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The effect of pyrethroid based pesticides on fish

Vliv pyrethroidových pesticidů na ryby

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Czech Republic, Vodňany, 2016

I dedicate this work to my father who helped me until the last moments of his life.

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Pyrethroids are synthetic analogues of the natural pyrethrins, extracts of the ornamental *Chrysanthemum cinerariaefolium* and its related species. Dried and ground pyrethrum chrysanthemum flowers were even noted to be powerful insecticides in ancient China (He et al., 2008). Pyrethrins had been used for decades as botanical insecticides. Pyrethroids have gradually replaced natural pyrethrins especially due to their better stability in sunlight. The first commercial synthetic pyrethroid, allethrin, was produced in 1949, followed in the 1960s by others (IPCS, 1998). The use of pyrethrins and pyrethroids has increased with the declining use of organophosphate pesticides which are more acutely toxic to birds and mammals than pyrethroids.

Decades of research and development by the agrochemical industry and by academic research laboratories have resulted in a wide range of pyrethroid structures and a multitude of uses in agricultural, veterinary, medical and household pest control (Soderlund et al., 2002). Due to the long-term biological hazards associated with the use of organochloride, organophosphate and carbamate pesticides, the use of pyrethroids as insecticidal and antiparasitic formulations has markedly increased as a viable substitute and nowadays they account for over 30% of insecticides used globally (Prasamthi et al., 2005). Public health management uses applications of pyrethroids to control cockroaches, mosquitoes, ticks, and flies, which may act as disease vectors. Veterinary use of cypermethrin and deltamethrin has even extended to fish aquaculture production from the 1990s onwards (Hart et al., 1997; Denholm et al., 2002). Products based on deltamethrin are used in salmon farming against sea lice mainly in Scandinavian countries and Canada (Pike and Wadsworth, 1999; Fairchild et al., 2010). Cypermethrin was also implemented in the treatment of Atlantic salmon (*Salmo salar*) successfully (Hart et al., 1997). Nowadays there are over 3500 registered pyrethrins and pyrethroids products, many of them are used widely.

Most pyrethrins and some pyrethroids products are formulated with chemicals, such as piperonyl butoxide and MGK-264, to enhance the pesticide properties of the product. They are called synergists and have no pesticidal effects of their own, but enhance the effectiveness of active substances (US EPA, 2015a). The importance of synergist addition to products grows as the pest resistance increases. They suppress metabolism-based resistance of agents (Pasay et al., 2009). Other synergists of pyrethroids could be piperonyl sulfoxide or sesamex. Synergists prevent some enzymes from breaking down pyrethroids. Technical-grade (concentrated) pyrethroids are usually mixed with carriers or solvents to produce commercial-grade formulated products. The formulated products contain many inert ingredients that can increase the toxicity of the product when compared to technical-grade material (ATSDR, 2003).

The chemical structure of pyrethroids is based on chrysanthemic acid linked to an aromatic alcohol through an ester linkage. The physical properties display a highly nonpolar nature of low water solubility, low volatility, high octanol-water partition coefficients, and have high affinity for soil and sediment particulate matter (Lastowski, 2002). Synthetic pyrethroids are chiral compounds containing stereoisomers. Due to the presence of 2 or 3 stereogenic centers they form a pesticide group with one of the highest chirality. Thus, each pyrethroid contains 2 or 4 enantiomer pairs, or 2 or 4 diastereomers (Liu and Gan, 2004).

Pyrethroids are divided into type I and type II, based on their structure, chemical and neurophysiological properties and toxicological action. This division has been widely adopted in the literature and is often used in a manner parallel to the T/CS nomenclature, so that type-I compounds are generally considered to produce the T syndrome and type-II compounds are considered to produce the CS syndrome. But this nomenclature is only based on intoxication

produced in mammals (Soderlund et al., 2002). The T (tremor) or CS (choreoathetosis with salivation) intoxication syndrome after intravenous or intracerebral administration to rodents have been described (Lawrence and Casida, 1982). T syndrome intoxication presents symptoms like aggressive sparing behaviour, increased sensitivity to external stimuli, fine tremors, prostration, coarse body tremor, increase of body temperature. CS syndrome intoxication causes chewing, profuse salivation, pawing and burrowing, coarse body tremor, increased startle response, abnormal locomotion of posterior limbs, sinuous writhing (choreoathetosis) and clonic and tonic seizures (Bradbury and Coats, 1989a). Although this classification system is widely employed, it has several shortcomings for the identification of common toxic effects. In particular, it does not reflect the diversity of intoxication signs found following oral administration of various pyrethroids (Soderlund et al., 2002). Moreover, there are "mixed-type pyrethroids" such as esfenvalerate and fenpropathrin, which exhibit effects between I and II pyrethroids. They delay the repolarization of neuronal tissues intermediate between I and II pyrethroids and elicit mix behaviours of both T and CS syndrome (US EPA, 2011a). A study on terrestrial insects described restlessness, incoordination, prostration and paralysis after exposure of type I pyrethroids whereas incoordination, convulsions and intense hyperactivity after exposure of type II in cockroaches (Gammon et al., 1981). It is difficult to differentiate between type-I and type-II syndromes in fishes. Both types of pyrethroids cause similar neurological symptoms and fish generally become inactive before death (Bradbury and Coats, 1989a).

1.1.1. Cypermethrin

Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate] is a racemic mixture of eight isomers, which has halogens in a cyclopropane side chain. Cypermethrin is a typical chiral compound with multiple asymmetric positions. The presence of three chiral centers in the formula of cypermethrin results in eight possible stereoisomers. Four diastereoisomers (pairs of enantiomers) exist, and each member has a diastereomeric relationship with each member of the other enantiomeric pairs (Díaz et al., 1998). Two of the eight isomers, 1R-cis-alphaS and 1R-trans-alphaS, contribute for almost all the toxicity in the racemate, while the other six enantiomers are inactive in relation to aquatic toxicity (Liu et al., 2005). All eight isomers consist of two groups, four with a cis orientation across the cyclopropyl ring of the dichlorovinyl and ester groups and four with a trans orientation.

Cypermethrin was initially synthesized in 1974 and first marketed in 1977 as a highly active synthetic pyrethroid insecticide, effective against a wide range of pests in agriculture, public health, and animal husbandry (IPCS, 1992). Animal husbandry and veterinary use also comprises the control of ectoparasite infestation (*Lepeophtheirus salmonis* and *Caligus elongatus*) in marine cage culture of Atlantic salmon (*S. salar*) (Treasurer and Wadsworth, 2004). This synthetic pyrethroid is available as cypermethrin technical, cypermethrin technical concentrates, cypermethrin emulsifiable concentrates, cypermethrin wettable powders, and cypermethrin ultra low volume liquids for protection of plants (FAO, 1995). Cypermethrin is available in numerous commercial formulations in many countries. It may be formulated mixed with other pesticides such as chlorpyrifos (FAO, 2008). It is among the most effective pyrethroid preparations. It blocks sodium channels of nerve filaments and moreover affects gama-amino butyric acid (GABA) receptors in nerve filaments (Bradbury and Coats, 1989a).

Total cypermethrin use in agricultural and non-agricultural settings in the United States is approximately 1.0 million pounds (453.6 tonnes) of active ingredient per year. In agricultural settings it is used primarily on cotton with minor use on pecan and broccoli. But the great

majority of cypermethrin use occurs in non-agricultural settings. The amount of outdoor use for control of subterranean termites and other insect pests accounts for nearly 750000 pounds (340.2 tonnes). While agricultural products are "restricted use", industrial, residental, and commercial products are "general use" (US EPA, 2006). Usage of cypermethrin as active substance in the Czech Republic was 21150 kg in 2013 and 21181 kg in 2014. It was used to protect cereals, maize, legumens, beet, potatoes, and mainly oil plants. Usage of alpha-cypermethrin as active substance in the Czech Republic was 2600 kg in 2013 and 2097 kg in 2014 (UKZUZ, 2013, 2014).

Different cypermethrins for agicultural use are registered in many countries. Four different cypermethrin products (alpha-, beta-, theta-, and zeta-cypermethrin) have different ratios in their eight isomers. Zeta-cypermethrin is an S-enantiomer enriched formulation of cypermethrin, which is not distinguished from cypermethrin by the analytical enforcement method, and the toxicological endpoints are the same for both cypermethrin and zeta-cypermethrin (US EPA, 2006). Alpha-cypermethrin consists essentially of two of the four cis isomers comprising cypermethrin. It is a racemic mixture of (S)-α-cyano-3-phenoxybenzyl-(1R,3R)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate and (R)-α-cyano-3-phenoxybenzyl-(1S,3S)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate (WHO, 2013). Ratio of isomers comprised in different cypermethrins is presented in Table 1.

Table 1. *Comparison of alpha-cypermethrin and zeta-cypermethrin (FAO, 2008).*

Isomer	cypermethrin	alpha-cypermethrin	zeta-cypermethrin
1R, cis-R	14	–	3
1S, cis-S	14	–	22
1R, cis-S	11	50	22
1S, cis-R	11	50	3
1R, trans-R	14	–	3
1S, trans-S	14	–	22
1R, trans-S	11	–	22
1S, trans-R	11	–	3

High acute toxicity for fish was reviewed and cited as 96 h LC50 of cypermethrin 0.9–1.1 $\mu g.l^{-1}$ for carp (*Cyprinus carpio*), 1.2 $\mu g.l^{-1}$ for brown trout (*Salmo trutta*), 0.5 $\mu g .l^{-1}$ for rainbow trout (*Salmo gairdneri*), 0.4 $\mu g.l^{-1}$ for rudd (*Scardinius erythrophthalmus*), and 2.2 $\mu g.l^{-1}$ for tilapia nilotica (*Oreochromis niloticus*) (Bradbury and Coats, 1989b). While 96 h LC0, 96 h LC50, and 96 h LC100 for common carp (*C. carpio*) were noted 1.82, 2.91, and 4.64 $\mu g.l^{-1}$, respectively (Velisek et al., 2011). The 24 h LC50 of alpha-cypermethrin was cited 4.5 $\mu g.l^{-1}$ for common carp (*C. carpio*) (Stephenson, 1990).

1.1.2. Cyhalothrin

Cyhalothrin (S)-α-cyano-3-phenoxybenzyl(1R,3R)-3-[(Z)-2-chlor-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate is a derivative of chrysanthemic acid which contains two kinds of halogens. Although, theoretically, it could be a mixture of 16 enantiomers, this number has been reduced to 4 in actual practice. A more active pair of enantiomers is produced separately as lambda-cyhalothrin (IPCS, 1990). Lambda-cyhalothrin consists of the cis-1R-alphaS and cis-1S-alphaS enantiomeric pair of cyhalothrin. Cyhalothrin comprises about 50%

lambda-cyhalothrin. One of the two enantiomers of lambda-cyhalothrin is the insecticidally active gamma-cyhalothrin (FAO, 2007). Gamma-cyhalothrin is a single, resolved isomer of the pyrethroid cyhalothrin, and as such shares physical, chemical and biological properties with both cyhalothrin and lambda-cyhalothrin, which are mixtures of 4 and 2 isomers respectively. Gamma-cyhalothrin is the most insecticidally active isomer of cyhalothrin/lambda-cyhalothrin, and thus the technical gamma-cyhalothrin product may be considered a refined form of cyhalothrin/lambda-cyhalothrin in that it has been purified by the removal of less active and inactive isomers (Federal Register, 2004).

Both gamma- and lambda-cyhalothrin were registered at the end of the 1980s and both are "restricted use" broad-spectrum insecticides. Due to their structure and relative toxicities it is possible to combine their toxicity databases for purpose of risk assessment (US EPA, 2011b). Research studies are still mainly devoted to lambda-cyhalothrin, but some indicate differences between lambda- and gamma-cyhalothrin. Summarized data from extensive databases of toxicity tests and field studies indicate that gamma-cyhalothrin causes effects at approximately one-half the concentration at which lambda-cyhalothrin causes similar effects. But because gamma-cyhalothrin has twice the biological activity, application rates needed to control insects are correspondingly lower and result in the same risk for aquatic species (Giddings et al., 2009).

Cyhalothrin is type II pyrethroid due to the presence of α-cyano moiety at the benzylic carbon of the alcohol portion of the ester. On the other hand, non specific effect of intoxication as a result of acute neurotoxicity test on Alderley Park rats does not support inclusion of this pyrethroid into either category T/CS syndrome (Barmmer, 1999; Soderlund et al., 2002).

Usage of gamma-cyhalothrin as an active substance in the Czech Republic was 676 kg in 2013 and 888 kg in 2014. It was used to protect cereals mainly. Usage of lambda-cyhalothrin as an active substance was 760 kg in 2013 and 741 kg in 2014 (UKZUZ, 2013, 2014).

Gamma-cyhalothrin caused 96 h LC50 0.17 $\mu g.l^{-1}$ while lambda-cyhalothrin 0.19 $\mu g.l^{-1}$ in rainbow trout (*Oncorhynchus mykiss*) (Machado, 2001). Another described result presents 96 h LC50 of lambda-cyhalothrin for rainbow trout (*O. mykiss*) 0.24 $\mu g.l^{-1}$ and 0.5 $\mu g.l^{-1}$ for common carp (*C. carpio*) (Maund et al., 1998).

1.2. PYRETHROIDS AS A THREAT FOR AQUATIC ENVIRONMENT

Environmental residues of pyrethroids and pyrethrins are degraded by hydrolysis, and mainly pyrethrins by photolysis, and so they do not accumulate in most ecosystems. Since they undergo photolysis in the atmosphere, they are also degraded by this mechanism in sunlit surface waters. Photosensitizing agents found in natural waters such as fulvic and humic acids increase the rate of photolysis. Pyrethroid compounds also undergo hydrolysis in the environment at varying rates depending upon pH and temperature. Pyrethroids are readily degraded by environmental microorganisms (ATSDR, 2003).

The main environmental hazard associated with pyrethroid use is the contamination of fresh water (Ray, 2005). Pyrethroids are normally absent in natural water unaffected by human activities. But the worldwide usage of pyrethroid pesticides produces pollutants that may reach and influence aquatic ecosystem. They can enter aquatic ecosystem by a number of various routes. Generally pesticides get into the water directly due to incorrect application (contact with water during the treatment of cultures under strong wind or use of aerial application treatment of cultures, leakage of pesticides during the pumping of water into the washer), they also get into the water during the disposal of unused residues (due to pouring the rest of pesticides or during rinsing the tanks with water, due to incorrect handling with packages), or due to accidents during transport. Pesticides also get into the water indirectly

(runoff from surrounding treated products) (Svobodova et al., 2003). Fish and other aquatic organisms are at higher risk of pyrethroid intoxication than birds and mammals.

Residues of pyrethroids in water and/or sediment have been detected near agricultural or urban areas worldwide. The positive correlation between concentrations of pesticides in water and land use practices was demonstarted (Pionke and Glotfelty, 1989). Weston and Lydy (2010) studied agricultural land water in California, USA and described that effluent of wastewater treatment plants contained up to 0.02 $\mu g.l^{-1}$ of bifenthrin. Because other authors found similar concentrations in the waters of this area, these values may be considered as environmentally relevant in California (DeGroot and Brander, 2014). The major contributor to pesticide contamination is reported by several studies to be drainage water/sub-surface runoff, which strongly coincides with high levels of precipitation (Mathiessen et al., 1995; Liess and Schulz, 1999; Schulz and Liess, 1999a). Seasonal influence of dry and wet periods on the concentration of several pesticides in canal water in rice fields in Malaysia was studied. No statistical difference of concentrations of cypermethrin between these periods was shown. Detected concentration was higher during time when the farmers applied cypermethrin to control pests in their rice fields (Ismail et al., 2012). Stream water concentrations of pyrethroids more than 0.1 $\mu g.l^{-1}$ were detected in 20% of the water samples collected in relation to high precipitation incidents in three Danish streams draining agricultural areas with up to 0.66 $\mu g.l^{-1}$ found on one occasion (Wiggers, 1999). Lambda-cyhalothrin was the most commonly detected insecticide in sediment in drainage of agricultural areas in Denmark. Sediment samples from Danish streams revealed λ-cyhalothrin concentration up to 0.02 $\mu g.g^{-1}$ of sediment (Friberg et al., 2003; Kronvang et al., 2003). The residues of cypermethrin and λ-cyhalothrin have been widely detected in water and sediment samples from streams and river draining canals major agricultural districts in Greece. The median concentrations of cypermethrin 0.038 $\mu g.l^{-1}$ and λ-cyhalothrin 1.141 $\mu g.l^{-1}$ were among the highest from monitored pesticides. Furthermore, the distribution width of concentrations within quartiles for cypermethrin was the biggest during monitored years 2006–2008. Despite the absence of cypermethrin and λ-cyhalothrin in the river some pesticides including pyretroid bifenthrin were detected with maximum concentration 0.17/0.15 $\mu g.l^{-1}$ (riparian drainage canal/river) (Vryzas et al., 2011). Residues of λ-cyhalothrin have been detected in runoff resulting from agricultural, public health, and residential applications in California, USA (He et al., 2008). Bifenthrin is also a commonly detected pyrethroid in this region. Concentrations of bifenthrin in northern California were summarized by Forsgren et al. (2013) and ranged from 0.0046 to 0.034 $\mu g.l^{-1}$. Residues of pyrethroids are also a problem outside streams and canals adjacent to the areas where they are used; study in South America found 0.376 $\mu g.l^{-1}$ of cypermethrin in rainwater (Laabs et al., 2002).

Only rare occurrences of cypermethrin in watercoarses have been proven in the Czech Republic. The highest detected concentration was 0.2 $\mu g.l^{-1}$ in the spring of 2015 and other increased concentrations were one order lower. Hundreds of control samples were measured during the last years. The concentration of most of the control samples from the Czech rivers did not exceed the limit of quantification during years of monitoring. (Povodi Labe, Povodi Moravy, Povodi Ohre, Povodi Vltavy, 2016).

The possible threat to ecosystems could easily become a reality if pyrethroids are not well maintained. Examples of ecocatastrophes exist. Fish mortality in Lake Balaton, Hungary in 1991 and 1995 was caused in connection with deltamethrin used to control mosquitos. Deltamethrin was detected in different animal species, i.e. eel (*Anguilla anguilla*), bream (*Abramis brama*), pike perch (*Stizostedion lucioperca*), and the common gull (*Larus canus*) and in sediment samples from the lake. It was proven that deltamethrin contributed to massive eel devastation (Balint et al., 1997; Nemcsok et al., 1999).

To minimize risk for aquatic ecosystem, registrants have been required to modify pyrethroid use labels according to higher-tier data through the years. Label restrictions include distances for ground and aerial applications, when used directly adjacent to water courses. Other recommendations to reduce spray drift and runoff have also been added to the use labels to promote safe uses (Solomon et al., 2001). Many products containing pyrethroids are "restricted use products", which are not available for purchase or use by the general public. It can be used only by certified applicators or someone under the certified applicator's direct supervision. Updated list of restricted use product is available due to US EPA (US EPA, 2015b). Products containing small amounts of pyrethroids for uses around the home are still classified as general use pesticides; however, emulsified or granular concentrate formulations that are applied to fields were classified as restricted use pesticides by the EPA in 1995.

High toxicity of pyrethroids was demonstrated in standard, clean-water, laboratory studies, but when more environmentally realistic exposure conditions are taken into account, potential risks from pyrethroid use are substantially reduced (Maund et al., 1998). Situation under natural conditions is considerably different from artificially controlled constant conditions in the course of laboratory tests. Pyrethroids are degraded fairly rapidly in the soil and plants in the environment. They persist only for a short time in the water column due to ability of adsorption by organic matter and degradation (Maund et al., 2002). Rapid and substantitial adsorption to plants, sediments and organic matter leads to signifiant reduction in exposure in the water column and decrease of potential risk in natural conditions. Adsorption to sediment leads to a signifiant decrease of bioavailabaility (Maund et al., 1998). A buffering capacity of natural waters reduced up to one order of magnitude cypermethrin toxicity to fish as it was evaluated in the Rolling Pampas region, Argentina, where soybeans are protected by cypermethrin regularly. Protective capacity was mainly associated with the organic mater content in the dissolved and particulate fractions (Carriquiriborde et al., 2007).

However, tendency of pyrethroids to adsorb to suspended particulate materials in the water column, including clay particles and organic matter, leads to formation of the primary vectors for transport through aquatic systems. The greatest risk to non-target aquatic organisms would be through exposure to contaminated sediments (He et al., 2008). Pyrethroids were found at low levels in sediment samples collected from five tributaries (primarily urban creeks) of the San Francisco Bay, California, and the highest concentration was 0.0176 $\mu g.g^{-1}$ (Woudneh and Oros, 2006). Cypermethrin was found in sediment after spraying and after a rainfall event in the Rolling Pampas region repeatedly. The highest concentration was 1.075 $\mu g.g^{-1}$ after a rainfall event of 57 mm occurring 19 days after spraying soybean crops (Marino and Ronco, 2005). Concentrations 0.183–0.197 $\mu g.g^{-1}$ were detected in sediment together with detection of cypermethrin in water samples and in different fish tissues in rivers Indus and Ravi (Jabeen et al., 2015; Mahboob et al., 2015).

1.3. PYRETHROIDS TOXICITY MECHANISMS

Pyrethroids are neurotoxicants. They influence both central and peripheral parts of the nervous system. Their neuroexcitatory effects are associated with the onset of signs of acute intoxication. These neuroexcitatory effects are consistent with the modification of cellular excitability on voltage-dependent ion channels. Voltage-sensitive sodium channels mediate the transient sodium permeability of the cell membrane that is associated with the production of action potentials in nerves (Soderlund et al., 2002). Pyrethroids affect the sodium channels of nerve filaments in general. They extend the time of opening and closing of sodium channels and extend their depolarisation phase. Moreover, pyrethroids with α-cyano moiety in their strucure affect the GABA receptors in the nerve filaments and affect chloride and calcium

channels (Bradbury and Coats, 1989a.; Hayes, 1994; Burr and Ray, 2004; Modra et al., 2008). As pyrethroids bind to the channels and prevent them from closing normally, it results in continuous nerve stimulation and tremors. Poisoned organisms lose control of their nervous system and are unable to produce coordinated movement (He et al., 2008). Pyrethroids cause the neuronal damage which is also related to acethylcholinesterase activity inhibition resulting in neurotransmission impairments (Kumar et al., 2009; Moshen et al. 2012). The toxicity of the pyrethroids is influenced by the isomeric properties of the compound. For pyrethroids possessing the cyclopropane moiety, the trans isomers show less toxicity in mammals than cis isomers. Pyrethroids that contain the alpha-S-cyano phenoxybenzyl alcohol moiety demonstrate considerably greater toxicity compared to the R configuration (Dorman and Beasley, 1991). Mixtures of pyrethroids with other pollutants amplify their toxic effects (Bacchetta et al., 2014). Possibility of the formation of such mixtures in water can not be ignored mainly in agricultural areas.

Pyrethroids have been shown to be up to 1000 times more toxic to fish than mammals and birds at comparable concentrations (Edwards et al. 1986; Bradbury and Coats, 1989a). The high toxicity of pyrethroids to fish is caused by the combination of three factors: a more sensitive fish central nervous system, rather slow hydrolytic detoxification, and the route of exposure (direct absorption via the gills into the bloodstream) (Edwards et al., 1987). Lethal concentration varies with fish species, size, and environmental factors, such as temperature or feeding (Bradbury and Coats, 1989b; Haya, 1989). Water hardness and salinity can influence the rate of pyrethroid toxicity to fish too (Dyer et al., 1989). Body weight and developmental stage must be also taken account (Von Westernhagen, 1988; Foekema et al., 2008; Kammann et al., 2009).

Aquatic organisms, in particular insects, crustaceans and fish are highly sensitive to pyrethroid insecticides. Crustaceans such as amphipods belong to the most sensitive taxa. Acute toxicity to fish and aquatic invertebrates is generally observed at concentrations bellow 1 $\mu g.l^{-1}$. Sublethal toxic effects have been reported at levels far below and use $ng.l^{-1}$ units. Sublethal concentration of pyrethroids could cause altered behaviour and reduced growth. It influences the immune, reproductive, and endocrine systems. Laboratory examination could reveal histopatological and biochemical changes in organisms (Werner and Moran, 2008).

Sublethal concentration of pyrethroid influences fish physiology. Exposure of cypermethrin affects spawning behaviour of brown trout (*Salmo trutta*) or Atlantic salmon (*S. salar*) male parr due to abolished ability of the olfactory epithelium of parr to detect prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from females (Moore and Waring, 2001; Jaensson et al., 2007)). Another study suggests that behavioral effects of cypermethrin are primarily mediated through more complex pheromone detection system using female urine and ovarian fluid not only $PGF_{2\alpha}$. Exposed males had lower volumes of milt. Moreover, pyrethroids affect blood plasma levels of sex hormones of male parr (Jaensson et al., 2007). Potential for endocrine disruption and gonadal dysfunction of bifenthrin on steelhead (*O. mykiss*) was described in both sexes. Significant amount of unhealthy ovarian follicles and changes in plasma sex steroid levels in female steelhead was obvious. While testicular tissue was unaffected, sex steroid levels in male were relatively unaffected, and gonadosomatic index was changed only in freshwater conditions (Forsgren et al., 2013). Effects on gonads, gonadotrophic cells and steroid levels was also described in catfish (*Heteropneustes fossilis*) (Singh and Singh, 2008). Effect on thyroid hormones levels and induction of thyroid endocrine disruption were described in zebrafish (*Danio rerio*) embryos (Tu et al., 2016). Changes of biochemical and haematological profiles were summarized by Richterova and Svobodova (2012).

1.4. TOXICITY TESTS

Toxicity tests are necessary in water pollution evaluations. They supplement chemical and physical tests to assess potential effects in aquatic organisms. Acute toxicity tests and early-life toxicity tests belong to the most common toxicity tests in fish. Acute toxicity test according to the Organisation for Economic Cooperation and Development (OECD) determines median lethal concentration (LC50) which is equal to the concentration of substance at which 50% of the fish die within an exposure period maximally 96 h (Rufli, 2012). Early life stage toxicity test defines the lethal and sub-lethal effects of chemicals on the early life stages of the species tested. The test starts when fertilised eggs are placed in the test chambers and continues at least until all the control fishes are free-feeding (OECD, 2013).

Recently, much work has resorted to fish of early life stages for ethical, practical, and financial reasons. Above all, the periods of intensive pyrethroid application in agriculture may coincide with the spawning season of multiple fish species. Consequentely fish could be exposed at embryolarval period in water nearby treated areas (Jin et al., 2009). Some researchers prefer embryo assays, because they are regarded to be pain-free in vivo tests and embryonic development is sensitive to environmental stress (Shi et al., 2011). Fish embryos are not provided protection under various governmental definitions. Animal welfare organisations followed by public and politicians urge that fish test should be replaced with such alternatives. Lammer et al. (2009) compared fish embryo tests and acute fish toxicity tests, confirmed that they are neither better nor worse, and offered them as reasonable alternative. But, Machova et al. (2010) demonstrated unequivocally higher sensitivity of embryolarval tests compared to embryonic tests. It is due to longer duration of exposure of organisms and inclusion of critical period of fish development. It means transfer from endogenous to exogenous nutrition and change from embryonic to larval stage.

1.5. OXIDATIVE STRESS

Aquatic environments receive an increased number of agricultural and industrial chemicals including pyrethroids, which being taken up by organisms may perturb free radical processes. Free radicals are atoms, molecules, or ions with unpaired electrons. They are capable of independent existence and seek another electron to achieve an electron bond of stable configuration. They have ability of high reactivity and limited time of existence. Free radicals of oxygen and nitrogen are the most important in organisms. They undergo transformations and may cause the formation of different substances, which however, do not have an unpaired electron. These new substances together with the original free radicals are referred to as reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Racek and Holecek, 1999). When ROS overwhelm the cellular defenses, they cause damage to proteins, membranes, and DNA. This disruption of the antioxidant defence, which leads to potential damage, is summarised as oxidative stress (Winston and Di Giulio, 1991). The activation of oxidative manifestations leads to the response of antioxidants, activation of expression of genes encoding antioxidant enzymes and elevation of the concentration of ROS scavengers (Stoliar and Lushchak, 2012). All organisms try to reduce damage from oxidative stress by using an antioxidant defence system. The antioxidant system in aquatic animals comprises both – low molecular mass and high molecular mass antioxidants (Livingstone, 2001). Low molecular mass antioxidants described by Lushchak (2011) include water-soluble compounds such as reduced glutathione (GSH), ascorbic acid (vitamin C), and lipid-soluble ones such as carotenoids (including β-carotene), retinol (vitamin A), α-tocopherol (vitamin E). High molecular mass antioxidant group consists of specific or non-specific proteins. Antioxidant

enzymes or enzymes providing needed cofactors are specific proteins of defence. Proteins that prevent ROS-induced damage by binding to transition metal ions belong to the group of non-specific proteins (Lushchak, 2011).

The practical use of oxidative stress markers in fish is connected to significant difficulties. For example oxidative stress markers differ in fish due to the seasons. Moreover, animals can adapt to low pollution conditions (Stoliar and Lushchak, 2012). Additionally, depending on the source of pollutant, steady-state ROS concentration can be enhanced transiently or chronically (Lushchak, 2011). In spite of this, oxidative stress and changes in antioxidant defence are proposed as biomarkers of aquatic pollution. Lipid peroxidation or oxidation of polyunsaturated fatty acids, measured usually as a level of thiobarbituric acid reactive substances (TBARS), is very frequently used to analyse the effect of pollutants (Livingstone, 2001). GSH is the most frequently studied scavenger in the field studies. The hepatic ratio of oxidized to reduced glutathione (GSSG/GSH) may be an appropriate biomarker of oxidative stress. The increase of the ratio of GSSG/GSH in fish could be expected. Antioxidant enzymes are commonly used in environmental polution assessments. Superoxide dismutase (SOD), catalase (CAT), and at least one of GSH-related enzymes are also frequently included in studies (Stoliar and Lushchak, 2012). Use of antioxidant enzymes and non enzymatic parameters has been proved to be useful tools as biomarkers of environmental pollution in aquatic organisms (Javed et al., 2016).

1.6. AIMS OF THE THESIS

The main aim of this work was to assess the sensitivity of early developmental stages of common carp (*Cyprinus carpio* L.) to Cyperkill 25 EC (alpha-cypermethrin 250 $g.l^{-1}$) and Nexide (gamma-cyhalothrin 60 $g.l^{-1}$). These chemicals are commonly used in agriculture and their residues have been already detected in surface water as mentioned above. Sensitivities of fish were examined in several increasing concentrations during embryolarval period and the effect was assessed with respect to

- Hatching and mortality
- Behaviour
- Biometric parameters (length, weight, and Fulton's condition factor)
- Ontogenetic development
- Histopathology
- Parameters of oxidative stress and antioxidant defence

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CHAPTER 2

PYRETHROIDS INFLUENCE ON FISH

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Review Article

PYRETHROIDS INFLUENCE ON FISH

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Summary: Pyrethroids belong to the most commonly used pesticides worldwide. Their massive expansion is a threat to the natural environment including the aquatic ecosystems. Although pyrethroids are rapidly degraded in soil and plants, they are extremely toxic to fish because of fish high sensitivity to them.
Pyrethroids are divided by characteristic into type I and type II. Both types cause similar neurological symptoms. They affect sodium channels of nerve filaments and type II pyrethroids even affect chloride and calcium channels. Critical in fish pyrethroid intoxication is slower elimination than in birds and mammals. Pyrethroids are absorbed by fish gills readily. After distribution to bile, liver, kidney and red blood cells, they are metabolized by hydrolysis, hydroxylation and conjugation to glucuronides and sulphates. Disorders of movement and breathing during acute poisoning are followed by death. Chronic effects of pyrethroids induce behaviour changes, blood profile changes, histopathological changes, decreased growth, immune system effects and endocrine effects. Both types of toxicity reduce reproductive potential. Toxicity of pyrethroids depends on many external and internal factors.

Key words: pyrethroids; fish; neurotoxicity; sensitivity; physiological disturbances

Abbreviations & Units: ALP - alkaline phosphatase; ALT - alanine transaminase; AST - aspartate transaminase; ATP - adenosine triphosphate; CA - carbonic anhydrase; CK - creatine kinase; EPA - Environmental Protection Agency; GABA - gamma-aminobutyric acid; GDH – glutamate dehydrogenase; Hb - haemoglobin; HSP - heat shock protein; LDH - lactate dehydrogenase; MCH - mean cell haemoglobin; MCHC - mean corpuscular hemoglobin concentration; MCV - mean cell volume; mRNA - messenger ribonucleic acid; PCV – packed cell volume, haematocrit; PGF2α – F-type prostaglandin; RBC - erythrocyte counts

Introduction

Pyrethroids are synthetic analogues of the natural pyrethrins, extracts of the ornamental *Chrysantemum cinerariaefolium* and its related species. Pyrethrins had been used for decades for control of insects. They were selective, safe and had short half lives. Although they were acutely toxic to fish, very few accidental poisoning occured because they were not registered for aquatic use and they seldom had enough persistence to reach water from normal application (1).

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The 1st generation of pyrethroids was developed in the 1960s, the 2nd generation was developed in 1970s. Many of pyrethroids have been produced with improved physical properties (involatility, lipophilicity) and greater insecticidal activity (knockdown) since then (2). Pyrethroids disrupt the insect nervous system and this determines them to protect food grains and other agricultural products against pests. They began to be used as ectoparasiticides in veterinary and human medicine too (3, 4). They have replaced natural pyrethrins especially due to their better photostability gradually. Pyrethroids use has increased rapidly in the past three decades. Pyrethroids are thermostable and photostable, slightly soluble in

water and highly soluble in fats. The presence of halogens in some pyrethroids contributes to the greater persistence and provides better residual activity against insect together with higher potential negative effects on the environment (5).

Classification of pyrethroids

Pyrethroids are divided into type I and type II, based on their structure, chemical and neurophysiological properties and toxicological action. Type I pyrethroids are without a cyano moiety at the α-position (i.e. permethrin, bifenthrin, allethrin, tetramethrin, resmethrin, phenothrin, bioresmethrin, etofenprox, prallethrin, tefluthrin), while type II pyrethroids have an α-cyano moiety at the benzylic carbon of the alcohol portion of the ester (i.e. cypermethrin, cyfluthrin, deltamethrin, cyphenothrin, flumethrin, cycloprothrin, fenvalerate, fluvalinate). Type II pyrethroids are more effective (6). All pyrethroids affect the sodium channels of nerve filaments. They extend time of opening and closing of sodium channels and extend their depolarisation phase. Moreover, type II pyrethroids affect the GABA receptors in the nerve filaments and affect chloride and calcium channels (6-9). Type I pyrethroids cause a type I poisoning called *"T syndrome"*, whereas type II pyrethroids induce a type II poisoning, known as *"CS syndrome"* in mammals (2). T- syndrome mainly includes symptoms like aggressive sparing behaviour, increased sensitivity to external stimuli, fine tremors, prostration, coarse body tremor, increase of body temperature. Pyrethroids that induce a "choreoathetosis with salivation" response are called CS-syndrome pyrethroids and result in a broader range of toxic events due enhanced neurotransmitter release. Their main symptoms are: chewing, profuse salivation, pawing and burrowing, coarse body tremor, increased startle response, abnormal locomotion of posterior limbs, sinuous writhing (choreoathetosis) and clonic and tonic seizures (7). They cause cardiac contractions (3).

Summarized all pyrethroids interfere with nerve cell function by interacting with ion channels. Pyrethroids also modulate the release of acetylcholinesterase in the brain (10) and can inhibit ATP-ases (11). They can disrupt hormon-releated functions. But their effects on the endocrine system are not described uniformly (12).

Presence in the aquatic environment

Pyrethroids are absent in natural water normally. They may contaminate aquatic ecosystems as pollutants, because they are an important group of pesticides. The contamination of surface waters by pesticides used in agriculture is a problem of worldwide importance (10, 13). Ecological catastrophes following application of deltamethrin for mosquito control have already been in 1991 and 1995. Deltamethrin exposure have been one of main causes of massive eel (*Anguilla Anguilla L.*) devastation in Lake Balaton, Hungary (14). Pesticides are also very important in veterinary medicine as ectoparasiticides. They are popular due to their strong and extended insecticidal and simultaneously acaricidal effects. Pyrethroids are also used as antiparasitic drugs in human medicine and they are used extensively in urban settings to control several medically important insects that vector diseases. In aquaculture, pyrethroids are applied to control some parasitic diseases caused by, for example, *Lepeophtherius salmonis* or other sea lice in salmon farming. These products mainly based on deltamethrin are used in Scandinavian countries or Canada (15, 16). In addition to the recent increased interest in introduction of using deltamethrin in warm waters too, there are encouraging therapeutic results against isopoda with no side effects on the sea bass (*Dicentrarchus labrax L.*) (17).

Aquatic organisms can be affected by pesticides during their improper application or improper handling. Pesticides can get into the water directly due to the incorrect application. They can get into the water during the disposal of unused residues or due to accidents during transport. Pesticides also can get into the water indirectly after running off from surrounding treated products (18). The residues of cypermethrin have been widely detected in water and sediment samples from streams and rivers draining major agricultural districts (19).

Toxicity in the aquatic environment

Pyrethroids are fairly rapidly degraded in soil and plants in the environment (2). Pyrethroids induce rapid onset of poisoning symptoms but persist only for a short time in the water column due to ability of adsorption by organic matter and deg-

radation (20). The major degradation processes are ester hydrolysis and oxidation at various sites of the molecule. Pyrethroids have high hydrophobicity and they are rapidly and strongly adsorbed into particulate material (21). The pyrethroids are strongly adsorbed on soil and sediments. Pyrethroids are widely recognized as being strongly lipophilic, and thus highly hydrophobic (21-23), adsorbing almost exclusively to organic carbon molecules in water sediment slurries within 24 hours (24). Furthermore, pyrethroids have shorter chemical half-lives than their organophosphate predecessors, ranging from several days (22) to around one month in aerobic sediments (25). Sediment organic carbon plays a critical factor in determining the bioavailability of a given pyrethroid in a particular aquatic system, and accordingly, the pyrethroid's potential toxic effects (24). Microbial biodegradation of pyrethroids in aquatic system (in the sediment and water column) has been acknowledged to play an important role in the degradability and the persistence of the residues (26).

Fish sensitivity

Pyrethroids have been shown to be up to 1000 times more toxic to fish than to mammals and birds at comparable concentrations (5, 27). Fish sensitivity to pyrethroids may be explained by their relatively slow metabolism and slow elimination of these compounds (7, 28). It may be explained as a result of exposure of toxicokinetic (i.e. absorption, biotransformation, distribution and elimination) and toxicodynamic (i.e. biochemical and physiological effects) factors (7). Unlike most animals, in which pyrethroids have a short life and are readily metabolized, fish are reported to be deficient in enzymes that hydrolyze these insecticides (1, 29-31).

The hypersensitivity of fish to pyrethroid intoxication is due partly to species specific differences in pyrethroid metabolism, but second important factor is higher sensitivity of the piscine nervous system to these pesticides. Fish brain seems to be more susceptible to pyrethroids than mammal and bird brains are (1, 32). The third factor is route of exposure. Pyrethroids are absorbed directly via the gills into the blood stream (31).

Pyrethroids are inhibitors for fish carbonic anhydrase enzymes, and might cause undesirable results by disrupting acid–base regulation as well as salt transport. The most potent inhibitor is deltamethrin. The most affected CA enzymes are in muscle tissue and the lowest inhibition of CA enzymes is in liver tissue (33).

Types of poisononig

Acute toxicity

Acute toxicity is defined as a significant reduction in survival of the exposed organisms within a relatively short time and is expressed as the species specific median lethal concentration (LC50) (12). The value 96 h LC50 is under 10μg/L in fish generally. Salmonid species are more susceptible than carp species (5, 7). The 96 h LC50 of cypermethrin is 3.14 μg/L in rainbow trout (*Oncorhynchus mykiss*) (34) and 4.0 μg/L in Indian carp (*Labeo rohita*) (10). But deltamethrin is described to be more toxic in common carp (*Cyprinus carpio*) than in rainbow trout on the contrary (35). Acute toxicity also influences viability of embryos and leads to significant increase of dead larvae even if concentarion is orders of magnitude less (31, 36).

Chronic toxicity

Chronic toxicity effects can occur at exposure levels far below the concentration that causes lethality. Sublethal biological responses include behavior changes, reduced growth, immune system effects, endocrine effects including decrease of reproductive success, histopathological and biochemical changes (12). Disturbance of the non-specific immune system is connected with decreased production of leucocytes. Changes of colours and integrity of body surface develop during the weeks of exposure (37). Early life stages are more susceptible to chronic toxicity of pyrethroids than adult fish (5, 12, 38). Fingerlings of Indian carp change shape of their bodies in sublethal exposure. They become lean towards the abdomen position compared to the control fish and they seem to be under stress, but this is not fatal (10).

Toxicokinetics

Fish in general are exposed to pyrethroids through their gills, which are multifunctional and complex organs with which fish make intimate contact with their ambient water (39). Py-

rethroids are attracted to the non-water soluble components of cells due to their lipophilicity and permeate through the gills easily, even from water containing low levels of pyrethroids. This is a contributing factor in the sensitivity of the fish to aqueous pyrethroid exposures (40, 41).

When rainbow trout body was studied, the greatest amount of radiolabeled fenvalerate residues were found in the bile, then in the fat deposits and followed by the liver, gill, kidney and red blood cells. Concentration in the brain was lower than in most other tissues (42).

Common way of detoxification is hydrolysis in liver and plasma of animals. The acid and alcohol components of pyrethroids that result from ester hydrolysis are of minimal toxicity to any animals (1, 4). Hydrolysis is followed by hydroxylation and conjugation to glucuronides and sulphates, which are excreted in urine (4). But fish treated by pyrethroids do not show significant levels of ester hydrolysis products in urine or bile. It seems that permethrin elimination from fish is quantitatively different from that reported in mammals and birds, with oxidative degradation predominating and ester hydrolysis constituting a minor reaction (7). Oxidation products are most common, primarily due to ring hydroxylation and side chain oxidation reactions in fish (1, 7). Because of lack of hydrolysis detoxification, products of ester hydrolysis are rarely found (1) and only low levels could be confirmed (7).

Toxicokinetic experiments indicate that fenvalerate elimination rate in rainbow trout is much slower than in birds and mammals (1). The half-lives for elimination of several pyrethroids by trout are all greater than 48 h, while half-lives of elimination in birds and mammals range from 6 to 12 h (7).

Toxicodynamics

Pyrethroids bind to a receptors at the sodium gate of neuron and prevent it from closing fully. The resulting steady leakage of sodium ions into the neuron creates a less stable resting state and the neuron is susceptible to repetitive firing of nerve, which leads to hyperactivity, tremors and tetany (43, 44).

Effect of pyrethroids in mammals and insects depends on stereospecificity highly. Some isomers demonstrate strong potency and their mirror image isomers show almost no toxicity. The available data for fish are not so uniform (1, 7). Fish seems to be equally sensitive to both cis and trans isomers of permethrin (1). In contrast stereospecific influence of fenvalerate toxicity on fish is similar to that of mammals. The 2S pair of isomers is 3.3 times more toxic to fathead minnow (*Pimephales promelas*) than technical mixture with all four isomers (1, 45). Recent research indicates stereoselectivity in the estrogenic activity of permethrin, which results from stereoselective biotransformation of the parent compound to more estrogenic metabolites. 1S-cis-permethrin has a higher activity than the 1R-cis enantiomer (46).

Synthetic pyrethroids have deleterious influence on Ca-ATPases and other ATPases in vertebrates and invertebrates so additional toxic effect must be considered (1). Fish treated by cypermethrin show inhibition of gill Na+/K+ -ATPase activity which induce osmotic imbalance and influence maintenance of osmotic and ionic homeostasis (11).

It is difficult to differentiate between type I and type II syndromes in fish. Both types of pyrethroids cause similar neurological symptoms and fish generally become inactive before death (7).

Clinical symptoms of poisoning

The following clinical symptoms are observed during acute toxicity tests on rainbow trout and common carp: accelerated respiration, loss of movement coordination, fish lay down at their flank and move in this position. Subsequent short excitation stage (convulsions, jumps above the water surface, movement in circles) changes into a resting stage, and another short excitation period follows again. In the end fish fall into damp, move mainly at their flank. Respiration is slowed down, the damp phase and subsequent agony are very long (34, 47). Similar neurological symptoms could be observed after 2 weeks of exposure to subacute concentration of deltamethrin (1.46 µg/L) on monosex Nile Tilapia (*Oreochromis niloticus*). It is accompanied by colour darkening of the body surface, slight erosions and/or rotting of fins and tail, slimness, general loss of fish scales, eye cataract and sometimes exophthalmia. Internally, there is general congestion of the liver, kidneys, gills and blood in the abdominal cavity (37). Loss of equilibrium, vertically hanging, gill flailing, erratic swimming, swimming at the water surface, air gulping from the water surface or staying mo-

tionless on the aquarium bottom are observed during tests of acute toxicity of deltamethrin on the fry rainbow trout. The toxicity and presence of symptoms depends on increasing concentration and exposure time. Colour darkening is observed at concentrations higher than 8 µg/L (48). Study of acute cypermethrin toxicity on rainbow trout describes the almost identical neurological symptoms again (gill flailing, hyperactivity, loss of buoyancy and inability to remain upright) (27) and on common carp abnormalities of movement again and hyperactivity are described especially (49). Necropsy after acute toxicity tests on rainbow trout and common carp reveales watery mucus on body surface, excess fluids in body cavity and congestion of visceral vessels (2). Acute toxicity of cypermethrin in silver catfish (*Rhamdia quelen*) causes loss of equilibrium, vertical hanging in water, rapid gill movement, erratic swimming, sudden swimming motion in a spiral fashion after long periods of inactivity and sudden movement after prolonged inactivity in the tank bottom (50). Respiration and movement abnormalities are described mainly (30, 51).

Endocrine and reproductive disruption

Cypermethrin reduces the fertilization success in atlantic salmon (*Salmo salar*). It inhibites ability of male salmon parr to detect and respond to the female salmon priming pheromone PGF2α. The increase in expressible milt and the levels of plasma sex hormones are reduced in the presence of the pyrethroid as the result of impaired olfactory detection of the priming pheromone (32).

Biochemical and haematological profiles

Reduction in hepatic glycogen accompanied by increased level of plasma glucose is a common reaction of fish against xenobiotic insult followed by metabolic stress (51-54).

In rainbow trout cypermethrin causes significantly decreased concentration of ALP and significantly increased concentration of ammonia, AST, LDH, CK and lactate in blood plasma (34). In common carp bifenthrin causes increased concentration of ammonia, AST and CK too (54). In silver catfish cypermethrin causes increasing of levels Na^+, K^+, Mg^{2+}, P, urea, glucose, cholesterol, creatinine, AST and ALP, whereas total protein, triglyceride and ALT levels are reduced (50). In common carp deltamethrin causes decreased concentration of total protein in blood plasma (47).

An increase of plasma ammonia level is supposed due to an increase of amino acids catabolism and due to an inability to convert the toxic ammonia to less harmful substances and failure of ammonia excretion. Decrease of the levels of free amino acids accompanied by increase of the activities of AST, ALT and GDH in the vital organs is seen, because the amino acid catabolism is one of the main mechanisms, which ensure immediate energy demand to the fish (55). An increase of AST and CK indicates tissue impairment based on the stress (56). The increase of LDH level is connected with metabolic changes, i.e. the glycogen catabolism and glucose shift towards the formation of lactate in stressed fish, primarily in the muscle tissue (52). Metabolic stress induced by pyrethroids is accompanied by changes in levels of enzymes of antioxidant defense (57, 58).

Studies of haematological parameters are inconsistent. In catfish (*Heteropneustes fossilis*) deltamethrin causes a significant increase in RBC, but a small decrease in Hb, MCV, MCH and PCV (59). In common carp acute intoxication of deltamethrin causes decrease in RBC, Hb and PCV and has no effect on MCV, MCH, MCHC, total leukocyte count and relative as well as absolute counts of lymphocytes, monocytes, neutrophil granulocytes and their developmental forms (47). In rainbow trout cypermethrin causes a significant increase in the levels of RBC and a significant decrease in the Hb, MCH, MCHC, thrombocyte count and erythrocyte sedimentation rate (60). But only significant decrease in count of developmental forms of myeloid sequence and the segmented neutrophilic granulocytes is described in another acute toxicity test with cypermethrin and any effect on the haematological indicators such as RBC, Hb, PCV, MCV, MCHC, MCH and leukocytes (34). Elevation of the relative and absolute monocyte counts is described in common carp treated by bifenthrin (54). Deltamethrin causes decreased lymphocyte and basophile percentages and decrease of total leukocyte and erythrocyte counts, Hb and PCV simultaneously with serious hypoproteinaemia, hypoalbuminaemia, hypercholesterolaemia, hyperglycaemia in Nile tilapia exposed to subacute concentration for weeks (37).

Post-mortem findings

Severe teleangioectasiae are revealed in secondary lamellae of gills, with the rupture of pillar cells in 50% of fish treated by bifenthrin (54). The most common gill changes of fish treated by deltamethrin are desquamation and necrosis. It is followed by the lifting of the lamellar epithelium, oedema, aneurism, hyperplasia of epithelial cells and fusion of the secondary lamellae. These changes are results of direct responses of gill to the action of deltamethrin and simultaneously defense responses of organism against toxicant to make it more difficult to access to blood stream (61).

Bifenthrin causes degeneration of hepatocytes, especially in periportal zones, in 40% of treated fish. Affected hepatocytes show pycnotic nuclei and many small or single large vacuoles in the cytoplasm. Vacuole shape is typical for fatty degeneration of liver. It can imply the influence of pyrethroids in the digestive tract. (54).

Deltamethrin destructive effects in fish kidney are characterized by degeneration in the epithelial cells of renal tubules, pycnotic nuclei in the haematopoetic tissue, dilatation of glomerular capillaries, degeneration of glomeruli, intracytoplasmatic vacuoles in epithelial cells of renal tubules with hypertrophied cells and narrowing of the tubular lumen (61).

Factors influencing pyrethroids toxicity

A lot of factors can modulate the toxicity. Many synthetic pyrethroids have their 96 h LC50 values under 1μg/L, while chronic toxicity can be recorded at one to two orders of magnitude lower than that (5). Fish toxicity studies vary widely in their methodology (e.g., static conditions vs. flow-through exposures, nominal concentrations added to the water vs. measured concentrations). A lot of studies in standardized water demonstrate extraordinary toxicity, however field trials show the pyrethroids to be less potent than expected from laboratory studies. It is determined that pyrethroids, with their extremely low water solubility and high affinity for particulate matter in solution, do not remain bioavailable for uptake by the fish in the field ponds. When the pyrethroids molecules bind to the suspended solids or the sediment, the resultant toxicity is orders of magnitude less than predicted by the clean water assays (1).

Currently available formulations of pyrethroids are oil based, emulsifiable concentrates (EC). The emulsifiable formulation keeps the pyrethroids in solution longer compared to the technical chemicals and the pyrethroids adsorb to the glass quickly. Pyrethroids tend to bind to the glass and plastic (62). EC formulations are usually two to nine times more toxic than the technical grade forms, most likely due to synergistic interactions (63).

The ionic characteristics of the water can exert influence on the toxicity of pyrethroids to fish. Water hardness (summary Ca^{2+} + Mg^{2+}) is shown to be a factor in bluegill (*Lepomis macrochirus*) susceptibility to fenvalerate. The LC50 values are twofold higher in very soft water, compared to hard water. Increased toxicity on bluegill fry is recorded when salinity raises (64). Pyrethroids are more toxic at lower temperatures and conversely fish are more susceptible at lower temperatures (1, 5, 13, 44). There is a possible increase in the toxic impact of pyrethroids on reproduction during spawning season in the cold water (32).

Pyrethroids appear to be generally more toxic to smaller fish than larger ones (5, 13, 51). Fish embryos appear to be less sensitive to pyrethroids than larvae (12).

Toxicity of pyrethroids is dramatically influenced by the presence of particulate matter in the water column, probably through adsorbtion of the very lipophilic toxicant molecules to the suspended matter, sediment and dissolved organic matter (40, 65). That is why adsorbtion of pyrethroids is more quick in system like farm ponds with organic matter than in typical streams (12).

Piperonyl butoxide is commonly added to pyrethroid products to enhance the toxic effects of the active ingredient. Piperonyl butoxide inhibites a group of enzymes, which are involved in pyrethroid detoxification (12).

Conclusion

Pyrethroids are predominant class of insecticides. Their widespread use represents an increasing threat of water pollution. Investigation of their properties in connection with environment, acute and chronic effects and potential bioaccumulation must continue thoroughly. Research on non target species including fish should be really detailed.

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VPLIV PIRETROIDOV NA RIBE

Z. Richterová, Z. Svobodová

Povzetek: Piretroidi spadajo med najbolj pogosto uporabljene pesticide po vsem svetu. Njihova masovna uporaba ogroža naravno okolje, vključno z vodnimi ekosistemi. Čeprav se piretroidi v tleh in rastlinah hitro razgradijo, so za ribe zelo strupeni. Glede na svoje značilnosti se piretroidi delijo v dve skupini, tip I in II. Oba povzročata podobne nevrološke simptome. Piretroidi vplivajo na delovanje natrijevih kanalčkov v živčnih celicah, piretroidi tipa II poleg tega vplivajo tudi na kloridne in kalcijeve kanalčke. Ključnega pomena pri zastrupitvi rib s piretroidi je njihovo počasnejše izločanje kot pri pticah in sesalcih. Piretroidi se hitro absorbirajo preko škrg, po krvi pridejo v žolč, jetra, ledvice in rdeče krvne celice, kjer se presnavljajo s hidrolizo, hidroksilacijo in vezavo na glukuronide in sulfate. Akutna zastrupitev rib se kaže z motnjami v gibanju in dihanju ter smrtjo. Kronična izpostavljenost piretroidom pri ribah povzroči spremembe v obnašanju, krvni sliki, histopatološke spremembe, zmanjšano rast ter vpliva na imunski in endokrini sistem. V obeh primerih pa je tudi prizadeta reprodukcijska sposobnost. Toksičnost piretroidov je odvisna od številnih notranjih in zunanjih dejavnikov.

Ključne besede: piretroidi; nevrotoksičnost; občutljivost; fiziološke motnje

CHAPTER 3

EFFECTS OF CYHALOTHRIN-BASED PESTICIDE ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

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Research Article

Effects of Cyhalothrin-Based Pesticide on Early Life Stages of Common Carp (*Cyprinus carpio* L.)

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The effects of Nexide (a.i. gamma-cyhalothrin 60 g L^{-1}) on cumulative mortality, growth indices, and ontogenetic development of embryos and larvae of common carp (*Cyprinus carpio* L.) were studied. Levels of oxidative stress parameters glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST), and lipid peroxidation were determined. Eggs of newly fertilised common carp were exposed to Nexide at concentrations 5, 25, 50, 100, and 250 μg L^{-1} (0.3, 1.5, 3, 6, and 15 μg L^{-1} gamma-cyhalothrin). All organisms exposed to concentrations higher than 50 μg L^{-1} died soon after hatching; at 25 μg L^{-1}, 95% mortality was recorded. Larvae exposed to 5 μg L^{-1} showed significantly lower growth and retarded ontogenetic development compared to control. Histological examination of the livers of larvae from the exposed group revealed dystrophic changes. The value of detoxification enzyme GST of organisms from the exposed group was significantly higher compared to the control and the value of defensive enzyme GPx was significantly lower compared to the control. The results of our investigation confirmed that contamination of aquatic environment by pesticides containing cyhalothrin may impair growth and development of early life stages of carp and cause disbalance of defensive enzymes.

1. Introduction

Pyrethroids are synthetic analogues of natural pyrethrins that occur in the daisy (*Chrysanthemum cinerariaefolium*) and related species. Pyrethroids have replaced natural pyrethrins as agricultural pesticides, primarily due to their greater photostability. They act through disruption of the insect nervous system, leading to hyperactivity, paralysis, and death. Pyrethroids are among the most commonly used pesticides worldwide and pose a threat to the natural environment including nontarget organisms, such as fish that are highly sensitive. Contamination of surface water by pesticides is widespread [1, 2] and median concentrations lethal to fish of the more commonly used pyrethroids are generally less than 10 μg L^{-1} [3].

Cyhalothrin is a pyrethroid that contains a cyano-3-phenoxybenzyl group. It blocks sodium channels of nerve filaments by lengthening their depolarization phase as well as affecting gamma-aminobutyric acid receptors that involve chloride and calcium channels in nerve filaments [4–7]. The presence of halogens in a formulation contributes to greater persistence and provides better residual activity against insects together with higher potential for negative effects on the environment [3].

Gamma-cyhalothrin is an insecticidal enantiomer of the synthetic pyrethroid lambda-cyhalothrin. Lambda-cyhalothrin consists of two of the four enantiomers of the cyhalothrin molecule. Different enantiomers of lambda-cyhalothrin show different toxicity to zebrafish (*Danio rerio*). The 24 h LC50 (−) enantiomer was reported as 2.03 μg L^{-1},

while 24 h LC50 of (+) enantiomer was $>1.2 \times 10^{-2}\ \mu g\ L^{-1}$ [8]. Lambda-cyhalothrin consists of approximately 50% gamma-cyhalothrin, and its biological activity against pests and ecotoxicity to aquatic communities is relevant to this ratio [9].

The aim of this study was to evaluate the effect of Nexide with its active ingredient gamma-cyhalothrin on early life stages of common carp (*Cyprinus carpio* L.) using an embryo-larval toxicity test.

2. Materials and Methods

2.1. Experimental Substances. Cyhalothrin (S)-α-cyano -3-phenoxybenzyl(1R,3R)-3-[(Z)-2-chlor-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate was tested in the form of the commercial preparation Nexide (Cheminova A/S, Denmark). Gamma-cyhalothrin as an active ingredient of this preparation was developed by Pytech Chemicals GmbH. The insecticide is a suspension of microcapsules containing $60\ g\ L^{-1}$ gamma-cyhalothrin in an aromatic solvent intended for dilution in water.

2.2. Experimental Animals. Fertilised eggs of common carp were obtained from the breeding station of the Department of Fish Genetics and Breeding of the Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Czech Republic. Eggs were produced according to standard methods of artificial reproduction [10].

2.3. Experimental Design

2.3.1. Early Life Stage Toxicity Test. The test was based on the methodology of the OECD 210 Guideline for Testing of Chemicals [11]. The method was modified, in that only fertilised eggs were selected for testing. One hundred fertilised eggs were inserted to each crystallisation dish at 24 h after fertilisation. The ingested volume of control and each Nexide concentration was 1 L, and tested concentrations were 5, 25, 50, 100, and $250\ \mu g\ L^{-1}$ (0.3, 1.5, 3, 6, and $15\ \mu g\ L^{-1}$ gamma-cyhalothrin). The trial was performed in duplicate. Dechlorinated tap water (pH 7.98, N-NH_4^+ $< 0.02\ mg\ L^{-1}$, NO_2^--N $0.006\ mg\ L^{-1}$, NO_3^--N $1.55\ mg\ L^{-1}$, PO_4^{3-}-P $0.09\ mg\ L^{-1}$, COD_{Mn}/chemical oxygen demand with oxidizing agent potassium permanganate/$0.6\ mg\ L^{-1}$, and oxygen saturation > 80%) was used for dilution of test concentrations and for control baths. Daily monitoring was conducted to maintain temperature at 21–23°C, pH 7.5–8.5, and dissolved oxygen >60%. Each dish was continuously gently aerated, and the water bath was renewed once a day. Observations of hatching, survival, anatomy, and behaviour were made daily. Unhatched eggs and dead larvae were removed. Beginning day 8, larvae were fed by freshly hatched brine shrimp (*Artemia salina*) nauplii *ad libitum*. The beginning of the test was considered to be one day after fertilization and was designated day 1. Hatching was mainly completed on day 4, and feeding with *A. salina* was initiated on day 8. The test was concluded on day 35 when the majority of the fish in the control dishes reached the juvenile stage. Samples for observations of ontogenetic development, malformations, total length, and weight were taken on days 5, 12, 19, 26, 33, and 35. Ten samples were taken from the control and from the lowest Nexide concentration. At higher concentrations, this number was lower as a result of higher mortality of organisms. These samples were fixed in 4% formalin and examined after completion of the trial. Developmental stages comprised nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1-J2) stages [12]. At the end of the test, samples of control and from the $5\ \mu g\ L^{-1}$ concentration were taken for histological examination. These samples were fixed in 10% formalin and processed using conventional paraffin techniques. Tissue sections were stained with haematoxylin and eosin. Slides of liver, intestine, kidney, and gill were examined at a magnification range 100–1000x by light microscopy.

2.3.2. Cumulative Mortality and Biometric Data. Cumulative mortality was recorded daily, and samples of embryos/larvae were measured and weighed. Weight to the nearest 0.1 mg was measured by an analytical balance, WAS 220/C/2. Total length of embryos/larvae was measured using a binocular loupe and a scale to the nearest 0.01 mm. Fulton's condition factor (FCF) of fish surviving at the end of the trial was calculated using the formula $FCF = W \times TL^{-3} \times 100$, where W is weight in g and TL is total length in cm.

2.3.3. Determination of Oxidative Stress. Ten control fish and ten fish exposed to $5\ \mu g\ L^{-1}$ for investigation of oxidative stress were taken on day 35 and placed immediately into liquid nitrogen for transport to a screening laboratory. Whole bodies were homogenized in a 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.4) and centrifuged at 11,000 g for 20 min at 4°C. The supernatant was pipetted into individual Eppendorf tubes and kept at −85°C until analysis. Supernatant was used for determination of glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST), and protein concentration. Noncentrifuged homogenate (stored at −85°C) was used to estimate lipid peroxidation. Protein concentration was quantified with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard [13]. Total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm [14]. Specific activity was expressed as the nmol of the formed product per min per mg of protein. The catalytic concentration of GR was determined by measuring NADPH oxidation at 340 nm [15]. The catalytic concentration of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm [16]. Specific activity of GR and GPx was expressed as nmol of NADPH consumption per min per mg of protein. The activity of CAT was determined by measuring H_2O_2 breakdown at 240 nm and expressed as μmol of decomposed H_2O_2 per min per mg of protein [17]. Lipid peroxidation was determined using the thiobarbituric acid-reactive substances (TBARS) method at 535 nm [18]. The concentration was expressed as nmol per g wet weight of

tissue. All parameters were measured spectrophotometrically using a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3.4. Determination of the Active Ingredient. Cyhalothrin in water samples was determined after extraction using isooctane by gas chromatography with electron capture detection (GC/ECD) [19]. Chromatography was performed on a column HP-5MS (60 m × 0.32 mm, 0.25 μm). The carrier gas was helium with a flow rate of 25 mL min^{-1} and a splitless injection volume of 2 μL and temperature of 250°C was used. Temperature column program was 100°C for 2 min, increased to 230°C at 14°C min^{-1}, increased to 285°C at 4°C min^{-1}, and held at 285°C for 40 min. This method was used to confirm the presence of cyhalothrin, the active substance at >80% throughout the test.

2.3.5. Statistical Analyses. The software program Statistica, v. 10.0, for Windows (StatSoft, Prague, Czech Republic) was used to compare differences among the test groups. Prior to analysis, all measured variables were checked for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, a one-way ANOVA was employed to determine whether there were significant differences in measured variables among experimental groups. When a difference was detected ($P < 0.05$), Dunnett's multiple-range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal-Wallis) was used. Normality of oxidative stress data was assessed by Shapiro-Wilk test; data were normally distributed. Test of homogeneity of variance (Levene's test) and an analysis of variance (ANOVA) test were performed, followed by multiple comparisons (Tukey-HSD test). Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Cumulative Mortality. Only small differences in mortality were observed among test groups and control days 1–5 (Figure 1). The first mortalities appeared on day 3 in 5, 25, and 250 μg L^{-1} concentrations. On day 6 delayed and reduced hatching along with posthatch mortality was observed with 1%, 1%, 6%, 3%, and 14% mortality at 5, 25, 50, 100, and 250 μg L^{-1}, respectively. No mortality occurred in the control group. Surviving embryos/larvae in the 250 μg L^{-1} exposure showed almost no movement, while control and those exposed to lower concentrations swam normally. Total mortality was observed in 50 and 250 μg L^{-1} concentrations on day 9 and in 100 μg L^{-1} on day 11. Day 18 of the test was accompanied by heavy mortality in the 25 μg L^{-1} group. At the conclusion of the trial, 93.5% larvae were viable in the 5 μg L^{-1} group and 5% in the 25 μg L^{-1} concentration, compared to a 95.5% survival rate in the control group.

3.2. Length and Weight Growth. Growth in total length and weight and ontogenetic developmental stage are recorded (Figures 2 and 3). Samples from all concentrations were taken

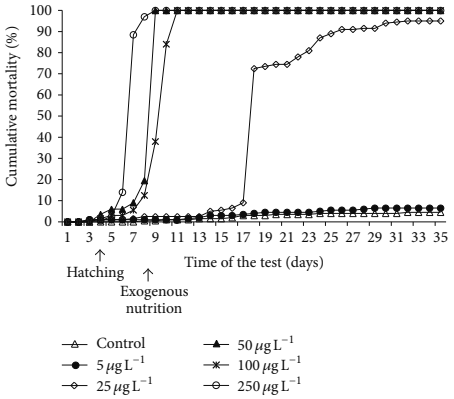


Figure 1: Cumulative mortality of common carp embryos and larvae during embryo-larval toxicity test with Nexide.

only on day 5, because no larvae survived beyond day 11 in 50, 100, and 250 μg L^{-1} exposure. Only the control and the 5 μg L^{-1} group were compared at the completion of the trial. Nineteen larvae/juvenile fish from the control group and ten larvae exposed to 5 μg L^{-1} were examined on day 35. Insufficient individuals survived in the 25 μg L^{-1} group to be included in growth comparisons.

FCF was calculated as an index of thriving in fish from the control group and the 5 μg L^{-1} concentration on day 35. The difference between control (1.174 ± 0.054) and concentration of the 5 μg L^{-1} (1.131 ± 0.085) was significant ($P < 0.05$).

3.3. Early Ontogeny. From day 26, developmental stages of control and the 5 μg L^{-1} group showed differences. No fish from the 5 μg L^{-1} concentration reached the juvenile stage by the end of the test, remaining at larval stage 6 (Table 1). Macroscopic morphological anomalies such as curvature of the spine, changes in yolk sac, and shortening of body were rare in both groups and could be considered chance occurrences. A higher incidence of deeper pigmentation was observed in fish from the 5 μg L^{-1} concentration.

3.4. Histopathology. Light microscopy revealed significant differences in steatosis dystrophy in liver of fish exposed to Nexide at concentration of 5 μg L^{-1}. The extent and degree of hepatodystrophic changes appeared clearly at magnitude 400x (Figure 4). Magnification to 1000x revealed rare mitotic structures in liver cells of fish exposed to Nexide at concentration of 5 μg L^{-1} (Figure 5). The intestine, kidney, and gill of 5 μg L^{-1} and control did not show significant differences.

3.5. Effect on Oxidative Stress. Levels of oxidative stress parameters of control and the 5 μg L^{-1} concentration on day 35 are summarized in Figures 6 and 7. A significant decrease

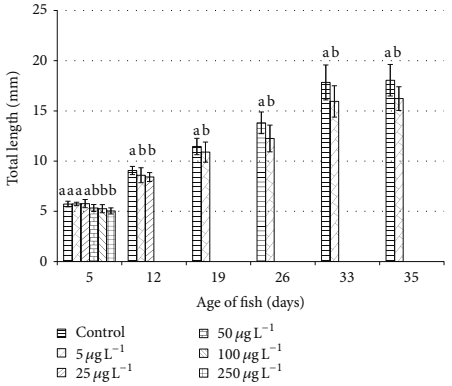


Figure 2: Effect of Nexide on total length (mean ± SD) of common carp larvae and juveniles during embryo-larval test. Significant differences ($P < 0.05$ on days 5–26 and $P < 0.01$ on days 33 and 35) between groups at each sampling time are indicated by different letters (a, b).

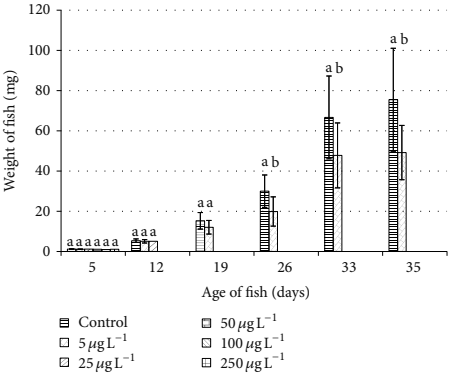


Figure 3: Effect of Nexide on weight (mean ± SD) of common carp larvae and juveniles during embryo-larval test. Significant differences ($P < 0.05$ except day 26 when $P < 0.01$) between groups at each sampling time are indicated by different letters (a, b).

($P < 0.01$) of GPx and significant increase ($P < 0.05$) of GST in 5 $\mu g\,L^{-1}$ group compared to control are shown in Figures 6 and 7.

Mean level of GR in control was 9.8 ± 1.36 compared to 11.3 ± 2.36 nmol of NADPH min^{-1} mg^{-1} of protein in 5 $\mu g\,L^{-1}$ group. Mean level of TBARS in control and in 5 $\mu g\,L^{-1}$ group was 12.1 ± 2.58 and 10.1 ± 4.19 nmol g^{-1} wet weight, respectively, and mean level of CAT was 31.4 ± 5.07 and 27.7 ± 7.32 μmol of H_2O_2 min^{-1} mg^{-1} of protein, respectively. There were no significant differences in GR, TBARS, and CAT levels between control and treated fish.

Table 1: Ontogeny of common carp exposed to Nexide at 5 $\mu g\,L^{-1}$ compared to control.

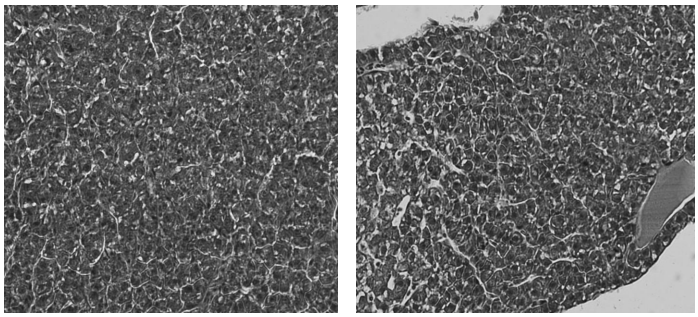
Sampling day	Developmental stages	
	Control	5 $\mu g\,L^{-1}$
Day 5	E8-E9	E8-E9
Day 12	L3-L4	L3-L4
Day 19	L4-L5	L4-L5
Day 26	L5-L6	L5
Day 33	L6-J1	L6
Day 35	L6-J1	L6

4. Discussion

The present study revealed significantly reduced growth, delayed and reduced hatching, and high mortality after hatching, especially at concentrations of 250 $\mu g\,L^{-1}$. Behaviour changes were observable as minimal movement of fish in 250 $\mu g\,L^{-1}$ preceding death. Histopathological examination revealed steatosis dystrophy in fish at the 5 $\mu g\,L^{-1}$ concentration. These findings are consistent with the toxic effects of common pyrethroids on fish [20]. Significantly lower FCF were observed in fish from concentration 5 $\mu g\,L^{-1}$ compared to control. Our results were in agreement with reports demonstrating a decline in condition factor in fish exposed to environmental pollutants [21], although this finding is not universal, as studies have reported no differences in common carp exposed to deltamethrin compared to control [22].

Exposure ≥50 $\mu g\,L^{-1}$ of Nexide resulted in death of all embryos/larvae. Lower concentrations did not cause 100% mortality but were associated with differences in biochemical parameters of oxidative stress and microscopic appearance of liver. The toxic effects of cyhalothrin described in literature are mainly associated with lambda-cyhalothrin, but the toxicity of isomers has been shown to differ. A comparison of 96 h LC50 values of gamma- versus lambda-cyhalothrin revealed respective levels of 0.047 $\mu g\,L^{-1}$ versus 0.149 $\mu g\,L^{-1}$ in bluegill (*Lepomis macrochirus*), 0.111 $\mu g\,L^{-1}$ versus 0.214 $\mu g\,L^{-1}$ in rainbow trout (*Oncorhynchus mykiss*), 0.17 $\mu g\,L^{-1}$ versus 2.3 $\mu g\,L^{-1}$ in guppy (*Poecilia reticulata*), and 0.27 $\mu g\,L^{-1}$ versus 0.64 $\mu g\,L^{-1}$ in zebrafish [9]. The 96 h LC50 lambda-cyhalothrin value for carp was reported at 0.5 $\mu g\,L^{-1}$ [23], and the 96 h LC50 lambda-cyhalothrin value for juvenile Nile tilapia (*Oreochromis niloticus*) was 2.901 $\mu g\,L^{-1}$ [24]. Thus we may presume that toxicity of lambda-cyhalothrin to early life stages of common carp may not be as significant as our results using gamma-cyhalothrin.

Oxidative stress parameters in whole body homogenates of larvae common carp in 5 $\mu g\,L^{-1}$ group revealed greater GST activity and lower GPx level compared to control, while differences in TBARS, GR, and CAT were not significant. Lambda-cyhalothrin was shown to lead to oxidative stress in liver of *O. niloticus* by increasing such indicators as lipid peroxidation, total glutathione (tGSH), GSH, TBARS content, and GST activity. An adaptive response was mounted by tGSH, GSH, and GSH-dependent enzymes. Oxidative



(a) (b)

Figure 4: Liver of common carp larvae and juveniles on day 35 of embryo-larval toxicity test (400x). Control group (a) and 5 μg L^{-1} Nexide (b) (photo by F. Tichý).

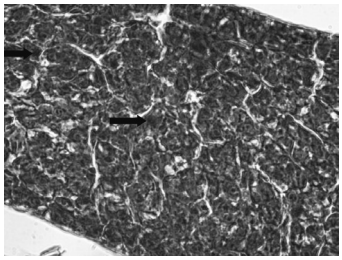


Figure 5: Rare mitotic structures (arrows) in liver of common carp larvae and juveniles exposed to 5 μg L^{-1} Nexide on day 35 of embryo-larval test (1000x) (photo by F. Tichý).

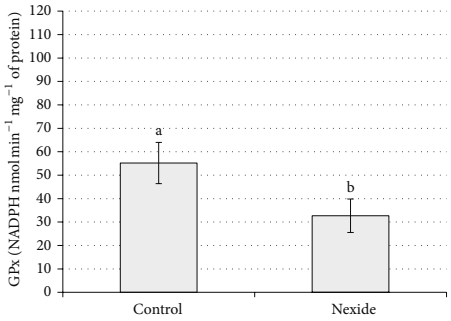


Figure 6: Effect of Nexide at 5 μg L^{-1} on GPx level in common carp larvae and juveniles on day 35 compared to control ($P < 0.01$).

stress is shown to upregulate GSH and GSH-related enzymes [24]. High levels of the antioxidant enzymes superoxide dismutase (SOD) and CAT followed exposure to cypermethrin in common carp. The enhanced lipid peroxidation in blood and tissue showed that cypermethrin-induced reactive oxygen species (ROS) were not completely scavenged by the antioxidant enzymes [25]. Many pesticides have been shown to be associated with production of oxidative stress in aquatic organisms, because they may induce the formation of ROS and alterations in antioxidant or free oxygen radicals scavenging enzyme systems [26–29]. Lambda-cyhalothrin has been reported to lead to oxidative stress by altering antioxidant systems and increasing lipid peroxidation in mammals [30, 31].

The present study did not show all pyrethroid effects previously reported, but influence on early developmental stages of common carp was clear. We found significant increase in mortality dependent on dose and duration of exposure. Acute toxicity tests of deltamethrin and cypermethrin on embryos and larvae of common carp have also shown dose-dependent decrease of hatching success [32, 33]. Our results agree that fish embryos appear to be less sensitive to pyrethroids than larvae [20].

5. Conclusion

Embryo-larval test on common carp with Nexide (containing 60 g L^{-1} of active substance gamma-cyhalothrin) revealed the following.

(i) Concentration of 250 μg L^{-1} caused 100% mortality of embryos after hatching.

(ii) Concentrations of 100 and 50 μg L^{-1} caused 100% mortality soon after beginning of exogenous nutrition.

(iii) Concentration of 25 μg L^{-1} caused 95% mortality of exposed organisms during 35 days.

(iv) The lowest tested concentration of Nexide (5 μg L^{-1}) caused slightly elevated mortality compared to

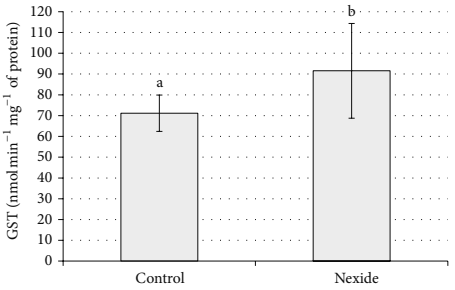


Figure 7: Effect of Nexide at 5 μg L^{-1} on GST level in common carp larvae and juveniles on day 35 compared to control ($P < 0.05$).

the control group, significantly lower growth (length and weight), and retarded ontogenetic development. Also dystrophy in liver, significantly greater activity of detoxification enzyme GST, and lower levels of defensive enzyme GPx compared to control were observed.

The results of our investigation confirmed that contamination of aquatic environment by pesticides containing cyhalothrin may impair growth and development of early life stages of carp and cause disbalance of defensive enzymes. That is why we recommend further attention to studies of long-term effects of pyrethroids on fish and focus the investigation on offspring quality and their susceptibility to infectious diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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CHAPTER 4

EFFECTS OF A CYPERMETHRIN-BASED PESTICIDE ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

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Effects of a cypermethrin-based pesticide on early life stages of common carp (*Cyprinus carpio* L.)

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ABSTRACT: The aim of this study was to assess the effects of Cyperkill 25 EC (a.i. cypermethrin 250 g/l) on cumulative mortality, growth indices, and ontogenetic development of embryos and larvae of common carp (*Cyprinus carpio* L.). An early-life stage toxicity test was used. Liver, intestine, kidneys, and gills of surviving larvae were examined, and the activity of the detoxifying and antioxidative enzymes glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST), as well as lipid peroxidation (TBARS) was determined. Eggs of common carp 24 h post-fertilisation were exposed for 35 days to Cyperkill 25 EC at concentrations of 7.2, 36, 72, 144, and 360 µg/l containing the active ingredient cypermethrin at concentrations of 1.8, 9, 18, 36, and 90 µg/l, respectively. All larvae exposed to concentrations higher than 144 µg/l showed signs of damage after five days and died in the next two days; at concentrations of 72 and 36 µg/l total mortality was observed several days after hatching. Larvae exposed to 7.2 µg/l survived to the end of the test but showed significantly lower growth ($P < 0.01$) and retarded ontogenetic development compared to controls. Examination of these larvae did not reveal histological changes. Activity of GST, GR, and GPx in the exposed group was significantly lower ($P < 0.01$), while CAT and TBARS did not show significant differences from controls. Exposure to Cyperkill 25 EC affected hatching and survival at tested concentrations above 7.2 µg/l. Alterations in oxidative stress parameters and retarded growth and ontogenetic development were evident at 7.2 µg/l.

Keywords: embryo-larva toxicity test; Cyperkill 25 EC; oxidative stress; pyrethroid; mortality; ontogenesis

Pyrethroids are synthetic analogues of the natural pyrethrins that occur in the ornamental pyrethrum daisy *Chrysanthemum cinerariaefolium* and related species. Synthetic analogues that show less rapid photodegradation have been developed to replace natural pyrethrins as agricultural pesticides. They are non-systemic insecticides that act through disruption of the nervous system leading to hyperactivity, paralysis, and death. The pyrethroids are also toxic to some non-target animals including crustaceans and fish. Median lethal concentrations of the more commonly used pyrethroids are generally less than 10 µg/l in fish, while birds and mammals show lower sensitivity (Bradbury and Coats 1989a). Pyrethroids are among the most commonly used pesticides worldwide, and pose a threat to the natural environment, including aquatic ecosystems (Richterova and Svobodova 2012). Contamination of surface water by pesticides is widespread (Hill 1985; Sibley and Kaushik 1991).

Cyperkill 25 EC contains 250 g/l cypermethrin. It is classified as a type II pyrethroid, which is more

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effective than type I (Narahashi 1986; Soderlund et al. 2002). Cypermethrin blocks sodium channels of nerve filaments by lengthening their depolarisation phase as well as affecting gamma-aminobutyric acid receptors and chloride and calcium channels in nerve filaments (Bradbury and Coats 1989b; Hayes 1994; Burr and Ray 2004). Cypermethrin contains chlorine atoms in a vinyl side chain of the compound. The presence of halogens contributes to greater insecticidal activity and higher stability, as well as providing better residual activity against insects. Further, halogen presence leads to a higher potential for negative effects on the environment (Brown at al. 1973; Bradbury and Coats 1989a). Cypermethrin is used to control pests including moths in cotton, fruits, and vegetable crops (Crawford et al. 1981). It is used in public health and animal husbandry, including in marine fish production to control ectoparasites (Treasurer and Wadsworth 2004).

The aim of this study was to assess the effect of synthetic cypermethrin, an active ingredient in the commonly used pesticide Cyperkill 25 EC, on early life stages of common carp using an embryo-larval toxicity test (EL test) supplemented by determination of oxidative stress parameters.

MATERIAL AND METHODS

Experimental substance. Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate] was tested in the form of Cyperkill 25 EC pesticide (Agriphar S.A., Belgium) containing 250 g/l active substance. Emulsifiable concentrate (EC) formulations of pyrethroids are usually two to nine times as toxic as the technical-grade pyrethroids, most likely due to synergistic interactions (Smith and Stratton 1986). Alpha-cypermethrin as an active ingredient of this emulsifiable concentrate formulation contains two isomers. The cis/trans ratio is 40 : 60.

Experimental animals. Fertilised eggs of common carp (*C. carpio* L.) were obtained from the Breeding Station of the Department of Fish Genetics and Breeding of the Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Czech Republic. Eggs were produced according to standard methods of artificial reproduction (Kocour et al. 2005). All experimental procedures involving animals were in accordance with European Community guidelines. The Law on Protection of Animals against Cruelty Act No 246/1992 Coll. adopted by The Czech National Council as amended was followed throughout the whole experiment.

Experimental design

Early-life stage toxicity test. This test followed the OECD 210 Guideline for Testing of Chemicals (OECD 2013), modified in that only fertilised eggs were selected for testing. One-hundred eggs at 24 h post-fertilisation were placed in crystallisation dishes containing 1 l of dechlorinated tap water (control) or 1 l aqueous Cyperkill 25 EC solution at 7.2, 36, 72, 144, or 360 µg/l representing cypermethrin concentrations of 1.8, 9, 18, 36, and 90 µg/l in solution, respectively. The beginning of the test was considered to be 24 h post-fertilisation and was designated Day 1. The test was conducted in two replicates. Water conditions were pH 7.98, NH_4^+, N < 0.02 mg/l; NO_2^-, N 0.006 mg/l; NO_3^-, N 1.55 mg/l, PO_4^{3-}, P 0.09 mg/l; COD_{Mn} 0.6 mg/l. During the test, water temperature ranged from 21 to 23 °C, pH values from 7.5 to 8.5, and oxygen saturation did not drop below 60%. Water in all dishes was continuously gently aerated, and was renewed once daily. Unhatched eggs and dead embryos and larvae were removed and recorded. Hatching was essentially complete on Day 4. Beginning on Day 8, larvae were fed with freshly hatched brine shrimp *Artemia salina* nauplii *ad libitum*. Samples of embryos/larvae for observation of length and weight growth, ontogenetic development, and malformations were taken from the control group and from the 7.2 µg/l concentration on Days 5, 12, 19, 26, 33, and 35. During these days, ten embryos/larvae were taken from the control group and from the experimental group. Sampled embryos/larvae were fixed in 4% formalin. The experiment was terminated on Day 35, when the majority of control fish reached the juvenile stage. Ontogeny stages comprised nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages (Penaz et al. 1983). At the end of the test, samples from the control and the 7.2 µg/l concentration were taken for histological examination, fixed in 10% formalin solution and processed using conventional paraffin techniques.

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Tissue sections were stained with haematoxylin and eosin. Samples of liver, intestine, kidneys, and gills were examined at magnification 100–1000× using light microscopy.

Cumulative mortality and biometric data. Cumulative mortality was recorded daily. The fixed samples of embryos/larvae were weighed to the nearest 0.1 mg using a WAS 220/C/2 analytical balance (RADWAG Balances & Scales, Poland). Total length was measured to the nearest 0.01 mm using a binocular loupe and a scale. Fulton's condition factor (FCF) was calculated at the end of the trial for nineteen fish from the control and the lowest concentration of cypermethrin using the formula

$$FCF = W \times TL^{-3} \times 100$$

where:

W = weight in g

TL = total length in cm

The software program Statistica, v. 10.0 for Windows (StatSoft, Prague, Czech Republic) was used to compare length and weight differences among the test groups.

Determination of oxidative stress. Samples of larvae/juveniles for investigation of oxidative stress parameters were taken on Day 35 and placed immediately into liquid nitrogen for transport to a screening laboratory. Whole bodies were homogenised in a 50mM potassium phosphate buffer with 1mM EDTA (pH 7.4) and centrifuged at 11 200 g for 20 min at 4 °C. The supernatant was pipetted into individual Eppendorf tubes and held at –85 °C until analysis. Supernatant was used for determination of GR (glutathione reductase), GPx (glutathione peroxidase), CAT (catalase), and GST (glutathione-S-transferase) activity and protein concentration. Non-centrifuged homogenate (stored at –85 °C) was used to estimate lipid peroxidation. Protein concentration was quantified with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard (Smith et al. 1985). Total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm (Habig et al. 1974). Specific activity was expressed as the nmol of the formed product per min/per mg of protein. The catalytic concentration of GR was determined by measuring NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidation at 340 nm (Carlberg and Mannervik 1975). The catalytic concentration of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm (Flohe and Gunzler 1984). Specific activity of GR and GPx was expressed as nmol of NADPH consumption per min/per mg of protein. The CAT activity was determined by measuring H_2O_2 breakdown at 240 nm and expressed as μmol of decomposed H_2O_2 per min/per mg of protein (Aebi 1984). Lipid peroxidation was determined using the TBARS method at 535 nm (Lushchak et al. 2005), with concentration expressed as nmol/g wet weight of tissue. All parameters were measured spectrophotometrically using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific). Obtained parameters of oxidative stress were checked for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, a one-way ANOVA was employed to determine whether there were significant differences in measured variables among experimental groups. When a difference was detected ($P < 0.05$), Dunnett's multiple-range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal-Wallis) was used. Obtained data of oxidative stress were controlled by the Shapiro-Wilk test for assessing normality of data, which determined that the data were normally distributed. Test of homogeneity of variances (Levene test) and an analysis of variance (ANOVA) were conducted, followed by a multiple comparison (Tukey-HSD test). Differences were considered to be significant when $P < 0.05$.

Determination of the active ingredient. The GC/ECD (gas chromatography/electron capture detector) method was used to control the level of active substance throughout the test (Kocourek and Hajslova 1989). Cypermethrin in water samples was determined after extraction using isooctane by gas chromatography with electron capture detection in the screening laboratory. Chromatography was performed on a column designated HP-5MS (60 m × 0.32 mm, film 0.25 μm). The carrier gas was helium with a flow rate of 25 ml/min a splitless injection volume of 2 μl, and a temperature of 250 °C was used. The temperature column program was 100 °C/2 min, increased to 230 °C at 14 °C/min, increased to 285 °C at 4 °C/min, and subsequently held for 40 min. This method confirmed the presence of cypermethrin at > 80% throughout the course of 24 h.

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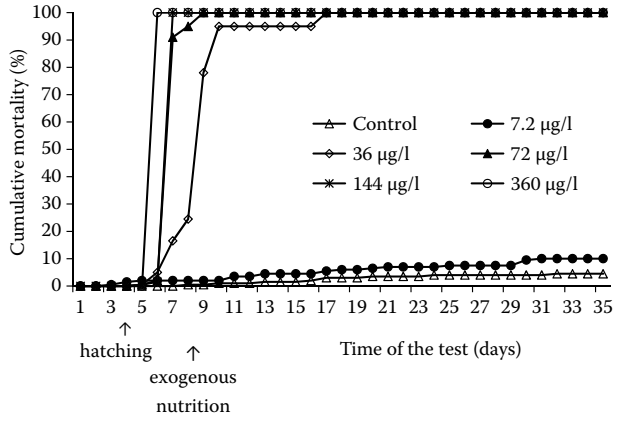

Figure 1. Cumulative mortality (%) of common carp embryos and larvae in EL toxicity test using Cyperkill 25 EC

RESULTS

Cumulative mortality

Mortality increased over the course of the trial (Figure 1). No significant differences in mortality were observed among test groups until Day 5. Total mortality was observed at 360 µg/l on Day 5 and at 144 µg/l on Day 6. Delayed and reduced hatching, along with post-hatch mortality, was observed at 72 µg/l. Only 5% of embryos from this group survived to initiation of exogenous nutrition, and total mortality was seen after several days. On Day 1 of feeding, 24% and 2% mortality was observed at 36 and 7.2 µg/l, respectively, with 0.5% mortality in the control. Total mortality at 36 µg/l was seen on Day 17. At the conclusion of the trial, 90% of larvae in the 7.2 µg/l group and 95.5% in the control group were viable.

Growth

Samples from all concentrations were taken only on Day 5, since insufficient numbers of larvae survived beyond Day 11 at higher concentrations. Only the control and the 7.2 µg/l group were compared at the completion of the trial. Survival at 36 µg/l concentration was not sufficient to include individuals in growth comparisons. Significant differences in weight and length growth were observed ($P < 0.01$) (Figures 2 and 3).

On Day 35, FCF was calculated as an index of thriving in fish from the control group and the 7.2 µg/l group. Values of 19 fish from each group were averaged (± SD). The mean FCF of control fish was 1.269 ± 0.3714 and mean FCF of the 7.2 µg/l concentration was significantly lower at 1.157 ± 0.0807 ($P < 0.05$).

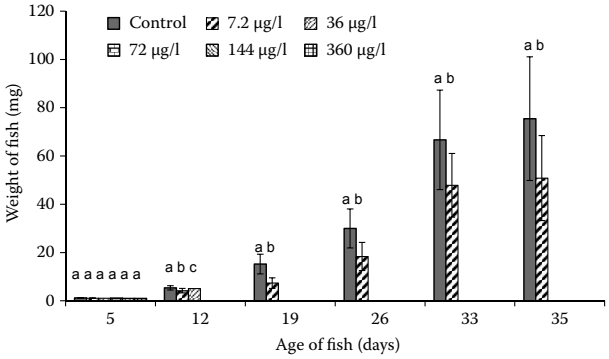

Figure 2. Effect of Cyperkill 25 EC on weight in mg (mean ± SD) of common carp larvae and juveniles in EL test. Significant differences ($P < 0.01$) between groups at each sampling time are indicated by different letters (a, b)

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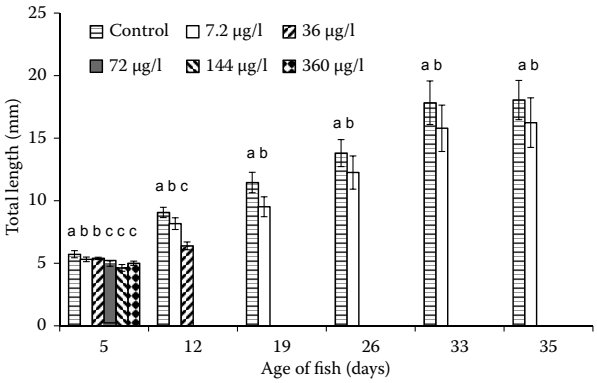

Figure 3. Effect of Cyperkill 25 EC on total length in mm (mean ± SD) of common carp larvae and juveniles. Significant differences ($P < 0.01$) between individual groups at each sampling time are indicated by different letters (a, b)

Early ontogeny

Ontogenetic developmental stages were investigated concurrently with growth indices (Table 1). Ten larvae on Day 5, 12, 19, 26, 33, and nineteen larvae on Day 35 from 7.2 µg/l and control groups were examined. From Day 19, developmental stages of controls and the 7.2 µg/l group showed visually observable differences. No fish from the 7.2 µg/l concentration had reached the juvenile stage by the end of the test with some remaining two stages behind. Morphological anomalies such as curvature of the spine, changes in yolk sac, and shortening of body were rare in both groups and could be considered chance occurrences. Deeper pigmentation was observed in 68% of the fish from the 7.2 µg/l concentration on Day 35. Larvae from the 36 µg/l that died later showed similar colour changes before death and no presence of food in the digestive tract.

Histology

Light microscopy did not reveal significant differences between 7.2 µg/l and controls in examined tissues.

Table 1. Ontogeny of common carp from control group and in response to exposure to Cyperkill 25 EC at 7.2 µg/l

Day	Developmental stages	
	control	7.2 µg/l
5	E8–E9	E8–E9
12	L3–L4	L3–L4
19	L4–L5	L3–L4
26	L5–L6	L5
33	L6–J1	L5–L6
35	L6–J1	L5–L6

Oxidative stress

Activity of GR, GPx, and GST of controls and the 7.2 µg/l concentration showed significant differences ($P < 0.01$) on Day 35 (Figures 4–6). The mean level of TBARS was 12.1 ± 2.58 and 10.7 ± 3.55 nmol/g of wet weight in control and treated fish, respectively, and mean CAT activity was 31.4 ± 5.07 and 24.5 ± 2.71 µmol H_2O_2/min/mg of protein, respectively. There were no significant differences in TBARs and CAT activity in control and treated fish.

DISCUSSION

The tests described here on the effects of Cyperkill 25 EC on common carp revealed low hatching rate, high mortality soon after hatching at concentra-

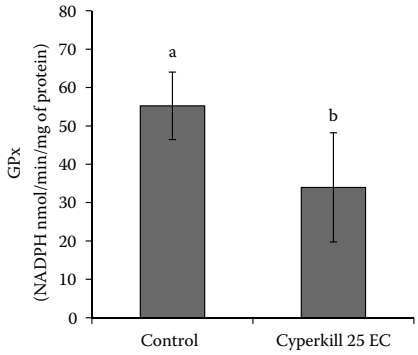

Figure 4. Effect of Cyperkill 25 EC at 7.2 µg/l on GPx activity in common carp on Day 35 of exposure compared to control ($P < 0.01$)

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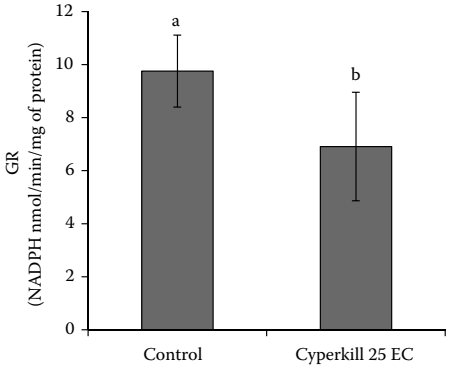

Figure 5. Effect of Cyperkill 25 EC at 7.2 µg/l on GR activity in common carp on Day 35 of exposure compared to control ($P < 0.01$)

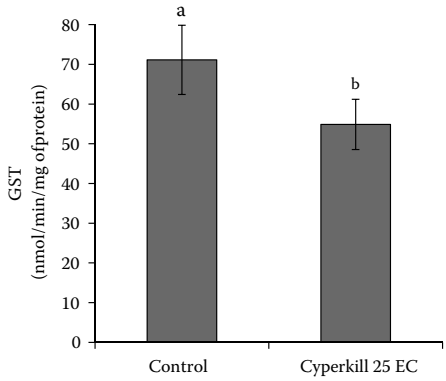

Figure 6. Effect of Cyperkill 25 EC at 7.2 µg/l on GST activity in common carp on Day 35 compared to control ($P < 0.01$)

tions of 36 µg/l (9 µg/l of cypermethrin) and higher, and significantly reduced growth and FCF in surviving larvae at a concentration of 7.2 µg/l (1.8 µg/l of cypermethrin). These larvae showed protracted ontogenetic development and colour changes.

Our results confirm that cypermethrin is highly toxic to common carp. High acute toxicity of cypermethrin to freshwater fish has been reported by many authors. The acute toxicity indicated for roho labeo (*Labeo rohita*) is 4.0 µg/l (Marigoudar et al. 2009). The 96 h LC50 value for cypermethrin in rainbow trout (*Oncorhynchus mykiss)* is 8.2 µg/l and in bluegill sunfish *(Lepomis macrochirus)* it is 1.8 µg/l (Bradbury and Coats 1989b). Similar values were found in other studies: 96 h LC50 for juvenile trout (mean weight 11.71 g and length 88.9 mm), 3.14 µg/l, and 96 h LC0, 96 h LC50, and 96 h LC100 for common carp were 1.82, 2.91, and 4.64 µg/l, respectively (Velisek et al. 2011). The 96 h LC50 for juvenile carp (mean weight 37.63 mg and length 13.85 mm) has been reported to be 1.38 µg/l (Stara et al. 2013a). We observed increased mortality dependent on concentration and duration of exposure. Similarly, acute toxicity tests of deltamethrin and cypermethrin in embryos and larvae of common carp have shown concentration-dependent decreases in hatching success (Koprucu and Aydin 2004; Aydin et al. 2005).

Larvae exposed to cypermethrin at 36 µg/l and higher often remained at the bottom and started to swim only upon stimulation. We did not observe curvature of the body as a result of spasms as was described, nor did we observe the spastic movement and 5–15 s immobility that was reported in zebrafish (*Danio rerio*) embryos exposed to cypermethrin (De Micco et al. 2010).

Treated larvae exhibited dark pigmentation at the end of the trial, as has been observed with exposure to pyrethroids (Ural and Saglam 2005; El-Sayed and Saad 2008; Richterova et al. 2014).

The reduced growth of fish observed in our test is in agreement with findings of reduced growth and feeding in fathead minnow (*Pimephales promelas*) larvae exposed to the pyrethroid esfenvalerate (Werner and Moran 2008). Decreased growth was described for mysid shrimp chronically exposed to cypermethrin at the very low concentration of 0.00078 µg/l (NOEC value) (Werner and Moran 2008).

Calculated FCF indicated reduced growth of exposed larvae compared with the control group. Significantly lower FCF values were observed in surviving larvae exposed to 7.2 µg/l compared to controls. These results were in agreement with reports demonstrating a decline in condition factor in fish exposed to pollutants (Khan 2003).

Although some authors report histopathologies in fish exposed to pyrethroids, we did not observe significant histological changes in liver, intestine, kidneys, or gill tissues of larvae exposed to cypermethrin. Degeneration of hepatocytes has been described after exposure of bifenthrin and cyhalothrin (Velisek et al. 2009a; Velisek et al. 2009b; Richterova et al. 2014), and damage of gill lamellae was de-

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scribed (Velisek et al. 2006; Velisek et al. 2009a). The concentration of 3.14 µg/l cypermethrin in the form of Alimethrine 10 EM was found to be associated with fatty degeneration of liver and severe teleangioectasiae of gills in juvenile rainbow trout (*O. mykiss*) (Velisek et al. 2006). It is possible that our tested concentration (1.8 µg/l of cypermethrin corresponding to 7.2 µg/l of Cyperkill 25 EC) was too low to cause histological damage.

The exposure of organisms to Cyperkill 25 EC was associated with differences in biochemical parameters of oxidative stress at a concentration of 7.2 µg/l. Activity of GR, GPx, and GST were decreased in our test. Many pesticides have been shown to be associated with induction of oxidative stress in aquatic organisms, via the formation of ROS (reactive oxygen species) and alterations in antioxidant or free oxygen radical scavenging enzyme systems (Uner et al. 2006; Slaninova et al. 2009; Lushchak 2011; Stara et al. 2012; Stara et al. 2013b). An increase or inhibition of antioxidant enzyme activity can depend on the intensity and the duration of exposure as well as on the susceptibility of the exposed fish (Oruc and Usta 2007). Manifestation of oxidative stress varies with fish species and organ (Slaninova et al. 2009). Exposure to 3 µg/l of cypermethrin for ten days caused increased activity of SOD (superoxide dismutase), CAT, and MDA (malondialdehyde) levels in the liver of common carp (*C. carpio*) and Nile tilapia (*Oreochromis niloticus*) while the level of GPx activity increased in tilapia but decreased in common carp (Uner et al. 2001). This study also found increases in SOD, GPx, CAT, and MDA in kidneys of common carp. The observed higher antioxidant enzyme activity in kidney suggested that this organ participates in the detoxification of cypermethrin or its metabolites (Uner et al. 2001). Significantly higher activity of SOD and GR and lower activity of CAT were observed in juvenile carp exposed to 1.38 µg/l of zeta-cypermethrin (Stara et al. 2013a). High levels of the antioxidant enzymes SOD and CAT followed exposure to cypermethrin in common carp, while the enhanced lipid peroxidation reported in blood and tissue demonstrated that cypermethrin-induced ROS were not totally scavenged by the antioxidant enzymes (Yonar 2013).

We can conclude that exposure to Cyperkill 25 EC (containing 250 g/l cypermethrin) may pose a risk for fish.

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CHAPTER 5

GENERAL DISCUSSION

Pyrethroids are the predominant class of pesticides. Their use has increased during the last decades. Widespread use of them represents an increasing threat of water pollution. They are used constantly and can be released into the environment. Threat of the influence on non-target organisms is growing continuously with the rate of pesticide use. Pyrethroids are commonly detected in water environment near agricultural and urban areas where these pesticides are applied normally. Ubiquitous presence of pyrethroids in aquatic system of such areas is documented. For example, repeated water and sediment sampling in creeks in Stanislaus County, California during seven months in 2007–2008 showed pyrethroid concentrations in water samples ranged from 0.005–0.021 $\mu g.l^{-1}$. Moreover, pyrethroids were detected more frequently in sediment samples. Cyfluthrin, bifenthrin, esfenvalerate, and λ-cyhalothrin were detected in sediment samples at concentrations ranging from 1.0 to 74.4 $ng.g^{-1}$ dry wet (Ensminger et al., 2011). Incorect use and accidents that increase risk also must be taken into consideration. The water environment is inhabited by many species of non-target organisms. Fish, water insects and chrustaceans belong to these organisms in aquatic ecosystem.

The experiments discussed in this thesis used fish as typical non-target species. Carp was chosen as a model fish due to its economical importance. Carp farming contributes about 90% of total fish production in the Czech Republic. Main producer countries include not only major part of Europe and Asia, but also an important part of South America, and even some African states. Global aquaculture production for *C. carpio* was 4080045 tonnes in 2013 (FAO, 2016).

We followed commonly used early life stage toxicity tests. Toxicity tests using early life cycle stages allow risk assessment of changes in growth, reproduction and surviving in polluted environment, and play an important role for good environmental monitoring. Early life stages were described as more sensitive to the adverse effect of pollutants (Kamman et al., 2009). Adult animals are not as sensitive as juveniles, and effects are not so readily detectible (Kristensen, 1994).

Fish are a good model for study of aquatic contamination generally. Altered water chemistry not only affects their behaviour and histology, but also their biochemical processes and physiology. The studies of the effect of pesticides on non-target organisms are important not only to ecologists but also to policy makers with a goal of developing guidelines that will safeguard ecosystem health and aquatic living resources (Olulah and Chineke, 2014).

HATCHING AND MORTALITY

Hatching is an important part of reproduction. Hatching of teleosts eggs is initiated with the release of the chorionase enzyme around the embryo. It is likely that some chemicals inhibit the release of this enzyme, thus prolonging the hatching span (von Westernhagen, 1988). Therefore, delay in hatching can be an adaptive response to avoid exposure to potentially noxious environments, since one of the roles of chorion is to protect the embryo (Blaxter, 1988). Such a protective response could be observed in both our studies. Exposure of pyrethroids showed delayed and reduced hatching. Both parameters were dependent on concentration and increased with higher concentration of pesticide. The influence on early survival success had been noted even in lower concentrations than we used. The 48 h LC50 value (95% confidence limits) of cypermethrin for common carp (*C. carpio*) embryos was found to be 0.909 (0.256–5.074) $\mu g.l^{-1}$. The 96 h LC50 value (with 95% confidence limits) of cypermethrin for common carp (*C. carpio*) larvae was estimated at 0.809 (0.530–1.308) $\mu g.l^{-1}$

(Aydin et al., 2005). Impact on concentration-dependent mortality of early life stages was evident during our study period. It is consistent with a study on zebrafish (*D. rerio*), which described dose and time-dependency on post-exposure mortality of permethrin and cypermethrin (Yang et al., 2014), and a study on rohu (*Labeo rohita*), which used cypermethrin in the range 0–140 $\mu g.l^{-1}$ (Dawar et al., 2016).

The most evident mortality onset at most concentrations was during transition from embryonic to larval stages in our studies. Developmental stages comprised nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages followed the description by Penaz et al. (1983) in our studies. Similar simultaneous mass mortality with the first exogenous nutrition, mixed nutrition, and completely exogenous nutrition was described after exposure to pesticide (Machova et al., 2010). It seems that first feeding is the most critical period relative to hatching.

We must take in account that negative hatching success in population could be amplyfied on concurrent environmental conditions. Water temperature or influence of mixture of pollutants play an important role. Single chemical risk assessment of pyrethroid is likely to underestimate the impact of insecticides on fish in aquatic ecosystems where mixtures appear (Bachetta et al., 2014). Toxic impact on reproduction may increase when water temperature is decreasing. It could influences cold water fish spawning season, because acute toxicity of synthetic pyrethroids to fish is negatively correlated to temperature (Kumaragura and Beamish, 1981). Moreover, pyrethroid action on breeding adults is also negative and operates in multiple ways in organisms (Mooore and Waring, 2001; Forsgren et al., 2013).

BEHAVIOUR

The changes of behaviour of animals are the first signs of toxic effect of xenobiotics. Behavioural observation is considered a promising tool in ecotoxicology. Since behaviour is not a random process, but rather a selective response that is constantly adapting through direct interaction with the environment, behavioural feedback provides valuable tools to discern and evaluate effect of exposure to environmental stressors. Therefore, fish behavioural alterations can provide important indices for aquatic ecosystem assessment (Kane et al., 2005).

Changes in behaviour caused by gamma-cyhalothrin were observed in our tests at the highest concentration. Minimal movement of embryos in 15 $\mu g.l^{-1}$ of gamma-cyhalothrin was apparent preceding death, which was totalled during several days after hatching. Alpha-cypermethrin caused behaviour changes in concentrations of 9 $\mu g.l^{-1}$ and higher in our study. Larvae from these concentrations often remained at the bottom and swam only upon stimulation. This result is consistent with study on adult guppy (*Poecilia reticulata*). The fish started to have less activity in the concentration of 8 $\mu g.l^{-1}$ 2 h after exposure. They also exhibited loss of balance, respiratory difficulties, became motionless, and attempted to obtain air from the surface (Yilmaz et al., 2004). Juveniles of common carp (*C. carpio*) changed their behaviour from the concentration of 5 $\mu g.l^{-1}$ of zeta-cypermethrin. Accelerated respiration, alternating with rest phases, loss of movement coordination, jerky movements, and seizures alternating with lethargy was described. Alternation of excitation and resting phases continued until fish stayed motionless on the tank bottom, moving mainly at the flank. Then respiration slowed and death followed (Stara et al., 2013).

Studies described exact beginning of behaviour changes in fish in variable concentrations of cypermethrin exists (Polat et al., 2002; Borges, 2007). Young silver catfish (*Rhamdia quelen*) showed loss of balance, swimming alteration, dyspnea (they kept their mouths and opercula open), upright swimming and sudden spiral swimming movements in 1500 $\mu g.l^{-1}$ of cypermethrin during the first hour of exposure (Cypermade 250 SC). They also attempted to

jump out of the aquariums in higher concentration. Before dying, the fish became less active, remained vertical in the water, and then, finally motionless at the bottom of the aquarium (Montanha et al., 2014).

All studies including our ones demonstrate neurotoxic effect on behaviour of fish. Various forms of swimming abnormalities, often accompanied by dyspnea, and concluded by apathy before death are typical behavioural signs of pyrethroid intoxication. The difference in effective concentration could be related to the different resistance of species, gender and size of animals. The difference may also be related both to the toxicity of the formulation of the tested active substance and the different acclimatizing period. Shorter period is considered a stressing factor to the animals (Montanha et al., 2014). Lastly we must take into account possible presence of mixture of xenobiotics in aquatic environment. Significant amount of crooked body and evidence of spasms were described on zebrafish (*D. rerio*) exposed to mixture of permethrin and cypermethrin compared to individual exposure of one pyrethroid (Yang et al., 2014).

BIOMETRIC PARAMETERS, EXTERNAL APPEARANCE, AND ONTOGENETIC DEVELOPMENT

Biometric parameters are considered to be general indicators of fish health and the quality of the aquatic environment. Our studies revealed significantly reduced growth both in length and weight. Concurrently investigated ontogenetical development was delayed too. The index of healthy fish surviving at the end of the trial was calculated. Fulton's condition factor (FCF) was used for expression. Individuals treated with 0.3 $\mu g.l^{-1}$ of cyhalothrin and 1.8 $\mu g.l^{-1}$ of cypermethrin had significantly lower FCF than individuals without presence of pyrethroids.

We saw deeper pigmentation in 68% of the individuals from the 1.8 and 9 $\mu g.l^{-1}$ of cypermethrin at the end of the trial. No fish from higher concentrations could be compared due to their early death. Cyhalothrin caused higher incidence of deeper pigmentation in 0.3 and 1.5 $\mu g.l^{-1}$ concentrations. No live individuals were available at the higher concentrations for comparison at the end of the trial. Similar colour changes after pyretroid exposure were described in literature. Colour darkening was shown on fry rainbow trout (*O. mykiss*) from concentration of 8 $\mu g.l^{-1}$ and higher (Ural and Saglam, 2005). Darkening of the body surface, slight erosions and/or rotting of fins and tail was seen on monosex Nile tilapia (*O. niloticus*) exposed to subacute concentration of deltamethrin (El-Sayed and Saad, 2007). Change of color in abdominal area and enlargement of the eyes were observed on adult guppy (P. reticulata) during acute toxicity test of cypermethrin (Yilmaz et al., 2004). Change of body surface pigmentation also was described to be darker in other studies. Guppies (*P. reticulata*) exposed to 15 $\mu g.l^{-1}$ of beta-cypermethrin showed colour darkening while those exposed to 50 $\mu g.l^{-1}$ changed to yellow in the abdominal area (Polat et al., 2002). Silver catfish (*R. quelen*) exposed to cypermethrin showed partial and in some cases even total loss of body surface pigmentation too. Hyperemic and ulcerated lesions, with hemorrhagic signs in several parts of the body, as well as barbel erosions and tails with a degenerative aspect, accompanied these lesions (Montanha, 2014). We suggest that loss of colour pigmentation in that study was the result of much higher concentration (1500 $\mu g.l^{-1}$) than we used. Irritating effects of high concentration of cypermethrin were observed.

Morphological anomalies were not commonly seen in our studies, they could be considered chance occurrences. Nevertheless, abnormalities such as eroded yolk, eroded margins of embryo and elongated yolk sac were reported in rohu (*L. rohita*) treated by 8.43 $\mu g.l^{-1}$ of cypermethrin solution prepared from analytical grade cypermethrin. Furthermore, there were larvae having abnormal yolk sac, elongated yolk sac, no eyes, blank eyes, and a short tail (Dawar et al., 2016).

Ontogenetic development showed one larval stage delay from the 19th day of exposure of 1.8 μg.l^{-1} of cypermethrin. No larvae from this concentration reached juvenile stage by the end of the trial. Some larvae were retarded by two stages. Development of individuals exposed to 0.3 μg.l^{-1} of cyhalothrin was delayed from the 26th day. All individuals from this concentration remained at larval stage six by the end of the trial, unlike the controls, which were able to reach juvenile stage. From this point of view it is important to report that we followed description of Penaz et al. (1983), because terminology in this branch of ichthyology is not standardized (Penaz, 2001). This description distinguished six larval stages, which individuals must undergo before juvenile stage. We can confirm the influence of both tested pyrethroid based products on the prolongation of fish development.

HISTOPATHOLOGY

Histopathology can be a powerful tool for monitoring anthropogenic contamination, because many tissue changes represent a biological endpoint of contaminant exposure. Fish histopathology is increasingly being used to indicate environmental stress, since it provides a definitive biological result of exposure to pollutants (Stentiford et al., 2003).

Our work showed histopathological alterations in liver of larvae and juveniles of common carp (*C. carpio*) exposed to cyhalothrin. Significant steatosis dystrophy was revealed in individuals exposed to 0.3 μg.l^{-1} of cyhalothrin, which was our lowest tested concentration. Hepatodystrophic changes were accompanied by rare mitotic structures in liver cells. These results are consistent with toxic effects of common pyrethroids on liver in fish (Velisek et al., 2006; Velisek et al., 2009a; Velisek et al., 2009b; Werner and Moran, 2008). We did not see changes of other examined tissues, such as intestine, gills and kidney after exposure to cyhalothrin.

In or study, no changes were observed in the examined tissues after exposure of cypermethrin. Nevertheless, histopathological changes caused by cypermethrin were noted in another study using acute toxicity of exposure (96 h LC50 2.91 μg.l^{-1}). This concentration resulted in hyperaemia and perivascular lymphocyte infiltration in skin, vacuolisation of pancreatic exocrine cells, mild hyperplasia of respiratory epithelium, and chloride cell activation in the gills of common carp (*C. carpio*) (Velisek et al., 2011). Degeneration of cytoplasm in hepatocytes, formation of vacuoles, rupture of blood vessels, necrosis and cytoplasmic vacuolization in liver started after 4 hours of exposure of 4 μg.l^{-1} of alpha-cypermethrin to Indian carp (*Catla catla*). Congestion of blood vessels, bulging, distortion filaments, erosion and disintegration of blood corpules and hyperplasia of epithelium were found simultaneously in gills (Muthuviveganandavel et al., 2013). Damage of fish gill lamellae was described after exposure of cypermethrin in several more studies. Severe teleangioectasiae of gills in juvenile rainbow trout (*O. mykiss*) was caused by concentration 3.14 μg.l^{-1} (Velisek et al., 2006; Velisek et al., 2011). Lifting of epithelial layer from gill lamellae and some necrosis were apparent in common guppy (*Lebistes reticulatus*) exposed to 15 μg.l^{-1} of zeta-cypermethrin (Caliskan et al., 2003). The concentration 1.8 μg.l^{-1} of cypermethrin that we tested might have been too low to alter gills, but we had no surviving individuals from higher concentrations. Changes in gill tissue are probably due to direct responses of gill to the action of pyrethroid and simultaneously defensive responses of organism against toxicant to make it more difficult to access the blood stream (Cengiz, 2006).

Histopathological changes of gills caused by other pyrethroids also were described. Severe teleangioectasiae were revealed in secondary lamellae of gills, with the rupture of pillar cells in fish exposed to bifenthrin (Velisek et al., 2009a). Desquamation and necrosis followed by the lifting of the lamellar epithelium, oedema, aneurisms, hyperplasia of epithelial cells, and fusion of the secondary lamellae was described related to deltamethrin (Cengiz, 2006).

Histopathological changes of other fish tissues were described as well. Kidney degeneration in the epithelial cells of renal tubules, pycnotic nuclei in the haematopoetic tissue, dilatation of glomerular capillaries, degeneration of glomeruli, intracytoplasmatic vacuoles in epithelial cells of renal tubules with hypertrophied cells, and narrowing of the tubular lumen was noted (Cengiz, 2006). Kidney tissue of silver crucian carp (*Carassius aureus gibelio*) exposed to 2 $\mu g.l^{-1}$ of deltamethrin showed similar changes (Staicu et al., 2007). The signs of tissue damage to the most affected organs were described after exposure of fenvalerate to mrigal carp (*Cirrhinus mrigala*). It was accompanied by atrophy of epithelial cells, necrosis of epithelial cells, desquamation of mucosal epithelium, and infiltration of lymphocytes into the lamina propria of the intestine (Velmurugan et al., 2007). Histopathological alterations of reproductive tissue both male and female fish were described (Staicu et al., 2007; Forsgren et al., 2013). Even, muscle alterations were documented (Muthuviveganandavel et al., 2013).

BIOCHEMICAL AND OXIDATIVE STRESS PARAMETERS

Changes in biochemical profile of fish due to metabolic stress caused by pyrethroids were described (Velisek et al., 2009a; Olulah and Chineke 2014). Metabolic stress induced by pyrethroids is accompanied by changes in the activities of enzymes of antioxidant defense (Uner et al., 2001, Sayeed et al., 2003). Oxidative stress arises due to a situation when a steady-state reactive oxygen species (ROS) concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation, and damaging cellular constituents. The steady-state concentration of ROS is a balance between production and elimination of these species, which are produced and eliminated continuously (Luschak, 2010). Oxidative stress leads to the formation of oxygen free radicals and other reactive oxygen species that cause damage to membrane lipids, DNA, and proteins. Changes are induced in antioxidant enzyme activities. It may result even in cell death (Chromcova et al., 2015). Like ROS, reactive nitrogen species (RNS) are derived from the interactions of biologically generated free radicals and result in multiple biological effects (Patel et al., 1999). Rates or amounts of ROS production can be increased by the presence of a wide range of natural and man-made xenobiotics (Livingstone, 2001). Activities of antioxidant enzymes appear to be a reliable indicator of oxidative stress induced by pollutants (Pavlovic et al., 2010).

Unbalance of specific parameters of oxidative stress and influence on detoxifying enzyme were evident in our studies. The activity of detoxification enzyme glutathione-S-transferase (GST) of organisms which survived 35 days of exposure 0.3 $\mu g.l^{-1}$ of gamma-cyhalothrin was significantly higher compared to the control and the activity of defensive enzyme glutathione peroxidase (GPx) was significantly lower compared to the control. Activities of glutathione reductase (GR), GST, and GPx in the surviving animals from group exposed for 35 days to 1.8 $\mu g.l^{-1}$ of alpha-cypermethrin were significantly lower. The same concentrations of cypermethrin and cyhalothrin did not cause significant changes in activities of catalase (CAT) and thiobarbituric acid reactive substances (TBARs) in our studies.

GPx is an enzyme that catalyzes the cleavage of the free radical hydrogen peroxide (H_2O_2) and simultaneously allows oxidation of cysteine-containing glutathione. It is necessary to regenerate the reduced glutathione form to the continuous ability of GPx to dispose H_2O_2. GR ensures this regeneration continuously (Racek and Holecek, 1999). GR is an important antioxidant playing a pivotal role in eliminating oxidative stress in aquatic animals (Hasspielar et al., 1994). Observed decrease of activity may be due to general "enzyme exhaustion" at a constant supply of a substrate. However, Elia et al. (2002) and Dawar et al. (2016) described increase of GR after pesticide exposure.

Influence on lipid peroxidation, induced by ROS was not observed in either of our studies, although it is a common oxidative stress biomarker of toxicants. The widely used analyses for lipid peroxidation is observation of malondialdehyde (MDA), a secondary product of lipid peroxidation and CAT, an enzymatic antioxidant. CAT scavenges ROS and converts them to less reactive species, thereby preventing lipid peroxidation (Kaviraj and Gupta, 2014). Enzyme CAT is located in peroxisomes and decomposes H_2O_2 into water and oxygen (Aebi, 1983). It acts on H_2O_2 in high concentrations to protect cells against the toxic effect of H_2O_2. It actually builds on work of superoxide dismutase (SOD), which is one of the key enzymes of antioxidant protection. SOD significantly accelerates dismutation of superoxide, which is the most common free radical in organisms. Oxidative stress, accompanied by the formation of superoxide induces arise of synthesis of SOD (Racek and Holecek, 1999). Study with 8.43 $\mu g.l^{-1}$ of cypermethrin derived from analytical grade described elevation of activity of CAT and higher lipid peroxidation reaction (Dawar et al., 2016). We assume that concentrations of cypermethrin and cyhalothrin in our studies were not high enough to influence oxidation of lipids expressed by CAT nor by TBARs.

Analysis of lipid peroxidation determined as TBARs uses thiobarbituric acid (TBA) (Lushchak et al. 2005). It reacts not only with MDA but also with many types of compounds, such as different aldehydes, amino acids, and carbohydrates (Lushchak, 2010). New approaches, more specific than TBARs measurement of the end products of lipid peroxidation could be recommended. They include high-performance liquid chromatography and immune techniques (Claeson et al., 2001). Lambda-cyhalothrin has been shown yet to lead to oxidative stress in liver of Nile tilapia (*O. niloticus*) by increasing lipid peroxidation and TBARS (Piner and Uner, 2012). Other studies still describe MDA levels nevertheless they use TBA for measurement. It is an example of cypermethrin, which was demonstrated to increase activity of MDA in the liver of common carp (*C. carpio*) and Nile tilapia (*O. niloticus*) together with the increase of MDA in common carp (*C. carpio*) kidneys (Uner et al., 2001). Increasing activities of MDA in whole body of rainbow trout (O. mykiss) fry according to increasing concentration of cypermethrin were determined with thiobarbituric acid too. The lowest tested and affecting concentration together was 1% in this 24-hour test (Sakin et al., 2011). Another study using 1.38 $\mu g.l^{-1}$ (96 h LC50) of zeta-cypermethrin did not reveal significant influence on TBARS activity in any tissue of juvenile carp (*C. carpio*). While SOD activity was higher in the intestine of experimental fish and GR was higher in gills, and lower in muscle. CAT activity decreased in the brain and the intestine, but increased in the liver (Stara et al., 2013).

Tissue specificity as a consequence of metabolite and anti-oxidative differences in various tissues was described (Ognjanovic et al., 2008). Moreover, the balance of antioxidants is different with respect to species, habitat, feeding behaviour, dose, and exposure time in tested organisms (Dobsikova et al., 2006; Stara et al., 2012).

Changes in activities of protective antioxidant enzymes (GPx, GR, and CAT) and detoxification enzyme GST confirm toxic effect of pyrethroids at the cellular level even in low concentrations in our studies. This negative effect could lead to the damage of tissues or organs by pyrethroids.

CONCLUSION

The contamination of aquatic ecosystem by pesticides including the widely used pyrethroid group has gained increasing attention in recent decades. The acute and chronic exposure and accumulation of these chemicals can result in tissue burdens that produce adverse effects, not only in the exposed organisms, but also in other organisms including humans. Organisms in the food chain and the balance of the ecosystem should be taken into account. So, it seems essential to study harmful effects of pyrethroids and try to formulate the strategies

for safe guarding aquatic organisms. Study with formulated commercial products, which are widely used, could offer a more realistic view than technical grades. Fish are commonly used as biological monitors of environmental levels of anthropogenic pollutants. The presented thesis aims to contribute to assessment of the toxicity and the effects of two representative pyrethroids in commercial form to early life stages of common carp. This species is crucially important to freshwater aquaculture and plays an important role in the water ecosystem.

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ENGLISH SUMMARY

The effect of pyrethroid based pesticides on fish

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Pyrethroids are some of the most used pesticides worldwides. They are synthetically produced, based on the insecticidal properties of natural pyrethrins. They differ from pyrethrins in having greater photostability and longer half-life. Pyrethroids are used to protect agricultural plants against pests. Also, public health and veterinary treatment of parasites involve the use of pyrethroids. The widespread use and high stability of pyrethroids leads to the assumption of that their occurrence in the environment could be quite common. They can reach water ecosystem as pollutants. Pyrethroids are absent in water ecosystems which are unaffected by human activities. But residues of pyrethroids are detected due to escapes from agriculture lands and urbanistic areas in different aquatic environments across the whole world. Residues of pyrethroids are not only present in the water column, but also in sediments and in fish tissues. Therefore, the presence of pyrethroids in the aquatic environment influences the hygienic quality of fish. Additionally, pyrethroids may adversely primarily and secondarily affect the growth of fish, their reproductive cycle, immunity and overall health.

Contamination of the water environment by pyrethroids has a negative effect on other non-target organisms, primarily crustaceans and water insects.

The goal of this thesis was to observe the impact of pyrethroids on early life stages of common carp (*Cyprinus carpio* L.). We tested two products, Cyperkill 25 EC and Nexide, both based on pyrethroids type II, that contain α-cyanogroup. Their active substances cypermethrin and cyhalothrin have widespread use worldwide. We used tests on early life stages of fish to evaluate the effects of cypermethrin and cyhalothrin. The assesed results were ontogenetic development, growth, and mortality. Histopathological examination and selective oxidative stress parameters assessment accompanied it as well.

The first study was devoted to the product Nexide containing 60 $g.l^{-1}$ of active substance gamma-cyhalothrin. Tested Nexide concentrations were 5, 25, 50, 100, and 250 $\mu g.l^{-1}$, which represented 0.3, 1.5, 3, 6, and 15 $\mu g.l^{-1}$ of gamma-cyhalothrin respectivelly. Fertilised eggs were exposed to various concentrations and development of embryos and larvae was observed until most of control individuals reached juvenile stage. The duration of the exposure was 35 days. There were significant mortalities in all concentrations except the lowest concentration 0.3 $\mu g.l^{-1}$ during the trial. Cumulative mortality was 100% in concentration of 3, 6, and 15 $\mu g.l^{-1}$ and 95% in concentration 1.5 $\mu g.l^{-1}$. The highest concentration tested caused total mortality immediately after hatching. Concentrations 6 and 3 $\mu g.l^{-1}$ caused total mortality soon soon after the beginning of exogenous feeding. Individuals from concentration of 1.5 $\mu g.l^{-1}$ lived until the 18th day of exposure without significant losses. Then, a significant mortality appeared and only 5% of individuals survived until the end of the trial. The lowest concentration tested 0.3 $\mu g.l^{-1}$ only caused a slightly increased mortality that did not exceed 7% and could be comparable with the control group. This lowest concentration influenced the growth in length and weight negatively, decelerated ontogenetic development, and darkened the body surface of the individuals. Survivors in concentration 0.3 $\mu g.l^{-1}$ were investigated in laboratory in detail. Histopathology revealed dystrophy in liver. Steatosis and mitotic structures were evident too. Examination of kidney, intestine and gills did not show significant histopathological differences compared with control. The evaluation of selected parameters of oxidative stress demonstrated a significantly higher ($P < 0.05$) activity of detoxification enzyme glutathione-S.transferase (GST) and a significantly lower ($P < 0.01$) activity of defensive enzyme glutathione peroxidase

(GPx) compared with the control group. The other examined parameters of oxidative stress such as catalase (CAT), glutation reductase (GR), and lipid peroxidation determined by using the thiobarbituric acid-reactive substances (TBARs) were comparable to the control group. Changes in oxidative stress parameters suggest that exposure of the organism to the product Nexide in the given concentration leads to dysbalance of defensive enzymes.

The second study was devoted to the product Cyperkill 25 EC containing 250 $g.l^{-1}$. The procedures of the trial were the same as in the preceding study and took 35 days of exposure too. Tested Cyperkill 25 EC concentrations were 7.2, 36, 72, 144, and 360 $\mu g.l^{-1}$, which represented 1.8, 9, 18, 36, and 90 $\mu g.l^{-1}$ of alpha-cypermethrin respectively. The development was observed from fertilised eggs until the most of control individuals reached juvenile stage. There were 100% mortalities in all concentrations except the lowest concentration 1.8 $\mu g.l^{-1}$ during the trial. Concentrations 90, 36, and 18 $\mu g.l^{-1}$ caused total mortality during 10 days. The highest concentration 90 $\mu g.l^{-1}$ caused 100% mortality on the 5^{th} day of the trial. Concentration 18 $\mu g.l^{-1}$ extended the period of hatching and decreased the number of succsessfully hatched individuals. Concentration 9 $\mu g.l^{-1}$ caused significant mortality of individuals during the period of transition to exogenous feeding. Only 5% of individuals from this concentration stayed alive after this time and total mortality was seen on the 17^{th} day. The lowest tested concentration 1.8 $\mu g.l^{-1}$ allowed 90% of individuals to stay alive until the end of experiment. That is combarable with the control group in which 95.5% survived of individuals. The lowest concentration influenced the growth in length and weight negatively and decelerated ontogenetic development compared with the control. Any individual exposed to this concentration did not reach juvenile stage until the end of the trial with some remaining two stages behind (last larval stage but one L5) compare with the control group. Dark pigmentation was visible in 68% of these exposed individuals on the last day. Similar darkening was also visible in individuals from higher concentrations shortly before death. The individuals who stayed alive in concentration 1.8 $\mu g.l^{-1}$ were investigated in laboratory in details. Histological examination did not revealed significant changes in intestine, liver, kidney, and gills compared with the control group. Evaluation of selected parameters of oxidative stress demonstrated significantly lower ($P < 0.01$) activities of GST, GR, and GPx. Activities of CAT and TBARS were comparable with the control group. Changes in oxidative stress parameters suggest that exposure of the organism to the product Cyperkill 25 EC in the given concentration could induce oxidative stress and interfere with the activities of antioxidant enzymes.

This thesis summarises data about pyrethroids and their influence on fish. The demonstrated effects confirm high susceptibility of early developmental stages of fish to tested pesticides. When interpreting the results, we have to take into account the fact that studies showed this risk even on single pyrethroid substances. But water organisms are exposed to many other more or less toxic products and substances in a real environment. These xenobiotics could react with each other and their mixture could even increase negative effects. Therefore, it is necessary to pay attention to these substances as well as mixtures, to monitor their presence in the environment and to continue laboratory studies. Both studies also clearly show the significant differences in the sensitivity of embryonic and embryolarval tests. In both, evaluations of tested products confirmed an icrease in sensitivity of the the early developmental stages of fish in the period of transition embryos to the larval stage of development. If the products were evaluated using the embryonic test only, it is obvious, that there would be a significant underestimation of the risks of that product for the reproduction of fish.

CZECH SUMMARY

Vliv pyrethroidů na ryby

Zuzana Richterová

Pyrethroidy patří celosvětově mezi nejčastěji používané pesticidy. Jsou to synteticky vyráběné látky, vycházející z insekticidních vlastností přírodních pyrethrinů. Oproti pyrethrinům jsou výrazně fotostabilnější a mají delší poločas rozpadu. Pyrethroidy jsou využívány k ochraně zemědělských produktů proti škůdcům a rovněž jako ektoparazitika v humánní i veterinární medicíně. Jejich široké použití a poměrně vysoká stabilita dávají předpoklad pro jejich výskyt v životním prostředí. Jako polutanty se následně mohou dostat až do vodního ekosystému. V přirozených, antropogenní činností nezatížených vodních ekosystémech, se pyrethroidy nevyskytují, ale díky únikům ze zemědělsky ošetřovaných ploch či urbanistických oblastí jsou jejich rezidua detekována již v mnoha vodních prostředích po celém světě. Vyskytují se nejen ve vodách a sedimentech, ale i v tkáních ryb. Jejich přítomnost ve vodním prostředí tedy negativně ovlivňuje hygienickou kvalitu ryb. Navíc pyrethroidy mohou negativně primárně i sekundárně ovlivňovat růst ryb, jejich reprodukční cyklus, imunitu i celkový zdravotní stav.

Kontaminace vodního prostředí má negativní vliv i na další necílové vodní organizmy, především korýše a vodního hmyz.

Cílem předložené práce bylo sledovat vliv pyrethroidů na raná vývojová stadia kapra obecného (*Cyprinus carpio* L.). K testům jsme použili dva přípravky – Cyperkill 25 EC a Nexide, které náleží do skupiny pyrethroidů druhého typu s obsahem α-kyanoskupiny. Jejich účinné látky, cypermethrin a cyhalothrin, mají široké uplatnění a používají se prakticky po celém světě. Účinky cypermethrinu a cyhalothrinu byly hodnoceny na základě výsledků embryolarválních testů, tj. ontogenetického vývoje, růstu a mortality a rovněž na základě histopatologického vyšetření a stanovení vybraných parametrů oxidativního stresu.

V prvním testu jsme sledovali vliv přípravku Nexide s účinnou látkou gamma-cyhalothrin v koncentraci 60 $g.l^{-1}$. Testovaným koncentracím Nexide 5, 25, 50, 100 a 250 $\mu g.l^{-1}$, což odpovídá koncentracím gamma-cyhalothrinu 0,3; 1,5; 3; 6 a 15 $\mu g.l^{-1}$, byly vystaveny oplozené jikry a poté byl sledován vývoj embryí a larev do doby, kdy většina kontrolních jedinců dosáhla juvenilního stadia vývoje. Celková délka expozice činila 35 dnů. V průběhu testu došlo ve všech koncentracích, s výjimkou nejnižší (0,3 $\mu g.l^{-1}$), k výraznému úhynu pokusných organizmů, přičemž v koncentracích 3, 6 a 15 $\mu g.l^{-1}$ dosáhla mortalita 100 % a v koncentraci 1,5 $\mu g.l^{-1}$ 95 %. Nejvyšší testovaná koncentrace vyvolala úhyn bezprostředně po vykulení embryí a v koncentracích 6 a 3 $\mu g.l^{-1}$ došlo k výraznému úhynu embryí a larev v průběhu přechodu na exogenní výživu. Larvy vystavené koncentraci 1,5 $\mu g.l^{-1}$ přežívaly bez větších ztrát do 18. dne pokusu. Poté došlo v této koncentraci k masivnímu úhynu a na konci testu přežívalo pouze 5 % larev. Mortalita embryí a larev v nejnižší testované koncentraci a v kontrole byla srovnatelná a nepřesáhla 7 %. Nejnižší testovaná koncentrace 0,3 $\mu g.l^{-1}$ výrazně negativně ovlivnila délkový i hmotnostní růst a vyvolala zpomalení ontogenetického vývoje a ztmavnutí povrchu těla. U přeživších jedinců bylo provedeno histologické vyšetření, při kterém byly v játrech zaznamenány změny dystrofického charakteru. Kromě steatózy byly potvrzeny i itotické změny. Histologické vyšetření vzorků ledvin, střev a žaber neprokázalo patologické změny. Vyšetřením vybraných parametrů oxidativního stresu byla prokázána statisticky významně vyšší ($P < 0,05$) aktivita detoxifikačního enzymu glutathion-S-transferásy (GST) a nižší ($P < 0,01$) aktivita obranného enzymu glutathionperoxidásy (GPx) ve srovnání s kontrolní skupinou. Další sledované parametry oxidativního stresu, katalása (CAT), glutathionreduktása (GR) a oxidace lipidů vyjádřená thiobarbiturovým číslem (TBARS), byly beze změn. Změny

parametrů oxidativního stresu naznačují, že expozice organizmu přípravku Nexide může v dané koncentraci vést k dysbalanci obranných enzymů.

V testu s přípravkem Cyperkill 25 EC s účinnou látkou alpha-cypermethrin v koncentraci 250 $g.l^{-1}$ bylo postupováno stejným způsobem jako v předešlém testu. I v tomto případě byly pokusné organizmy vystaveny po dobu 35 dnů (od stadia oplozené jikry do dosažení juvenilního stadia vývoje v kontrole) koncentrační řadě uvedeného přípravku (7,2; 36; 72; 144 a 360 $\mu g.l^{-1}$, tedy 1,8; 9; 18; 36 a 90 $\mu g.l^{-1}$ účinné látky). S výjimkou nejnižší testované koncentrace došlo ke 100% úhynu embryí a larev ve všech testovaných koncentracích. V koncentracích 18, 36 a 90 $\mu g.l^{-1}$ došlo k totálnímu úhynu již během prvních 10 dnů pokusu, při čemž v nejvyšší koncentraci byl zaznamenán 100 % úhyn již pátý den pokusu. Koncentrace 18 $\mu g.l^{-1}$ prodloužila dobu kulení a způsobila vysokou úmrtnost čerstvě vykulených jedinců. V koncentraci 9 $\mu g.l^{-1}$ došlo k výraznému úhynu testovaných organizmů v období přechodu na exogenní výživu. Nadále v této koncentraci přežívalo 5 % larev, které však uhynuly 17. den pokusu. V nejnižší testované koncentraci (1,8 $\mu g.l^{-1}$) dosáhlo přežití v závěru testu 90 %, což je hodnota srovnatelná s kontrolou, kde přežití činilo 95,5 %. Nejnižší testovaná koncentrace však výrazně negativně ovlivnila délkový i hmotnostní růst jedinců ve srovnání s kontrolou a vedla ke zpomalení ontogenetického vývoje. Žádný jedinec z této koncentrace nedosáhl v den ukončení studie juvenilního stadia a někteří zůstali vývojově v larválním stadiu o dva stupně pozadu (předposlední larvální stadium L5) oproti kontrole. U 68 % embryí z této koncentrace byla patrná výraznější pigmentace, která byla pozorována i u jedinců z vyšších koncentrací krátce před jejich úhynem. Přeživší jedinci z koncentrace 1,8 $\mu g.l^{-1}$ byli podrobeni dalšímu laboratornímu vyšetření. Výsledky histologického vyšetření jater, střeva, ledvin a žaber neprokázalo žádné výrazné změny oproti kontrolním jedincům. Při vyšetření vybraných parametrů oxidativního stresu bylo prokázáno statisticky významné snížení ($P < 0,01$) katalytických koncentrací GST, GR a GPx ve srovnání s kontrolou. Aktivita CAT a TBARS byly srovnatelné s kontrolou. Změny parametrů oxidativního stresu naznačují, že expozice organizmu přípravku Cyperkill 250 EC může v dané koncentraci indukovat oxidativní stres a zasahovat do aktivit antioxidačních enzymů.

Předložená dizertační práce shrnuje aktuální údaje o vlivu pyrethroidů na ryby. Zjištěné účinky potvrzují vysokou vnímavost ryb v období raného vývoje k testovaným pesticidům. Při interpretaci výsledků je nutné vzít v úvahu, že pro testování byly použity jednosložkové přípravky, které již samy toto riziko představují. V reálném prostředí jsou však vodní organizmy vystaveny celé škále látek a přípravků v potenciálně toxických koncentracích. Tyto spolu mohou reagovat a jejich kombinací se mohou škodlivé účinky ještě zesilovat. Proto je nutno věnovat pozornost těmto látkám, monitorovat jejich výskyt v životním prostředí a laboratorními testy prohlubovat znalosti o jejich účincích včetně jejich směsí. Provedené testy také jednoznačně ukazují významné rozdíly v citlivosti embryonálních a embryolarválních testů. V obou případech bylo při hodnocení testovaných přípravků potvrzeno, že citlivost testu na raných vývojových stadiích ryb výrazně stoupá s přechodem embryí do larvální fáze vývoje. Pokud by byl k hodnocení přípravků použit pouze embryonální test, je jednoznačně zřejmé, že by došlo k výraznému podcenění rizik, které přípravek představuje pro reprodukci ryb.

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LIST OF PUBLICATIONS

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Richterova, Z., Machova, J., Stara, A., Tumova, J., Velisek, J., Sevcikova, M., Svobodova, Z., 2015. Effects of a cypermethrin-based pesticide on early life stages of common carp (*Cyprinus carpio* L.). Vet. Med. 60, 423–431. (IF 2015 = 0.560)

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INTERNATIONAL ABSTRACTS AND CONFERENCE PROCEEDINGS

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