimental batches were used to examine the effect of the pesticide marketed under the name of Sevin® and the active substance, carbaryl, on the method of laying eggs in *Hyla chrysoscelis* species. Their results have shown that unpolluted ponds are preferred by amphibians for laying eggs, while

the presence of Sevin® and carbaryl in the environment reduces the process, while volatile chemicals have no influence at all.

Gurushankara et al. [119] investigated the effect of malathion in various concentrations on the survival, growth, and food consumption in *Limnonectus limnocharis*. Exposure of tadpoles to malathion changed all parameters. Thus, there was a decrease in survival rate, body size in parallel with the increase in malathion concentration in the environment as well as a reduction in the amount of food consumed.

Bioaccumulation of malathion and its impact on *Ambystoma tigrinum* was studied by Henson-Ramsey et al. [120]. The toxic was administered by two methods: soil contamination with malathion in a concentration of 50 and 100 μg/cm^2 and food—the administration of earthworms that lived on a malathion-contaminated soil in a concentration of 200 μg/cm^2.

The amount of xenobiotic substances was determined by gas chromatography in liver, muscle, skin, and bones. Malathion was higher in muscle, skin, and bones after 1 day of exposure and in viscera, after 2 days of exposure. To determine the biological impact of malathion on *Ambystoma tigrinum*, cholinesterase activity was measured in the brain, with a reduction in its activity. Animals exposed to toxic action did not show clinical signs of toxicosis.

Aquatic organisms are exposed to repeated pesticide applications over time. Repeated and long-term (79 days) administration of low concentrations of malathion (10–250 μg/l)—the most widely used insecticide, affects the entire aquatic community of zooplankton, phytoplankton, periphyton, and amphibian larvae [121]. This caused the decline of zooplankton followed by an increase in the biomass of the phytoplankton and the decline of periphyton after repeated treatments. The decline of the periphyton has had little influence on the development of *Rana sylvatica* species because it has a short time of metamorphosis, but has strongly influenced *Rana pipiens* larvae which have a longer time of metamorphosis with a high mortality level. In conclusion, malathion did not directly kill amphibian larvae but caused changes in the trophic chain and caused their mortality in an indirect way.

Toxicity caused by endosulfan (an organochlorine chemical compound used as an insecticide in vegetable, fruit, cotton, and coffee) was studied on *Bombina orientalis*, by Kang et al. [122]. They found a decrease in the survival rate of the tadpoles in parallel with an increase in the toxic concentration. The surviving tadpoles showed morphological changes consisting of tail dysplasia, presence of vesicles in the pectoral and ventral side, tail bending, and cephalic dysplasia; the higher the toxic concentration, the more significant the morphological changes.

Similar changes have also been reported in intoxication of larvae and *Bombina orientalis* tadpoles with molinate, a thiocarbamate used as an herbicide [123]. The change in survival rate was not significant until the blastula stage. A significant decrease of this parameter was observed in the tadpole stage even in low doses of molinate. The surviving tadpoles showed many malformations: trunk and tail bending, tail and eye dysplasia, and cephalic dysplasia. All these data suggest that the molinate herbicide influenced the larval development of this species.

Relyea and Jones [124] set LC50 after 96 h for glyphosate (Roundup) for various North American amphibian species. There were used nine species of larval anura Groser 25 belonging to *Raniidae*, *Bufonidae*, and *Hylidae* families, as well as four larval urodeles from two families: *Salamandridae* and *Ambystomatidae*. LC50 was between 0.8 and 2 mg acid/l for the nine anura species after 96 h and ranged from 2.7 to 3.2 mg acid/l for the four larval urodele. This work brings new data on the sensitivity of amphibians to the action of glyphosate.

Endosulfan insecticide appears to be highly toxic in low doses for amphibian populations and also exhibits long-term toxic effects. Toxicological studies

conducted by Jones et al. [125] established LC50 in 4 days (LC504-d) for endosulfan insecticide on tadpoles of nine amphibian species belonging to three families (*Bufonidae*, *Hylidae*, and *Ranidae*). LC504-d value was between 1.3 and 120 ppb, which places it in the category of highly toxic pesticides. At the end of the treatment, experimental animals were kept in clean water for 4 more days. There has been a significant increase in mortality depending on the family. Bufonides were the least sensitive, hylides were moderately sensitive, and ranidae were the most sensitive.

Atrazine is a widely used herbicide for weed control in corn crops. Its residues could be detected in the soil 1 year after application. Numerous studies have tracked the effect of atrazine on embryonic development of amphibians in the environment and reported its ability to induce an enzyme—aromatase, which appears to convert testosterone to estrogen [126–132].

Some studies followed the effect of atrazine on juvenile and adult amphibians. Storrs et al. [133] followed the effect of atrazine administered directly on the soil on the behaviour of *Bufo americanus* species. They also studied various ways to absorb, accumulate, and eliminate this toxic from the body using ^{14}C-labeled atrazine. Their results have shown that amphibians do not avoid atrazine-enriched soils. This pesticide is rapidly absorbed through the skin, and accumulated in the intestine, especially in the gallbladder.

Small quantities of herbicides used in agriculture often affect the surface of water and can become a stress factor for aquatic organisms. Williams and Semlitsch [134] studied the effect of 4 herbicides (the active substance atrazine, S-metolachlor and glyphosate) on the amphibian larvae of 3 species: *Bufo americanus*, *Pseudacris triseriata* and *Hyla versicolor*. The glyphosate under the trade name Roundup WeatherMax (containing 572 ppb equivalent glyphosate acid) determines the increase in mortality of *Pseudacris triseriata* tadpoles, while Roundup OriginalMax (containing 572 ppb equivalent glyphosate acid) determines the extension of the larval period in *Bufo americanus* species.

Chronic exposure to atrazine and S-metolachlor has no side effects on survival, metamorphosis, or larval period in the organisms. These results show the importance of adjuvants in the preparation of pesticides and their different effects on organisms even in the case of similar products.

Budischak et al. [135] followed how some parasites of amphibians can influence the toxicity of some pesticides. They used two batches of *Rana palustris*: tadpoles infected with *Echinostoma trivolvis* trematode, then intoxicated with malathion and uninfected tadpoles, and intoxicated with the same dose of malathion. It has been shown that the parasite does not influence susceptibility to pesticide action.

The influence of six pesticides (Reldan 40EC, Actara 25WG, Tilt 250EC, Champion 50WG, Fusilade Forte, and Dual Gold 960EC) on physiological indices of *Pelophylax ridibundus* was studied by Paunescu et al. [136]. The chronic pesticides exposure can lead to alteration of various indices (increased hepatosomatic index, decrease in erythrocytes number, leucopenia, hyperglycaemia, and decrease in cholesterol levels), as well as to hepatic lesion.

The fact that some species of amphibians survive in agroecosystems is due to their special plasticity. Thus, irrigation canals represent new habitats that amphibians colonize, and in the absence of predators, which are less mobile (fish), they become metapopulations of altered landscapes [137–139].

5. Conclusions

As nontarget species for pesticides in the environment, ferns can be used in their gametophyte stage, young or mature sporophyte in different biotests to evaluate

the risk associated with these substances. The biochemical, hemathological, and histopathological changes recorded in both fish and amphibians can be considered biomarkers of pesticide pollution.

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References

[1] Sharpe JM, Mehltretter K, Walker LR. Ecological importance of ferns. In: Mehltretter K, Walker LR, Sharpe JM, editors. Fern Ecology. Cambridge: Cambridge University Press; 2010. pp. 1-21. DOI: 10.1017/CBO9780511844898.002

[2] Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG. Fern classification. In: Ranker TA, Haufler CH, editors. Biology and Evolution of Ferns and Lycophytes. Cambridge: Cambridge University Press; 2008. pp. 417-467

[3] Rodríguez-Romero AJ, Sedeño-Díaz JE, López-López E, Esteban M, Quintanilla LG, Catalá M. Recent advances in the use of mitochondrial activity of fern spores for the evaluation of acute toxicity. In: Fernándes H, editor. Current Advances in Fern Research. Cham: Springer International Publishing; 2018. pp. 481-498. DOI: 10.1007/978-3-319-75103-0

[4] García-Cortés H, Catalá M, Rodríguez Gil JL. Update on the assessment of chronic phytotoxicity using fern spore biomarkers. In: Fernándes H, editor. Current Advances in Fern Research. Cham: Springer International Publishing; 2018. pp. 499-515. DOI: 10.1007/978-3-319-75103-0

[5] Ehrendorfer F. Pteridophyta. In: Sitte P, Ziegler H, Ehrendorfer F, Bresinsky A, editors. Strasburger—Lehrbuch der Botanik. 34 Auflage ed. Berlin: Spektrum Akademischer Verlag Heidelberg; 1999. pp. 652-684

[6] Banks JA. Gametophyte development in ferns. Annual Review of Plant Physiology and Plant Molecular Biology. 1999;**50**:163-186. DOI: 1040-2519/99/0601-0163$08.00

[7] Catalá M, Esteban M, Rodríguez-Gil JL, Quintanilla LG. Development of a naturally miniaturised testing method based on the mitochondrial activity of fern spores: A new higher plant bioassay. Chemosphere. 2009;**77**:983-988. DOI: 10.1007/s10646-011-0790-2

[8] Droste A, Brizola Cassanego MB, Windisch PG. Germination and sporophytic development of *Regnellidium diphyllum* Lindm. (Marsileaceae) in the presence of a glyphosate-based herbicide. Revista Brasileira de Biociências. 2010;**8**:174-178

[9] Soare LC, Dobrescu CM, Popescu M, Boeru AG. The effects of some pesticides on spore germination and gametophyte differentiation in *Athyrium filix-femina* (L.) Roth. and *Polypodium vulgare* L. Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 2013;**41**: 458-462. DOI: 10.15835/nbha4129088

[10] Soare LC, Dobrescu CM, Popescu AG. The influence of a copper-containing funcicide on the gametophyte of some non-target pteridophyte species. Acta Horti Botanici Bucurestiensis. 2013;**40**:49-56. DOI: 10.2478/ahbb-2013-0006

[11] Soare LC, Dobrescu CM, Burtescu L. Research on the influence of two insecticides on the gametophyte of some leptosporangiate pteridophytes. Analele Ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi, Biologie Vegetală. 2013;**59**:5-12

[12] Aguilar-Dorantes K, Mehltreter K, Mata-Rosas M, Vibrans H, Esqueda-Esquivel V. Glyphosate susceptibility of different life stages of three fern species. American Fern Journal. 2015;**105**:131-144. DOI: 10.1640/0002-8444-105.3.131

[13] Raja W, Rathaur P, John SA, Ramteke PW. Endosulfan induced changes in growth rate, pigment composition and photosynthetic activity of mosquito fern *Azolla microphylla*.

Journal of Stress Physiology & Biochemistry. 2012;**8**:98-109

[14] El-Shahate RM, El-Araby MMI, Eweda EW, El-Berashi MN. Evaluation of the effect of three different pesticides on *Azolla pinnata* growth and NPK uptake. Journal of American Science. 2011;**7**:1020-1031

[15] Sooda A, Pabbib S, Uniyala PL. Effects of paraquat on lipid peroxidation and antioxidant enzymes in aquatic fern *Azolla microphylla*. Russian Journal of Plant Physiology. 2011;**58**:667-673. DOI: 10.1134/S1021443711040170

[16] Rowntree JK, Sheffield E. Effects of asulam spraying on non-target ferns. Canadian Journal of Botany. 2005;**83**:1622-1629. DOI: 10.1139/b05-127

[17] Otludil B, Cengiz EI, Yildirim MZ, Unver O, Unlu E. The effect of endosulfan on the great ramshorn snail *Planorbarius corneus* (Gastropoda: Pulmonata): A histological study. Chemosphere. 2004;**56**:707-716. DOI: 10.1016/j.chemosphere.2004.04.027

[18] Wang C, Lu G, Cui J, Wang P. Sub-lethal effects of pesticide mixtures on selected biomarkers of *Carassius auratus*. Environmental Toxicology and Pharmacology. 2009;**28**:414-419. DOI: 10.1016/j.etap.2009.07.005

[19] Köprücü K, Aydın R. The toxic effects of pyrethroid deltamethrin on the common carp (*Cyprinus carpio* L.) embryos and larvae. Pesticide Biochemistry and Physiology. 2004;**80**:47-53. DOI: 10.1016/pestbp.2004.05.004

[20] Jin M, Zhang X, Wang L, Huang C, Zhang Y, Zhao M. Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. Aquatic Toxicology. 2009;**95**:347-354. DOI: 10.1016/j.aquatox.2009.10.003

[21] Fulton MH, Moore DW, Wirth EF, Chandler GT, Key PB, Daugomah JW, et al. Assessment of risk reduction strategies for the management of agricultural nonpoint source pesticide runoff in estuarine ecosystems. Toxicology and Industrial Health. 1999;**15**:201-214. DOI: 10.1177/074823379901500118

[22] Grant IF. Insecticides for tsetse and trypanosomiasis control: Is the environmental risk acceptable? Trends in Parasitology. 2001;**17**(1):10-14. DOI: 10.1016/S1471-4922(00)01848-1

[23] Austin B. The effects of pollution on fish health. Journal of Applied Microbiology Symposium Supplement. 1999;**85**:234S-242S. DOI: 10.1111/j.1365-2672.1998.tb05303.x

[24] U.S. Environmental Protection Agency. Pesticide Fact Sheet Number 199: Cypermethrin. Washington, D.C.: Office of Pesticides and Toxic Substances; 1989

[25] Davey RB, Meisch MV, Carter FL. Toxicity of five ricefield pesticides to the mosquitofish, *Gambusia affinis*, and green sunfish, *Lepomis cyanellus*, under laboratory and field condition in Arkansas. Environmental Entomology. 1976;**5**:1053-1056. DOI: 10.1093/ee/5.6.1053

[26] Velisek J, Dobsikova R, Svobodova Z, Modra H, Luskova V. Effect of deltamethrin on the biochemical profile of common carp (*Cyprinus carpio* L.). Bulletin of Environmental Contamination and Toxicology. 2006;**76**:992-998. DOI: 10.1007/s00128-006-1016-9

[27] Velisek J, Jurcikova J, Dobsikova R, Svobodova Z, Piackova V, Machova J, et al. Effects of deltamethrin on rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Pharmacology. 2007;**23**:297-301. DOI: 10.1016/j.etap.2006.11.006

[28] Clark JR, Partick JM, Middaugh DP Jr, Moore JC. Relative sensitivity of six estuarine fishes to carbophenothion, chlorpyrifos, and fenvalerate. Ecotoxicology and Environmental Safety. 1985;**10**:382-390. DOI: 10.1016/0147-6513(85)90083-1

[29] Envirofacts. Cyproconazole The Active Ingredient in Alto® One of the Active Ingredients in Quadris Xtra®. Vol. 2. Greensboro, NC: Syngenta Crop Protection Inc; 2005. pp. 7419-8300

[30] Van Wijngaarden R, Leeuwangh P, Lucassen WG, Romijn K, Ronday R, Velde R, et al. Acute toxicity of chlorpyrifos to fish, a newt, and aquatic invertebrates. Bulletin of Environmental Contamination and Toxicology. 1993;**51**:706-712. DOI: 10.1007/BF00201650

[31] Ferrando MD, Andreu-Moliner E. Lindane induced changes in carbohydrate metabolism in *Anguilla anguilla*. Comparative Biochemistry and Physiology. 1992;**74B**:750-799. DOI: 10.1016/0742-8413(92)90300-V

[32] McCormic SD, Moyes SD, Ballantynes JS. Influence of salinity on the energitics of gill and kidney of Atlantic salmon (*Salmo salar*). Fish Physiology and Biochemistry. 1989;**6**: 243-254. DOI: 10.1007/BFO1875027

[33] Bakthavathasalam R. Effects of lindane and carbofuran on the lactic acid levels of the fish, *Anabas testudineus* at submerged condition and on exposure to air. Environment and Ecology. 1988;**6**:32-37

[34] Cebrián C, Andreu-Moliner ES, Fernández-Casalderrey AS, Feraldo MD. Acute toxicity and oxygene consumption in the gills of *Procambarus clarkia* in relation to chlorpyrifos exposure. Bulletin of Environmental Contamination and Toxicology. 1992;**49**:145-149. DOI: 10.1007/BF00193353

[35] Bradbury SP, Coats JR, McKim JM. Toxicokinetics of fenvalerate in rainbow trout *Salmo gairdneri*. Environmental Toxicology and Chemistry. 1986;**5**:567-576. DOI: 10.1002/etc.5620050609

[36] Bratosin D, Estaquier J, Slomianny C, Tissier JP, Quatannes B, Bulai T, et al. On the evolution of erythrocyte programmed cell death: Apoptosis of *Rana esculenta* nucleated red blood cells involves cysteine proteinase activation and mithochondrion permeabilization. Biochimie. 2004;**86**(3):183-193. DOI: 10.1016/j.biochi.2004.03.003

[37] Svobodova Z, Luskova V, Drastichova J, Svoboda M, Zlabek V. Effect of deltamethrin on haematological indices of common carp (*Cyprinus carpio* L.). Acta Veterinaria Brno. 2003;**72**:79-85. DOI: 10.2754/avb200372010079

[38] Sopinska A, Guz L. Influence of permethrin on phagocytic activity of carp. Medycyna Weterynaryjn. 1998;**54**:126-128

[39] Dorucu M, Girgin A. The effects of cypermethrin on some haematological parameters of *Cyprinus carpio*. Aquaculture International. 2001;**9**: 183-187. DOI: 10.1023/A:1014297528028

[40] Jayaprakash C, Shettu N. Changes in the hematology of the freshwater fish, *Channa punctatus* (Bloch) exposed to the toxicity of deltamethrin. Journal of Chemical and Pharmaceutical Research. 2013;**5**(6):178-183

[41] Velisek J, Svobodova ĆZ, Machova ĆJ. Effects of bifenthrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Fish Physiology and Biochemistry. 2009;**35**:583-590. DOI: 10.1007/s10695-008-9258-6

[42] Atamanalp M, Yanýk T, Haliloûlu I, Aras MS. Alterations

in the hematological parameters of rainbow trout (*Oncorhynchus mykiss*) exposed to cypermethrin. Israeli Journal of Aquaculture = Bamidgeh. 2002;**54**:99-103. DOI: http://hdl.handle.net/10524/19056

[43] Ahmad F, Ali SS, Shakoori AR. Sublethal effects of Danitol (Fenpropathrin), a synthetic pyrethroid, on freshwater Chinese grass carp, *Ctenopharyngodon idella*. Folia Biologica (Kraków). 1995;**43**:151-159

[44] Galloway T, Handy R. Immunotoxicity of organophosphorous pesticides. Ecotoxicology. 2003;**12**:345-363. DOI: 10.1023/A:1022579416322

[45] Beaman JR, Finch R, Gardner H, Hoffmann F, Rosencrance A, Zelikoff JT. Mammalian immunoassays for predicting the toxicity of malathion in a laboratory fish model. Journal of Toxicology and Environmental Health. 1999;**56**:523-542. DOI: 10.1080/00984109909350175

[46] Hontela A. Interrenal dysfunction in fish from contaminated sites: *In vivo* and *in vitro* assessment. Environmental Toxicology and Chemistry. 1998;**17**: 44-48. DOI: 10.1002/etc.5620170107

[47] Tang Y, Boutilier PG. White muscle intracellular acid-base and lactate status following exhaustive exercise: A comparison between freshwater and sea water adapted rainbow trout. Journal of Experimental Biology. 1991;**156**:153-171

[48] Perry SF, Walsh PJ. Metabolism of isolated fish gill cells: Contribution of epithelial chloride cells. Journal of Experimental Biology. 1989;**144**:507-520

[49] Schulte PM, Mayes CD, Hochachka PW. Intergrating metabolic pathways in post exercise recovery of white muscle. Journal of Experimental Biology. 1992;**166**:181-1854

[50] Weber JM. Pathways for oxidative fuel provision to working muscles. Ecological consequences of maximal supply limitations. Experientia. 1992;**48**:551-564. DOI: 10.1007/BF01920239

[51] Rojik I, Nemso K, Borose L. Morphological and biochemical studies on the liver, kidney, and gill of fishes affected by pesticides. Acta Biologica Hungarica. 1983;**34**(1):81-92

[52] Ganathy V, Reddy SD, Reddy SL, Shankaraiah V. Effect of hexachlorocyclohexane on biochemical composition of the fish *Channa punctatus*. Journal of Ecotoxicology and Environmental Monitoring. 1994;**4**(1):15-20

[53] Bálint T, Szegletes T, Szegletes Z, Halasy K, Nemcsók J. Biochemical and subcellular changes in carp exposed to the organophosphorus methidathion and the pyrethroid deltamethrin. Aquatic Toxicology. 1995;**33**:279-295. DOI: 10.1016/0166-445X(95)00029-4

[54] Jee LH, Masroor F, Kang JC. Responses of cypermethrin-induced stress in haematological parameters of Korean rockfish, *Sebastes schlegeli* (Hilgendorf). Aquaculture Research. 2005;**36**:898-905. DOI: 10.1111/j.1365-2109.2005.01299.x

[55] Datta M, Kaviraj A. Ascorbic acid supplementation of diet for reduction of deltamethrin induced stress in freshwater catfish *Clarias gariepinus*. Chemosphere. 2003;**53**:883-888. DOI: 10.1016/S0045-6535(03)00557-5

[56] Van Leeuwen CJ, Maas-Diepeveen JL, Overbeek HCM. Sublethal effects of tetramethylthiuram disulfide (thiram) in rainbow trout (*Salmo gairdneri*). Aquatic Toxicology. 2002;**9**(1):13-19. DOI: 10.1016/0166-445X(86)90003-2

[57] Kirby MF, Morris S, Hurst M, Kirby SJ, Neall P, Tylor T, et al. The use of cholinesterase activity In flounder (*Platichthys Flesus*) muscle tissue As a

biomarker of neurotoxic contamination in UK estuaries. Marine Pollution Bulletin. 2000;**40**(9):780-791. DOI: 10.1016/S0025-326X(00)00069-2

[58] Carr RL, Strauss DL, Chambers JE. Inhibition and aging of catfish brain acethylcholinesterase following exposure to two phosphorothionate insecticides and their active metabolites. Journal of Toxicology and Environmental Health. 1995;**45**:325-336. DOI: 10.1080/15287399509531999

[59] Costin D, Staicu AS, Dinu D, Huculeci R, Costache A, Dinischiotu A. Biochemical and histological effects of deltamethrin exposure on the gills of *Carassius auratus gibelio* (*Pisces Cyprinidae*), Lucrări ştiinţifice zootehnie şi biotehnologii. Timişoara. 2007;**40**(1):23-28

[60] Clark JM, Matsumura F. Two different types of inhibitory effects of pyrethroids on nerve Ca- and Ca+Mg ATPase in the squid, *Loligo pealei*. Pesticide Biochemical Physiology. 1982;**4**:232-238. DOI: 10.1016/0048-3575(82)90104-3

[61] Li ZH, Grabic R, Li P, Machova J, Velisek J, Randak T. Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na^+-K^+-ATPase activity in brain of rainbow trout, *Oncorhynchus mykiss*. Aquatic Toxicology. 2010;**98**(3):297-303. DOI: 10.1016/j.aquatox.2010.02.017

[62] Cengiz EI. Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. Environmental Toxicology and Pharmacology. 2006;**21**:1093-1096. DOI: 10.1016/j.etap.2006.03.006

[63] Cengiz EI, Unlu E. Sublethal effects of commercial deltamethrin on the structure of the gill, liver and gut tissues of mosquitofish, *Gambusia affinis*: A microscopic study. Environmental Toxicology and Pharmacology. 2006;**21**:246-253. DOI: 10.1016/j.etap.2005.08.005

[64] Skidmore JF. Toxicity of zinc compounds to aquatic animals with special reference to fish. The Quarterly Review of Biology. 1964;**39**:227-248. DOI: 10.1086/404229

[65] Burton DT, Jones AH, Cairns J. Acute zinc toxicity to rainbow trout (*Salmo gairneri*): Confirmation of the hypothsis that death is related to tissue hypoxia. Journal of the Fisheries Research Board of Canada. 1972;**29**:1463-1466. DOI: 10.1139/f72-225

[66] Muir DCG, Rawn GP, Townsend BE, Lockhart WL. Bioconcentration of cypermethrin, deltamethrin, fenvalerate and permethrin by *Chironomus tentans* larvae in sediment and water. Environmental Toxicology and Chemistry. 1985;**4**:51-61. DOI: 10.1139/f72-225

[67] Smith TM, Stratton GW. Effects of synthetic pyrethroid insecticides on nontarget organisms. Research Reviews. 1986;**97**:93-119. DOI: 10.1007/978-1-4612-4934-4_4

[68] Mello GC, Donatti L, Rudniki CA, Fanta E. Hepatic alterations in the fish *Rhamdia quelen* contaminated with Folidol 600®. Ecotoxicology and Environmental Safety. 2008;**71**(3):821-829. DOI: 10.1016/j.ecoenv.2008.02.001

[69] Sarkar B, Chatterjee A, Adhikari S, Ayyappan S. Carbofuran- and cypermethrin-induced histopathological alterations in the liver of *Labeo rohita* (Hamilton) and its recovery. Journal of Applied Ichthyology. 2005;**21**:131-135. DOI: 10.1111/j.1439-0426.2004.00590.x

[70] Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B, Lakra WS. Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel

electrophoresis. Food and Chemical Toxicology. 2009;**47**(3):650-656. DOI: 10.1016/j.fct.2008.12.021

[71] Di Giulio RT, Washburn PC, Wenning RJ, Winston GW, Jewell CS. Biochemical responses in aquatic animals: A review of determinants of oxidative stress. Environmental Toxicology and Chemistry. 1989;**8**: 1103-1123. DOI: 10.1002/etc.5620081203

[72] Bainy ACD, Saito E, Carvalho PSM, Junqueira VBC. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquatic Toxicology. 1996;**34**:151-162. DOI: 10.1016/0166-445X(95)00036-4

[73] Banaee M, Sureda A, Zohiery F, Hagi BN, Garanzini N. Alterations in biochemical parameters of the freshwater fish, *Alburnus mossulensis*, exposed to sub-lethal concentrations of Fenpropathrin. International Journal of Aquatic Biology. 2014;**2**(2):58-68

[74] Sayeed I, Parvez S, Pandey S, Bin-Hafeez B, Haque R, Raisuddin S. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus Bloch*. Ecotoxicology and Environmental Safety. 2003;**56**:295-301. DOI: 10.1016/S0147-6513(03)00009-5

[75] Abdollahi M, Ranjbou A, Shadnic S, Nikfar S, Rezaice A. Pesticides andoxidative stress: A review. Medical Science Monitor. 2004;**10**:141-143

[76] Ambali S, Akanbi D, Igbokwe N, Shittu M, Kawu M, Ayo J. Evaluation of subchronic chlorpyrifos poisoning on hematological and serum biochemical changes in mice and protective effect of vitamin C. The Journal of Toxicological Sciences. 2007;**32**:111-120. DOI: 10.2131/jts.32.111

[77] Dandapam ZA, Kavima MR, Jayaseelan L, Dommen A. Oxidative damage in intermediate syndrome of acute organophosphorous poisoning. Indian Journal of Medical Research. 2003;**17**:253-259

[78] Fabra A, Angelini J, Donolo A, Permigiain M, Castro S. Biochemical alteration in Bradyrhizobium Sp USDA 3187 induced by the fungicide mancozeb. Antonie van Leeuwenhoek. 2004;**73**(3):223-228. DOI: 10.1023/A:1000987524112

[79] Edwards R, Millburn P, Hutson DH. Comparative toxicity of cys-cypermethrin in rainbow trout, frog, mouse and quail. Toxicology and Applied Pharmacology. 1986;**84**:512-522. DOI: 10.1016/0041-008X(86)90256-5

[80] Levine SL, Oris JT, Denison MS. Modulation of CYP1A expression in rainbow trout by a technical grade formulation of propiconazole. Environmental Toxicology and Chemistry. 1999;**18**:2565-2573. DOI: 10.1002/etc.5620181125

[81] Mauck WL, Olson LE, Marking LL. Toxicity of natural pyrethrins and five pyrethroids to fish. Archives of Environmental Contamination and Toxicology. 1976;**4**:18-29. DOI: 10.1007/BF02221012

[82] Macek KJ, Hutchinson C, Cope OB. The effects of temperature on the susceptibility of bluegills and rainbow trout to selected pesticides. Bulletin Environmental Contamination and Toxicology. 1969;**4**:174-183. DOI: 10.1007/BF01560960

[83] Marinescu AG, Drăghici O, Ponepal C, Păunescu A. The influence of fungicide (Dithane M-45) on some physiological indices in the prussian carp (*Carassius auratus* gibelio Bloch). In: Proceedings 35th IAD Conference; 19-23 April; Novi-Sad, Serbia and Montenegro; Limnological Reports. Vol. 35, 2004. pp. 209-214

[84] Marienescu AlG, Draghici O, Ponepal C, Păunescu A. Research on the changes of some physiological parameters in several fish species under the action of the Thiametoxame insecticide. In: Proceedings of the 36th International Conference IAD; 4-8 September 2006, Klosterneuburg Vienna; 2006. pp. 163-167

[85] Ponepal MC, Păunescu A, Drăghici O, Marinescu AG. Research on the changes of some physiological parameters in several fish species under the action of the thiametoxame insecticide. In: Proceedings 36th International Conference of IAD; 4-8 September; Vienna; 2006. pp. 163-167

[86] Ponepal MC, Păunescu A, Marinescu AlG, Drăghici O. Research on the changes of some physiological parameters in Prussian carp under the action of the Reldan insecticide. In: Proceedings of the Second International Conference "Research People and Actual Tasks on Multidisciplinary Sciences"; 10-12 June; Lozenec, Bulgaria. Vol.1. 2009. pp. 209-212

[87] Ponepal MC, Păunescu A, Drăghici O, Marinescu AG. Research on the changes of some physiological parameters under the action of the Tiradin fungicide in prussian carp and crayfish. Annals. Food Science and Technology. 2009;**10**(1):267-270

[88] Ponepal MC, Păunescu A, Marinescu AG, Drăghici O. The changes of some physiological parameters in Prussian carp under the action of the tilt fungicide. Bulletin USAMV Animal Science and Biotechnologies. 2009;**66**(1-2):47-52

[89] Ponepal MC, Păunescu A, Marinescu AG, Drăghici O. Effect of the Fungicide Chlorothalonil (Bravo) on some physiological parameters in Prussian Carp. Lucrări Ştiinţifice USAMV Iaşi, Seria Horticultură. 2009;**52**:1157-1162. http://www.uaiasi.ro/revista_horti/arhiva.php?an=2009

[90] Ponepal MC, Păunescu A, Marinescu Al G, Drăghici O. Research on the changes of some physiological parameters in several fish species under the action of the Talstar insecticide. Analele Universităţii din Oradea, Fascicula Biologie. Tom. 2010;**XVII**(1):175-179

[91] Ponepal MC, Păunescu A, Drăghici O, Marinescu Al G. Changes of some physiological parameters in Prussian carp under the action of some fungicides. Analele Universităţii Ovidius, Seria Biologie-Ecologie. 2010;**14**:83-88

[92] Ponepal MC. Investigaţii ecofiziologice şi ecotoxicologice la unele specii de peşti din râul Argeş [thesis]. Universitatea din Pitesti. 2011

[93] Schuytema GS, Nebeker AV. Effects of ammonium nitrate, sodium nitrate and urea on red-legged frogs: Pacific treefrogs and African clawed frogs. Bulletin of Environmental Contamination and Toxicology. 1999;**63**:357-364

[94] Pollet I, Bendell-Young LI. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. Environmental Toxicology and Chemistry. 2000;**19**:2589-2597. DOI: 10.1002/etc.5620191027

[95] Marco A, Cash D, Belden LK, Blaustein AR. Sensitivity to urea fertilization in three amphibian species. Archives of Environmental Contamination and Toxicology. 2001;**40**:406-409. DOI: 10.1007/s002440010190

[96] Bridges CM. Predator-prey interactions between two amphibian species: Effects of insecticide exposure. Journal Aquatic Ecology. 1999;**33**:205-211. DOI: 10.1023/A:1009922825444

[97] Bridges CM. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Journal Archives of

Environmental Contamination and Toxicology. 2000;**39**:91-96

[98] Sampath K, Kennedy IJJ, James R. Pesticide impact on excretory physiology of the common frog, *Rana tigrina* (Daud) tadpoles. Bulletin of Environmental Contamination and Toxicology. 2002;**68**:652-659

[99] Broomhall S, Shine R. Effects of the insecticide endosulfan and presence of congeneric tadpoles on australian treefrog (*Litoria freycineti*) tadpoles. Archives of Environmental Contamination and Toxicology. 2003;**45**:221-226

[100] Tomizawa M, Casida J. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annual Review of Entomology. 2002;**48**:339-364. DOI: 10.1146/annurev.ento.48.091801.112731

[101] Tomizawa M, Casida J. Neonicotinoid insecticide toxicology: Mechanisms of selective action. Annual Review of Pharmacology and Toxicology. 2004;**45**:247-268.DOI:10.1146/annurev.pharmtox.45.120403.095930

[102] Gendron AD, Marcogliese DJ, Barbeau S, Christin MS, Brousseau P, Ruby S, et al. Exposure of leopard frogs to a pesticide mixture affects life history characteristics of the lungworm *Rhabdias ranae*. Journal Oecologia. 2003;**135**: 469-476. DOI: 10.1007/s00442-003-1210-y

[103] Greulich K, Pflugmacher S. Uptake and effects on detoxication enzymes of cypermethrin in embryos and tadpoles of amphibians. Journal Archives of Environmental Contamination and Toxicology. 2004;**47**:489-495

[104] Howe CM, BeMGill M, Pauli BD, Helbing CC, WeMGy K, Veldhoen N. Toxicity of glyphosate-based pesticides to four north American frog species. Environmental Toxicology and Chemistry. 2004;**23**(8):1928-1938

[105] Cy C, Hathaway KM, Folt CL. Multiple stress effects of vision herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. Environmental Toxicology and Chemistry. 2004;**23**(4):823-831

[106] Relyea RA. Growth and survival of five amphibian species exposed to combinations of pesticides. Environmental Toxicology and Chemistry. 2004;**23**(7):1737-1742

[107] Shaolong F, Zhiming K, Xinming W, Lirong Z, Pingan P. Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell*. Chemosphere. 2004;**56**:457-463. DOI: 10.1016/j.chemosphere.2004.02.010

[108] Relyea RA. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecological Applications. 2005;**15**:1118-1124

[109] Relyea RA, Schoeppner NM, Hoverman JT. Pesticides and amphibians: The importance of community context. Ecological Applications. 2005;**15**(4):1125-1134

[110] Hofer R, Lackner R, Lorbeer G. Accumulation of toxicants in tadpoles of the common frog (*Rana temporaria*) in High Mountains. Archives of Environmental Contamination and Toxicology. 2005;**49**:192-199. DOI: 10.1007/s00244-004-0188-8

[111] Seifert J, Stollberg J. Antagonism of a neonicotinoid insecticide imidacloprid at neuromuscular receptors. Environmental Toxicology and Pharmacology. 2005;**20**(1):18-21. DOI: 10.1016/j.etap.2004.09.011

[112] Honda H, Tomizawa M, Casida J. Neonicotinoid metabolic activation and inactivation established with coupled nicotinic receptor-CYP3A4 and -aldehyde oxidase systems. Toxicology Letters. 2006;**161**(2):108-114

[113] Zafeiridou G, Geronikaki A, Papaefthimiou C, Tryfonos M, Kosmidis EK, Theophilidis G. Assessing the effects of the three herbicides acetochlor, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid on the compound action potential of the sciatic nerve of the frog (*Rana ridibunda*). Chemosphere. 2006;**65**:1040-1048. DOI: 10.1016/j.chemosphere.2006.03.032

[114] Casco VH, Izaguirre MF, Marín L, Vergara MN, Lajmanovich RC, Peltzer P, et al. Apoptotic cell death in the central nervous system of Bufo arenarum tadpoles induced by cypermethrin. Cell Biology and Toxicology. 2006;**22**:199-211. DOI: 10.1007/s10565-006-0174-1

[115] Relyea RA. The effects of pesticides, pH, and predatory stress on amphibians under mesocosm conditions. Journal Ecotoxicology. 2006;**15**:503-511. DOI: 10.1007/s10646-006-0086-0

[116] Thompson DG, Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR. Chemical and bio-monitoring to assess potential acute effects of vision herbicide on native amphibian larvae in forest wetlands. Environmental Toxicology and Chemistry. 2004;**23**:843-849

[117] Edginton AN, Rouleau C, Stephenson GR, Boermans HJ. 2,4-D butoxyethyl ester kinetics in embryos of *Xenopus laevis*: The role of the embryonic jelly coat in reducing chemical absorption. Archives of Environmental Contamination and Toxicology. 2007;**52**:113-120. DOI: 10.1007/s00244-005-0215-4

[118] Vonesh RJ, Buck CJ. Pesticide alters oviposition site selection in gray treefrogs. Oecologia. 2007;**154**:219-226

[119] Gurushankara HP, Krishnamurthy SV, Vasudev V. Effect of malathion on survival, growth, and food consumption of indian cricket frog (*Limnonectus limnocharis*) tadpoles. Archives of Environmental Contamination and Toxicology. 2007;**52**:251-256. DOI: 10.1007/s00244-006-0015-5

[120] Henson-Ramsey H, Kennedy-Stoskopf S, Levine JF, Taylor SK, Shea D, Stoskopf MK. Acute toxicity and tissue distributions of Malathion in *Ambystoma tigrinum*. Archives of Environmental Contamination and Toxicology. 2008;**55**:481-487. DOI: 10.1007/s00244-007-9091-4

[121] Relyea RA, Diecks N. An unforeseen chain of events: Lethal effects of pesticides on frogs at sublethal concentrations. Ecological Applications. 2008;**18**(7):1728-1742

[122] Kang HS, Gye MC, Kim MK. Effects of endosulfan on survival and development of *Bombina orientalis* (Boulenger) embryos. Bulletin of Environmental Contamination and Toxicology. 2008;**81**:262-265. DOI: 10.1007/s00128-008-9475-9

[123] Kang HS, Park CJ, Gye MC. Effects of molinate on survival and development of *Bombina orientalis* (Boulenger) embryos. Bulletin of Environmental Contamination and Toxicology. 2009;**82**:305-309. DOI: 10.1007/s00128-008-9602-7

[124] Relyea RA, Jones D. The toxicity of roundup original MAX to 13 species of larval amphibians. Environmental Toxicology and Chemistry. 2009;**28**(9):2004-2008. DOI: 10.1897/09-021.1

[125] Jones DK, Hammond JI, Relyea RA. Very highly toxic effects of endosulfan across nine species of tadpoles: Lag effects and family level sensitivity. Environmental Toxicology and Chemistry. 2009;**28**(9):1939-1945. DOI: 10.1897/09-033.1

[126] Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA. Hermaphroditic, demasculinized frogs after exposure to

the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**:5476-5480. DOI: 10.1073/pnas.082121499

[127] Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. Atrazine induced hermaphrodism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environmental Health Perspectives. 2003;**111**:568-575. DOI: 10.1289/ehp.5932

[128] Hecker M, Giesy JP, Jones PD, Jooste AM, Carr JA, Solomon KR. Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*Xenopus laevis*) from South Africa. Environmental Toxicology and Chemistry. 2004;**23**:1996-2007

[129] Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA. Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. Ecotoxicology and Environmental Safety. 2005;**62**:160-173. DOI: 10.1016/j.ecoenv.2004.10.010

[130] Hecker M, Kim WJ, Park JW, Murphy MB, Villeneuve D, Coady KK. Plasma concentrations of estradiol and testosterone, gonadal aromatase activity and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol or atrazine. Aquatic Toxicology. 2005;**72**:383-396. DOI: 10.1016/j.aquatox.2005.01.008

[131] Murphy MB, Hecker M, Coady KK, Tompsett AR, Jones PD, du Preez LH. Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. Aquatic Toxicology. 2006;**76**:230-245. DOI: 10.1016/j.aquatox.2005.09.010

[132] Storrs SI, Semlitsch RD. Variation in somatic and ovarian development: Predicting susceptibility of amphibians to estrogenic contaminants. General and Comparative Endocrinology. 2008;**156**:524-530. DOI: 10.10166/j.ygcen2008.03.001

[133] Storrs SI, Tillitt DE, Rittenhouse TA, Semlitsch RD. Behavioral response and kinetics of terrestrial atrazine exposure in American toads (*Bufo americanus*). Archives of Environmental Contamination and Toxicology. 2009;**57**:590-597. DOI: 10.1007/s00244-009-9292-0

[134] Williams BK, Semlitsch RD. Larval responses of three midwestern anurans to chronic, low-dose exposures of four herbicides. Archives of Environmental Contamination and Toxicology. 2010;**58**(3):819-827. DOI: 10.1007/s00244-009-9390-z

[135] Budischak AS, Belden KL, Hopkins AW. Relative toxicity of malathion to trematode-infected and noninfected *Rana palustris* tadpoles. Archives of Environmental Contamination and Toxicology. 2009;**56**:123-128. DOI: 10.1007/s00244-008-9167-9

[136] Paunescu A, Soare LC, Fierascu RC, Fierascu I, Ponepal MC. The influence of six pesticides on physiological indices of *Pelophylax Ridibundus* (Pallas, 1771). Bulletin of Environmental Contamination and Toxicology. 2018;**100**(3):376-383. DOI: 10.1007/s00128-018-22777-9

[137] Knutson MG, Richardson WB, Reineke DM, Gray BR, Parmelee JR, Weick SE. Agricultural ponds support amphibian populations. Ecological Applications. 2004;**14**:669-684

[138] Mazerolle MJ. Drainage ditches facilitate frog movements in a hostile landscape. Landscape Ecology. 2005;**20**:579-590. DOI: 10.1007/s10980-004-3977-6

[139] Herzon I, Helenius J. Agricultural drainage ditches, their biological importance and functioning. Biological Conservation. 2008;**141**:1171-1183. DOI: 10.1016/j.biocon.2008.03.005