Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
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ISBN 978-80-87437-93-3

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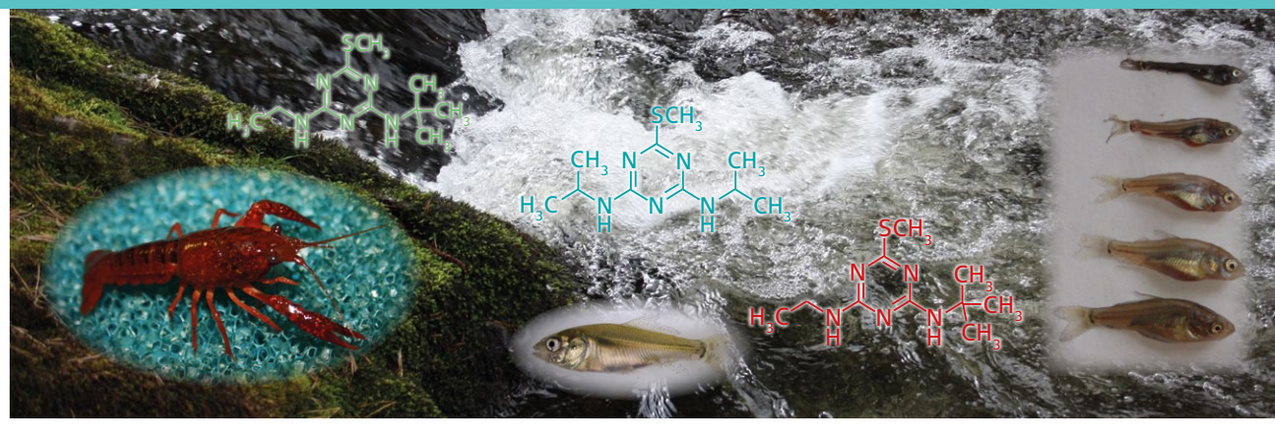
The effect of triazine based pesticides on fish

Vliv triazinových pesticidů na ryby



Alžběta Stará

Czech Republic, Vodňany, 2014

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Name: Alžběta Stará
Title of thesis: The effect of triazine based pesticides on fish

Ph.D. thesis, USB, FFPW, RIFCH, Vodňany, 2014, 111 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-87437-93-3

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Triazine

Anthropogenic pollution constitutes a worldwide problem of growing concern (Abrantes et al., 2010). Increased environmental pollution can be attributed to a variety of factors associated with industrial and agricultural technologies (Figueiredo-Fernandes et al., 2006). Agricultural development has been accompanied by parallel expansion in the use of chemical agents for pest and weed control (Bermudez-Saldana et al., 2005). The use of chemical pesticides in agriculture has undoubtedly increased crop yields and reduced post-harvest losses. As a consequence of the extensive use of these compounds, residual amounts of pesticides and their metabolites are found in all components of ecosystems, as well as in drinking water and foods, leading to risks for the environment and, consequently, for humans (Riahi et al., 2010).

Among pesticides, the most frequently reported in the environment are residuals of triazine herbicides. The triazines were discovered in 1954 (Modra and Svobodova, 2009). The structure of triazines includes a six-member ring containing three nitrogen atoms and three carbon atoms. The triazines can be symmetrical (atrazine, simazine, prometryne) with alternating nitrogen and carbon atoms around the ring, or asymmetrical with adjacent nitrogen atoms (metribuzine, metamitrone). No differences in toxicity or action appear to be linked to these structural differences (Kamrin, 1997).

Triazine compounds are used against a wide variety of weed species, primarily to control broadleaf and grassy weeds (Sanderson et al., 2001). Most are used in a selective weed control program (prometryne, simazine), others, such as prometron have non-selective (herbicide cannot differentiate between weeds you would like to kill and the plants you want unharmed) properties, which makes them suitable for use on industrial sites. They may be used alone or in combination with other herbicidal ingredients to increase the weed control spectrum (Fishel, 2012). Triazines have been used increasingly since the 1960s, particularly on maize crops in North America and Europe. The estimated use of atrazine alone in the United States was almost 35,000 tons in 1993 (Sanderson et al., 2001). However, the (US EPA, 2012) registers the amount of atrazine used up to the about 38,000 tonnes in recent years.

The chief mechanism of triazine action is inhibition of photosynthesis (Hogan, 2013). Most of the compounds interrupt photosystem II of photosynthesis, which utilizes chlorophyll to convert short-wavelength light (photons) into chemical energy for use by plants cells. Without photosystem II plants are unable to produce sufficient energy for growth. Susceptible plants, especially emergent seedlings, turn yellow and die. The compounds are applied to the soil, taken into the roots, and transferred to other parts of the plant. The effectiveness of triazines depends on their relative rates of uptake and breakdown in plants (Kamrin, 1997).

Triazines are among the most commonly used pesticides in the world. In recent years, concerns about the persistence, mobility, and toxicity of triazines and their metabolites have been mounting, owing to the detection these compounds and their residual concentrations in various environmental compartments (Chapadense et al., 2009). Detectable levels are found in drinking and ground water, foods, and fish (Solomon et al., 1996), and their metabolites are frequently found in aquatic ecosystems (Hogan, 2013).

In Italy and Spain, a considerable number of monitored aquifers are contaminated with simazine, terbuthylazine, and their de-ethylate metabolites, with concentrations greater than 0.1 $\mu g.l^{-1}$ (Grenni et al., 2009), which is the maximum admissible concentration in drinking water under EC legislation (EC 98/83EEC). Since the number of contaminated aquifers is increasing, there is a need to study the capacity of groundwater to recover from pesticide contamination (Grenni et al., 2009) along with the quality of surface water. Contamination by

triazines decreased significantly from 0.35 $g.l^{-1}$ in 1992 to 0.15 $g.l^{-1}$ in 1994 in the River Elbe (Pietsch et al., 1995). Triazines were the predominant pesticides detected in 1995 and 1996 in surface water of the Elbe and Želivka rivers, with concentrations of atrazine in the range 50 to 500 $ng.l^{-1}$. The concentration of triazines in drinking water in Prague in 1995/1996 were typically 50 $ng.l^{-1}$ to 100 $ng.l^{-1}$. Concentrations of triazines in water differ with location, from below the detection limit to hundreds of nanograms per litre (Pitter, 1999).

1.1.1. Prometryne

Prometryne (2,4-bis (isopropylamino)-6-methylthio-s-triazine) was the first effective herbicide for several crops, making it a pioneer herbicide in the thiomethyl triazine class of chemistry (LeBaron et al., 2008). Prometryne is classified as a selective herbicide of the s-triazine chemical family. It was first registered in the United States in 1964 by Ciba Crop Protection. It is available in dissolvable powder and liquid formulations, usually used as an aqueous spray, and has little to no odour (Kamrin, 1997). Prometryne has been used for pre- or post-emergence control of annual grasses and broadleaf weeds in a variety of crops, including cotton, celery, pigeon peas, and dill (US EPA, 1996a; Kamrin, 1997; Jiang and Yang, 2009). It rapidly penetrates and is absorbed by the foliage and roots and accumulates in growing shoots (Betse, 1983; US EPA, 1996a). Prometryne's mechanism of action affects plant photosynthesis by inhibiting electron transport (Erickson and Turner, 2002). Prometryne is metabolised in animals through N-dealkylation along with hydrolysis and/or amino acid conjugation (Yazgan and Tanik, 2005).

Prometryne has a soil half-life of 60 days and persists for up to 90 days. Following multiple annual applications of the herbicide, prometryne activity can persist for 12–18 months after the final application. Half-life in water is 500 days (US EPA, 1996b). Prometryne application is not permitted in the Europe Union, but is widely used in China (Zhou et al., 2009), Australia, Canada, New Zealand, South Africa, and the United States (Kegley et al., 2010). Over-use of this herbicide may lead to its accumulation in the ecosystem and cause chronic environmental toxicity (LeBaron et al., 2008).

Although prometryne was banned in the EU in 2004, it is still found in surface and groundwater. Prometryne commonly occurs at a concentration of 0.51 $\mu g.l^{-1}$ in Czech rivers (CHMI, 2011). Maximum reported concentration was 3.34 $\mu g.l^{-1}$ in River Cidlina in 2008 and a maximum concentration of 1.67 $\mu g.l^{-1}$ in groundwater was reported for 2009 (CHMI, 2014). In surface waters of Greece, prometryne has been detected at concentrations ranging from 0.190 to 4.40 $\mu g.l^{-1}$ (Vryzas et al., 2011) and in ground water at concentrations exceeding 1 $\mu g.l^{-1}$ (Papadopoulou-Mourkidou et al., 2004).

Prometryne presents a potential risk to the environment, especially to aquatic ecosystems and the non-target organisms that inhabit them (US EPA, 1996b). Prometryne is slightly to moderately toxic to aquatic organisms. Acute toxicity of prometryne to aquatic organisms is presented in Table 1.

Table 1. *Acute toxicity of prometryne to aquatic organisms.*

Species	Exposure time (h)	LC50/EC50 ($mg.l^{-1}$)	Reference
Green algae (*Selenastrum capricornutum*)	72	0.012 0.023	US EPA (1996a) Mischke and Avery (2013)
Mayfly (*Cloeon dipterum*)	48	40.0	Pesticide Ecotoxicity Database (2000)
Water flea (*Daphnia magna*)	48	9.7 18.59	Marchini et al. (1988) Mischke and Avery (2013)
Mysid shrimp (*Mysidopsis Bahia*)	96	1.7	Erickson and Turner (2002)
Signal crayfish (*Pacifastacus leniusculus*)	96	14.4	Velisek et al. (2013)
Goldfish (*Carassius auratus auratus*)	48	3.5 8.7	Extoxnet (1996a) Erickson and Turner (2002)
Zebrafish (*Danio rerio*)	96	3.0	Pesticide Ecotoxicity Database (2000)
Bluegill sunfish (*Lepomis macrochirus*)	96	7.9 10.0	Kegley et al. (2010) Mischke and Avery (2013)
Sheepshead minnow (*Cyprinodon variegatus*)	96	5.1 7.0	Kegley et al. (2010) US EPA (1996a)
Rainbow trout (*Oncorhynchus mykiss*)	96	2.5 7.2	Mischke and Avery (2013) US EPA (1996a)
Common carp (*Cyprinus carpio*)	96	8.0 9.0	Popova (1976) Extoxnet (1996a)

Commonly used herbicide products containing prometryne include Caparol® and Gesagard®. Prometryne is currently being progressively replaced by simetryn (LeBaron et al., 2008).

1.1.2. Simazine

Simazine (6-chlor-N2,N4-diethyl-1,3,5-triazin-2,4-diamin) is classified as one of the first triazine compounds. In addition to the three carbon and three nitrogen atoms in the six-membered ring, simazine possesses one chlorine and two ethylamine groups attached to the ring. Simazine was introduced by the Swiss company J. R. Geigy in 1956 and was registered in 1957 (Cremlyn, 1985; Kamrin, 1997). Simazine belongs to a group of selective herbicides and is used for a pre and post-emergence control of weeds in field crops as well as in non-crop areas. Primarily, simazine is used to control broadleaf weeds and annual grasses in crop fields such as fruit orchards. Simazine was used to control and kill submerged aquatic weeds and algae in ponds, aquaria, swimming pools, and cooling towers until 1992 (US EPA, 2006; Catalkaya and Kargi, 2009). From 1990 to 1993 it was among the most widely used herbicides in the US (US EPA, 2006). In the Czech Republic, Simazine, as the product Gesetop 90 WG(H), is used for the treatment of potatoes, alfalfa, tree fruits, grapes, hops, gooseberries, raspberries, blackberries, strawberries, trees and ornamental plants. The registration has expired, but remaining supplies may be used. It was distributed under the trade name Simazine 50WP. In 2000, application of Simazine in the Czech Republic decreased to 200 kg per year. To augment its effects, simazine is often combined with other pesticides including diuron, ametryne, and atrazine among others (Kleger, 2008).

When applied to the soil or plants, simazine is absorbed by leaves and roots, causing inhibition of photosynthesis in whole plants (Ahrens et al., 1994; Manahan 2005; US EPA, 2006). Its mechanism of action is similar to that of other triazines used in agriculture (Jerzykiewicz and Klobus, 2007). It is biodegradable, and metabolized in plants and soil through chemical and microbiological processes (LeBaron et al., 2008). Simazine is fairly resistant to physical and chemical dissipation processes in soil, and is persistent and mobile in the environment (WHO, 2006). Simazine in soil and groundwater is moderately persistent, with an average field half-life of 60 days (Kamrin, 1997). Soil half-lives of 28–149 days have been reported (Arndt, 2009). Residual activity may remain for a year after application at 2 to 4 $kg.ha^{-1}$ in high pH soils. Simazine is moderately to poorly bounds to soils (Wauchope et al., 1992). The average half-life of simazine in water, ponds, and cooling towers is 30 days, depending on the level of algae present, the degree of weed infestation, and other factors (Ahrens et al., 1994).

A major concern with any agricultural pesticide is its potential to leach from the soil into surface or ground water systems that are used as drinking water sources. The sorption and desorption of simazine in soils plays a fundamental role in the level of resulting water contamination and is affected by the heterogeneous nature of soil systems with respect to soil types and level of organic matter. Simazine as a contaminant in water systems is of primary concern, because ground and surface waters are used in many communities as the primary source of drinking water (Gunasekara, 2004). The excessive use of simazine to control weeds in field crops and aquatic vegetation in slowly flowing waters has led to the pollution of ecosystems, especially aquatic (Turner, 2003; Oropesa et al., 2009). Simazine, and its major degradation products, deisopropyl atrazine and diamino chlorotriazine, have been extensively monitored in surface and ground waters in California. Simazine was detected in concentrations ranging from 0.02 to 49.2 $\mu g.l^{-1}$ (US EPA, 1994; Gunasekara, 2004). In southwest Spain, simazine concentrations from 0.1 to 4.5 $\mu g.l^{-1}$ were detected in natural waters in 1997, 2001, and 2003. Residues of simazine were also detected in drinking water (Oropesa et al., 2008). Simazine was found in concentrations < 0.1 $\mu g.l^{-1}$ in groundwater in a clay-dominant soil area in Denmark (Spllid and Koppen, 1998). In sandy soil areas in Spain, groundwater wells were reported contaminated with simazine at concentrations below 0.1 $\mu g.l^{-1}$ (Gunasekara, 2004). In the Czech Republic, the highest concentration of simazine detected in surface water in 2006 was 6.3 $\mu g.l^{-1}$ in the Svatava River in the Sokolov District. The highest concentration of simazine detected in river water in the Czech Republic in the past five years was 0.51 $\mu g.l^{-1}$. Commonly occurring environmental concentrations in surface waters is 0.06 $\mu g.l^{-1}$, and maximum concentration in groundwater was reported as 0.51 $\mu g.l^{-1}$ in the Czech Republic (CHMI, 2014). Public concern arose, as these concentrations are higher than the maximum advisory legal limit for drinking water (0.1 $\mu g.l^{-1}$) (Council Directive 98/83/CE, 1998). Simazine had been identified as relevant in a study of the prioritization of substances dangerous to the aquatic environment in the member states of the European Community (European Commission, 1999).

Simazine may cause long-term adverse effects in the aquatic environment (Moraes et al., 2009) The acute toxicity effects of simazine are likely to be different for the various aquatic organisms in which it is moderately to slightly toxic (Turner, 2003). Highest toxicity is seen in invertebrates (Kamrin, 1997; Okland et al., 2005). Simazine acute toxicity to other aquatic animals is given in Table 2.

Table 2. *Acute toxicity of simazine on aquatic organisms.*

Species	Exposure time (h)	LC50/EC50 ($mg.l^{-1}$)	Reference
Bacteria (*Vibrio fisheri*)	1/2	> 6	Hernando a kol. (2007)
Plecoptera (*Pteronarcys reticulata*)	96	1.9	Johnson a Finley (1980)
Water flea (*Daphnia pulex*)	48	16–50	Fitzmayer et al. (1982)
Water flea (*Daphnia magna*)	48	1.1–10.0	Johnson and Finley (1980); Mayer and Ellersieck (1986); Kamrin (1997)
Freshwater shrimp (*Gammarus fasciatus*)	96	> 130	Mayer and Ellersieck (1986)
Signal crayfish (*Pacifastacus leniusculus*)	96	77.9	Velisek et al. (2013)
Rainbow fish (*Poecilia reticulata*)	48	3.5	Kamrin (1997)
Zebrafish (*Danio rerio*)	96	12.6	Rao a Dad (1979)
Mozambique tilapia (*Oreochromis mossambicus*)	96	3.1	Rao a Dad (1979)
Sheepshead minnow (*Cyprinodon variegatus*)	96	4.3	Pesticide Ecotoxicity Database (2000)
Bluegill sunfish (*Lepomis macrochirus*)	96	16	Turner (2003)
Crucian carp (*Carassius carassius*)	96	> 100	Kamrin (1997)
Goldfish (*Carassius auratus auratus*)	96	> 32	US EPA (2006)
Rainbow trout (*Oncorhynchus mykiss*)	96	25–100	Schoettger (1970); Kamrin (1997); Pesticide Ecotoxicity Database (2000);
Common carp (*Cyprinus carpio*)	48	4–40	Hashimoto and Nishiuchi (1981); Kamrin (1997); Pesticide Ecotoxicity Database (2000)

Simazine is included in the EU Priority Pollutants list and the US Environmental Protection Agency list. Simazine and plant protection products containing this substance were banned in 2004 by Commission Decision 2004/247/CE. The presence of simazine in the soil-water system in considered an environmental hazard, and, because of its estrogenic effect on various cell lines in laboratory experiments, it has recently become subject to control (Sanderson et al., 2001; Zorrilla et al., 2010).

1.1.3. Terbutryn

Terbutryn (2-t-buthylamino-4-ethylamino-6-methylthio-s-triazine) is a selective triazine herbicide. Terbutryn was first tested in 1952 in Switzerland and its herbicidal properties were first reported in 1965 by Ciba-Geigy (US EPA, 1986). Terbutryn is used as a pre-emergent and post-emergent control agent for most grasses and many annual broadleaf weeds in winter wheat, winter barley, sorghum, sugarcane, sunflowers, peas, and potatoes. However, it is also

used as an aquatic herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs, and fish ponds (Tomlin, 2003). Similarly to other triazines, it is absorbed by the roots and foliage and acts as an inhibitor of photosynthesis (Larsen et al., 2000).

Terbutryn degrades slowly, with half-life of 240 and 180 days in pond and river sediment, respectively (Muir and Yarechewski, 1982). The application of terbutryn has been banned in many countries (Rioboo et al., 2007). Use of preparations containing terbutryn has been banned since 2005 in the Czech Republic (Plhalova et al., 2010). Terbutryn has the potential to bioaccumulate in organisms (Rioboo et al., 2007). Its tendency to move from treated soils into water compartments through water runoff and leaching has been demonstrated, and residual amounts of terbutryn and its metabolites have been found in drinking water and industrial food products long after application (Villarini et al., 2000; Moretti et al., 2002; Konstantinov et al., 2006). It is currently detectible in the environment, especially in the aquatic environment (Rioboo et al., 2007; Plhalova et al., 2010).

In the Czech Republic, the highest reported concentration of terbutryn was 1.6 $\mu g.l^{-1}$ in the Bílina River in 2007 and 1.08 $\mu g.l^{-1}$ in groundwater in 2009. In the past five years, levels have not exceeded 0.66 $\mu g.l^{-1}$ in river water in the Czech Republic (CHMI, 2014). Maximum concentrations of up to 5.6 $\mu g.l^{-1}$ were determined in the Weschnitz River in Germany (Quednow and Puttmann, 2007). Terbutryn has been detected at concentrations ranging from 0.02 to 0.50 $\mu g.l^{-1}$ in surface waters in Europe (Arge Elbe, 1997; Maloschik et al., 2007; Ormand et al., 2008). Degradation products of terbutryn (hydroxy-terbutryn, terbutryn-sulfoxide, d N-deethyl terbutryn) are also detected in sediments and surface waters (Muir and Yarechewski, 1982).

Terbutryn is considered moderately toxic to fish (Meister, 1992). The organisms most sensitive to terbutryn are freshwater algae and cyanobacteria (Environmental Quality Standards, 2011). The degradation products of terbutryn show moderate toxicity to algae and low toxicity to crustacean and fish (Daho, 1994). Acute toxicity of terbutryn on aquatic organisms is presented in Table 3.

Table 3. *Acute toxicity of terbutryn to aquatic organisms.*

Species	Exposure time (h)	LC50/EC50 ($mg.l^{-1}$)	Reference
Green algae (*Selenastrum capricornutum*)	72	0.0024	De Vries et al. (1991); PPDB (2009)
Virginia oyster (*Crassostrea virginica*)	96	1.0	Pesticide Ecotoxicity Database (2000)
Brine shrimp (*Artemia salina*)	24	0.022	Pesticide Ecotoxicity Database (2000)
Water flea (*Daphnia magna*)	48	7.1	Marchini et al. (1988)
Signal crayfish (*Pacifastacus leniusculus*)	96	13.9	Velisek et al. (2013)
Zebrafish (*Danio rerio*)	96	5.20–6.13	Plhalova et al. (2010)
Bluegill sunfish (*Lepomis macrochirus*)	96	4	Weed Science Society of America (1983)
Rainbow trout (*Oncorhynchus mykiss*)	96	0.82	Mayer and Ellersieck (1986)
		3.0	Weed Science Society of America (1983)
Common carp (*Cyprinus carpio*)	96	4.0	Verschueren (2001)

1.1.4. Effect of triazines on non-target aquatic organisms – acute toxicity tests

Prometryne, simazine, terbutryn, and other s-triazines (atrazine, cyanazine, propazine, sebuthylzine, terbuthylazine) have been identified as relevant in the prioritization of substances dangerous to the freshwater aquatic environment in the member states of the European Community (Tolosa et al., 1996; European Commission, 1999). However, metabolites of these substances still occur in ecosystems, especially in the aquatic ecosystems (CHMI, 2014).

Pesticides based on triazines show differing toxicity to aquatic organisms (96hLC50 range is from 0.82–130 $mg.l^{-1}$). Copepods show greatest sensitivity and are up to 5 times more sensitive than fish. Clinical signs of poisoning fish these products are characterized mainly attenuation phases. Velisek et al. (2008, 2009) detailed the progression of clinical symptoms in rainbow trout after acute exposure to metribuzin. Initial behaviours included accelerated respiration, loss of movement coordination, and fish lying on their flanks and moving in this position. A subsequent short excitation stage including convulsions, jumping above the water surface, and movement in circles alternated with a resting stage, followed by another short excitation phase. Fish finally fell into an extended torpor phase with slowed respiration, moving mainly on the flanks, their respiration is slowed down, and the damp phase and subsequent agony are very long. Similar results were reported in goldfish (*Carassius auratus*) (Saglio and Trijasse, 1998), and zebrafish (*Danio rerio*) (Williams et al., 2012) after acute exposure to triazines. Necropsy of rainbow trout performed after acute exposure to metribuzin revealed increased amounts of watery mucus on body surface, with skin matte and dark in colour. Characteristic pathological-morphological signs of poisoning are reported to include the formation of transudate in the body cavity and in digestive tract. In addition, an increased injection of visceral vessels was observed. The presence of transudate led to an increase in body cavity size (Svobodova and Pecena, 1988; Velisek et al., 2008, 2009). Triazine

poisoning in fish is accompanied by a decrease in haemoglobin and a significant decrease in the concentration of plasma proteins (Svobodova et al., 2008). Acute exposure to triazines led to alterations in haematological indicators. Carp blood showed decrease in leukocyte and lymphocyte counts and in haematocrit. Changes in biochemical indices in exposed carp to triazines included increase in glucose, aspartate aminotransferase, ammonia, and lactate dehydrogenase, and a decrease in inorganic phosphorus, triglycerides, and chlorides (Dobsikova et al., 2011), along with changes in activity of alkaline phosphatase, glutamic-oxaloacetic transaminase, and glutamic-pyruvic tranaminase activities (Neskovic et al., 1993). The most common histopathological changes are observed in the caudal kidney, gills, and liver. Acute toxicity of atrazine to grass carp (*Ctenopharyngodon idella*) was reflected in gill epithelial lifting, partial cell proliferation, lamellar fusion, and aneurysm (Botelho et al., 2012). Similarly, in common carp, acute toxicity was characterized by hypertrophy and hyperplasia of gill epithelial cells with lamellar fusion, hyperaemia, and the presence of multiple foci of coagulation necrosis (Dobsikova et al., 2011). Gills tend to be one of the most affected organs due to direct contact with xenobiotics through the large interface between the fish external and internal environment (Oliveira Ribeiro et al., 2002). Histopathological changes in liver parenchyma typically include dystrophic lesions, morphological signs of initial cell injury represented by swelling and hydropic vacuolar degeneration of hepatocyte, focal steatosis, hyperaemia, necrosis, fatty dystrophy, glycogen inclusions, and vacuolisation (Dezfuli et al., 2006; Mikula et al., 2008; Dobsikova et al., 2011). The haematological and biochemical indicators and pathological alteration of tissues reflect the preparation's effect on the fish immune system and the stress reactions and alteration of metabolic pathways, especially in fish gill and liver (Dobsikova et al., 2011). Though, in acute toxicity tests on non-target aquatic organisms damaging effects of triazines were identified.

Reduced reproductive success has been reported in invertebrates with acute exposure to hexahydro-1,3,5-trinitro-1,3,5-triazine (Peters et al., 2009). Mutagenic and genotoxic effects of atrazine were observed in *Channa punctatus* (Nwani et al., 2011). Atrazine was also found to affect osmoregulatory capacities of the crab *Eriocheir sinensis* (Silvestre et al., 2002). Simazine and atrazine act as endocrine disruptors (Fan et al., 2007; Storek and Kavlock, 2010). In addition, atrazine was reported to show teratogenic effects and affect reproduction in amphibians and fish (Storek and Kavlock, 2010).

Although the effects of acute and sub-chronic exposure to triazines have been well documented in fish, most research in fish has focussed on effects of high concentrations for short durations of exposure. There is a dearth of data on the chronic toxicity of triazines at environmental concentrations on non-target aquatic organisms, especially freshwater fish and crayfish.

1.2. Aims of the thesis

- The main aim of this work was to investigate the effects of chronic exposure to triazines (prometryne, simazine, terbutryn) at concentrations commonly occurring in the environment on diferent developmental stages of common carp (*Cyprinus carpio* L.).
  The effect was assessed with respect to
  - behaviour and mortality,
  - biometric parameters (length and weight) especially on early life stages,
  - haematological parameters,
  - biochemical blood parameters,
  - histopathology,
  - indices of oxidative stress and antioxidant parameters.

- The effects of triazine (prometryne) on crayfish *Procambarus clarkii* as a non-target organism were also investigated with respect to
  - behaviour,
  - histopathology,
  - indices of oxidative stress and antioxidant parameters.

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CHAPTER 2

EFFECTS OF LOW-CONCENTRATIONS OF SIMAZINE ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Svobodova, Z., 2012. Effects of low-concentrations of simazine on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinology Letters 33, 90–95.

It was allowed by publisher on 24th January, 2014 to include the paper in this Ph.D. thesis.

ORIGINAL ARTICLE

Neuroendocrinology Letters *Volume 33 Suppl. 3 2012*

Effects of low-concentrations of simazine on early life stages of common carp (*Cyprinus carpio* L.)

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Submitted: 2012-09-01 Accepted: 2012-11-15 Published online: 2012-12-26

Key words: **Triazine; embryo-larval toxicity test; early development; histology**

Neuroendocrinol Lett 2012; **33**(Suppl.3):90–95 **PMID:** 23353850 NEL330912A13

Abstract

OBJECTIVES: The aim of this study is to assess the toxicity of simazine in different developmental stages of common carp (*Cyprinus carpio*) on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, and Fulton's condition factor during and at the conclusion of the test.
DESIGN: The toxicity tests were performed on carp according to OECD 210 methodologies. The developmental stages of carp were exposed to simazine at four concentrations, 0.06, (reported concentration in Czech rivers), 60, 600, and 3 000 μg.l^{-1} for 36 days and compared to carp in a non-treated control group.
RESULTS: Simazine in concentration 0.06 μg.l^{-1} had no effect on early life stages of carp. Simazine in concentration 600 and 3 000 μg.l^{-1} caused decrease of mass and total length of carp. Fish exposed to three highest levels of simazine showed alteration of tubular system of caudal kidney. On the basis of histopatological changes the values of LOEC = 60 μg.l^{-1}, NOEC = 0.06 μg.l^{-1} for simazine were estimated.
CONCLUSIONS: Chronic simazine exposure of early-life stages of common carp affected their growth rate, and histology. Some of the changes were observed only at higher exposures (600, 3 000 μg.l^{-1}), but change founded in caudal kidney was affected in fish exposed to the second lowest concentration tested (i.e., 60 μg.l^{-1}), which is about 10 μg.l^{-1} higher than reported in Colorado rivers in recent years. Concentrations of simazine in World rivers have been reported to generally vary in the range 0.0003–49.20 μg.l^{-1}.

Abbreviations:

NOEC	- no observed effect concentration
LOEC	- lowest observed effect concentration
48hLC50	- lethal concentration
ANOVA	- analysis of variance
ANC4.5	- acid neutralization capacity
CODMn	- chemical oxygen demand
FWC	- Fulton's weight condition factor
OECD	- Organization for Economic Cooperation and Development

To cite this article: **Neuroendocrinol Lett** 2012; **33**(Supp.3):90–95

INTRODUCTION

During the several centuries since the industrial revolution, humankind's activities have caused strong alterations in the structure and function of their environment. The increasing production of pesticides that enter the environment has become a real threat not only for plants or animals, but also for humans themselves. Negative consequences of anthropogenic activities include the input of toxic, persistent and bioaccumulative compounds to the environment (Haluzova *et al.* 2010; Mikulikova *et al.* 2011; Plhalova *et al.* 2011).

Simazine (6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine) was introduced by a Swiss company J. R. Geigy in 1956 and is a member of the triazine family compounds (a six-membered ring containing three carbon and three nitrogen atoms). It is a selective systemic herbicide used to control germinating annual grasses and broad-leaved weeds in a variety of crops. In addition, simazine is used on shelterbelts, nursery stock, woody ornamentals, woodlots, woodland and Christmas tree plantations, and site preparation for conifer planting (Arufe *et al.* 2004; Velisek *et al.* 2009).

Simazine is the second most commonly detected pesticide in surface and ground waters in the U.S., Europe, and Australia, presumably due to relatively high persistence in soil (up to 3 years) and water (half-life 50–176 days) and to a combination of solubility (3.5 mg.l^{-1} at 20 °C), low absorption (soil organic carbon-water partitioning coefficient – 135 ml.g^{-1}) (Inoue *et al.* 2006). Its degradation products are detected less frequently than atrazine and other triazine pesticide in the aquatic environment. The highest concentration reported in surface water in the Czech Republic is 0.06 µg.l^{-1} (Velisek *et al.* 2009). In Europe simazine levels can reach values up to 5.0 µg.l^{-1} (Beitz *et al.* 1994). In USA simazine levels can reach values up to 49.20 µg.l^{-1} (U.S. EPA 1994).

Currently simazine and another seven s-triazines have been identified as relevant in a study of the prioritization of substances dangerous to the aquatic environment in the member states of the European Community. Simazine is included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. According to Commission regulation (EU) No 196/2010 of 9 March 2010, amending Annex I to Regulation (EC) No 689/2008 of the European Parliament and of the Council concerning the export and import of dangerous chemicals simazine use is banned in the countries of the European Union. Although the effects of acute and sub-chronic exposure of fish to atrazine, another s-triazine herbicide, have been well-documented, there is a dearth of data on the sub-chronic toxicity of simazine at environmentally realistic concentrations in early life stages of common carp. The aim of the present study was to describe lethal and sublethal effects of simazine on embryos and larvae of common carp using a 36 day embryo larval toxicity test. Toxicity tests with early life stages of aquatic organisms have been proposed as a faster and more cost-efficient way of testing chemicals and environmental samples. Moreover, experience shows that these developmental stages of fishes are often the most sensitive to toxic effects, although the various embryonic and larval stages differ in their susceptibility as a result of physiological and biochemical differences (McKim 1995; Velisek *et al.* 2012a). Common carp was selected because it is the most often bred fish in the Czech Republic. That is why it is often used for the toxicity tests of chemicals and for the contamination monitoring of surface waters (Dobsikova *et al.* 2006). The toxicity of simazine was assessed on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, and Fulton's condition factor during and at the conclusion of the test.

MATERIALS AND METHODS

Experimental animals

Fertilized eggs of common carp (*Cyprinus carpio* L.) were obtained from the breeding station of the Research Institute of Fish Culture and Hydrobiology in Vodnany, University of South Bohemia (Czech Republic). Eggs were produced according to standard methods of artificial reproduction by mating 15 females with 25 males (full-factorial scheme of crossing) as described by Kocour *et al.* (2005).

Experimental protocol

The trial was carried out using the modified test design of Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals 210 (Fish, Early-life stage toxicity test). At 24 h post-fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into each crystallization basins containing tested solution of simazine (Sigma Aldrich, Czech Republic, chemical purity 99.5%) and also into the control dish. Four ascending concentrations of tested solutions and control were used, each with 100 fertilized eggs in triplicate groups. The concentrations were marked as follows: 0.06 µg.l^{-1} (reported environmental concentration in Czech rivers – group 1 – E1), 60 µg.l^{-1} (group 2 – E2), 600 µg.l^{-1} (group 3 – E3), and 3000 µg.l^{-1} (group 4 – E4). The simazine concentrations of 60 µg.l^{-1}, 600 µg.l^{-1}, and 3000 µg.l^{-1} corresponded to the 0.15% of 48-hour half lethal concentration (48h LC50), 1.5% 48h LC50 and 7.5% 48h LC50 for juvenile common carp (*Cyprinus carpio*).

Water parameters

Aerated tap water was used in the present study, with the following parameters: dissolved oxygen >93%, temperature 19.3–21.1 °C, pH 7.2–8.1, $ANC_{4.5}$ 1.00 mmol.l^{-1}, COD_{Mn} 1.0 mg.l^{-1}, total ammonia 0.01 mg.l^{-1}, NO_3^- 6.30 mg.l^{-1}, NO_2^- 0.02 mg.l^{-1}, sum of Ca^{2+}+Mg^{2+} 10.1 mg.l^{-1}. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature

were measured daily. Simazine concentrations were checked daily by high performance liquid chromatography. Water samples were assayed using the method of Katsumata *et al.* (2005). The values measured did not differ from the value stated for test purposes by more than 7%.

Experimental protocol

The basins were placed in a laboratory (open-air conditions) with the natural light exposure (16:8 h light: dark), and the arrangement of basins was random. The exposure water for each treatment was renewed twice daily by gently draining each chamber then adding new solution slowly to prevent disturbing the embryos and larvae. Observations of hatching, survival, and behavior were made twice daily and dead embryos and larvae were collected. When able to feed, larvae were given freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii ad libitum twice daily prior to water exchange. The nauplii were rinsed with tap water to avoid contaminating the exposure water with chloride. During and at the conclusion of the trial, samples of embryos and larvae were collected to monitor development, occurrence of morphological anomalies, rate of length and weight, Fulton's weight condition factor (FWC = body weight (g)/standard lenght (cm)$^3 \times 100$), and the length–weight relationship. Samples were collected at days 8, 19, 25, 32, and 36. Samples were fixed in 4% formalin, with 10 specimens per replicate (i.e., 30 per group).

Determination of developmental periods and stages followed Penaz *et al.* (1983), who described nine embryonic (E1–E9), six larval (L1–L6), and two juvenile stages (J1–J2). Final measurements included accumulated mortality, basic length parameters for fish with no cranial–skeletal deformities (TL, total length; SL, standard length), and mass. The length parameters were measured under a stereomicroscope (Olympus SZ61/SZ51) using a micrometer (accuracy of 0.01 mm). Weight, to 0.1 mg, was measured by using a Mettler-Toledo balance. The trial schedule was as follows: day 1, trial beginning (1 day before: fertilization of eggs); day 5–6, hatching completed; day 9, beginning of exogenous nutrition (*A. salina*); day 36, end of the trial (at that time, the majority of fish in the control group had reached the first juvenile stage).

The mean specific growth rate (SGR) for fish in each of the experimental groups was calculated for the period beginning at day 8 (the first sampling time) and ending at day 36 (end of the trial). The following equation was used (OECD 2000).

$$SGR = \frac{\overline{\ln w_2} - \overline{\ln w_1}}{t_2 - t_1} \cdot 100$$

where SGR = mean specific growth rate in the group, w_1 = mass of one fish at time t_1 individually (μg), w_2 = mass of one fish at time t_2 individually (μg), $\overline{\ln w_1}$ = mean value of the ln w_1 values, $\overline{\ln w_2}$ = mean value of the ln w_2 values, t_1 = time (days) – first sampling time, t_2 = time (days) – end of exposure.

The inhibition of specific growth rate in each experimental group was calculated using the following formula according to OECD number 215 (OECD 2000):

$$I_x[\%] = \frac{SGR_x\ (control) - SGR_x\ (group)}{SGR_x\ (control)} \cdot 100$$

where I_x = inhibition of specific growth in selected experimental group after x days of exposure, SGR_x (*control*) = mean specific growth rate in the control group, SGR_x (*group*) = mean specific growth rate in selected experimental group.

Statistical analysis

The statistical software program STATISTICA (version 8.1 for Windows, StatSoft) was used to compare differences among test groups. Prior to analysis, all measured variables were evaluated for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If these 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 conditions for ANOVA were not satisfied, a non-parametric test (Kruskal-Wallis) was used.

Evaluation of LOEC and NOEC

For the evaluation of LOEC, and NOEC values, the probit analysis was used on the basis of histopatological changes of cranial kidney in the test groups and the EKOTOX 5.1 software (Ingeo Liberec) was used.

Histopathology examination

Histopathology was evaluated in all experimental groups at the samples days. Five whole fish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, and stained with hematoxylin and eosin. Histological changes in samples of skin, gills, caudal and cranial kidney and liver were examined by light microscopy.

RESULTS

Hatching began 5 day after the onset of exposure, on days 5 to 6. The majority of eggs in all treatment groups hatched by day 6. No significantly negative effects of simazine on hatching and embryos viability were observed compared to control.

Accumulated mortality

Cumulative mortality of common carp samples exposed to simazine and the control sample was between 7–11% (Figure 1). Simazine in the tested concentrations had no negative influence on the early-life stages of common carp mortality.

Tab. 1. Growth rate and fish mortality results of the 36 day embryo-larval toxicity test on common carp after simazine exposure.

Fish Group	Control	E1	E2	E3	E4
Simazine (µg.l^{-1})	–	0.06	60	600	3000
m_8 (Mean ± SD, mg)	1.84±0.21	1.88±0.33	1.77±0.23	1.64±0.16	1.71±0.21
m_{36} (Mean ± SD, mg)	94.95±22.88	90.61±26.46	104.24±29.71	67.74±14.76*	65.59±20.78*
SGR	14.00	13.69	14.40	13.21	12.78
I (%)	–	2.21	–2.28	5.60	8.71
Total mortality (%)	11	8	7	8	7

m_8, m_{36} = Mean fish mass in selected group after 8 and 36 days exposure; SGR = mean specific growth rate in selected group; I = inhibition of specific growth in selected group; SD = standard deviation. *Experimental groups significantly (p<0.05) different from the control group.

Length and weight growth parameters

Mass and total length of fish related to simazine concentration in water are depicted in Figures 2 and 3. From 32 days of exposure, fish exposed to the two highest tested groups E3 – 600 µg.l^{-1}, and E4 – 3000 µg.l^{-1} simazine showed significantly (p<0.05) lower mass compared with controls. By day 36, fish exposed to the highest tested concentration of simazine showed significantly lower (p<0.05) total length compared with controls. Simazine in the tested concentrations had no negative influence on FWC. Specific growth rates and inhibition of growth were calculated for 36 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to the highest simazine concentration (3000 µg.l^{-1}) was 8.71 % compared to control.

Early ontogeny

No significant differences (p<0.05) in early ontogeny of carp embryos and larvae were noticed among test groups. Early ontogeny indicators of all tested groups were comparable to the control. Furthermore, no significant differences in the type and occurrence of morphological abnormalities were observed in tested fish embryos and larvae during the test.

Histopatology

The most histological changes were observed in caudal kidney in groups E2, E3 and E4 at all sampling time compared to control fish and group E1. Fish exposed to three higher levels of simazine showed alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules (Figure 4). No histopathological changes were demonstrated in skin, gills, liver and cranial kidney following exposure to simazine. On the basis of histopatological changes of cranial kidney in the experimental groups, values of LOEC = 60 µg.l^{-1} simazine, NOEC = 0.06 µg.l^{-1} simazine were estimated.

DISCUSSION

The most sensitive method of assessing the influence of chemicals on early life stages of fish, since fish are exposed through the all critical periods of early devel-

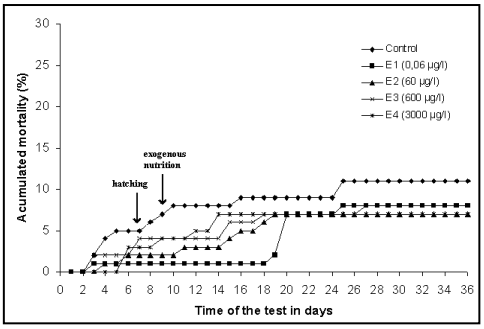

Fig. 1. Accumulated mortality (percentage) of common carp embryos, larvae, and juveniles after simazine exposure.

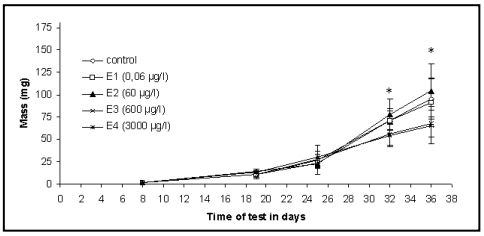

Fig. 2. Mean mass of common carp larvae (juveniles) after simazine exposure. *Experimental groups significantly (p<0.05) different from the control group.

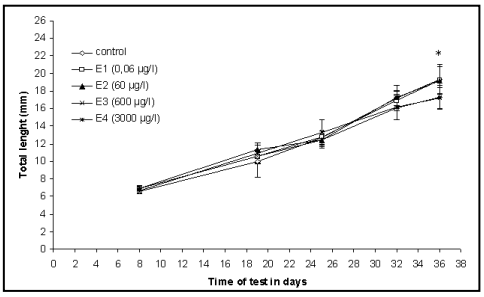

Fig. 3. Total length of common carp larvae (juveniles) after simazine exposure. *Experimental groups significantly (p<0.05) different from the control group.

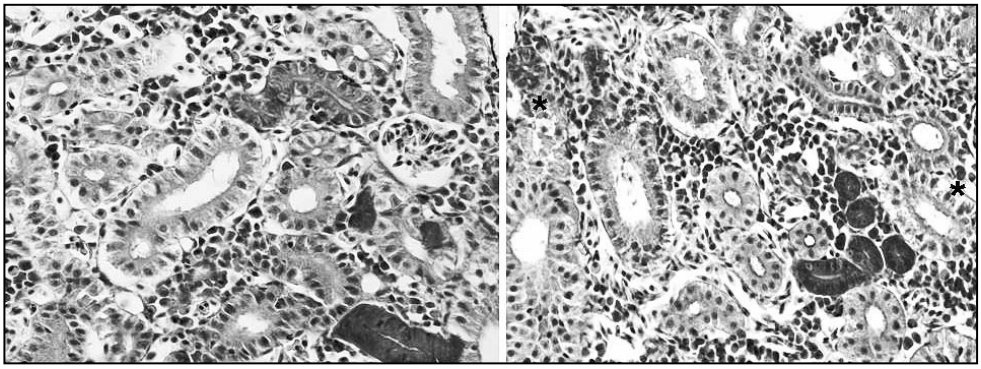

Fig. 4. Caudal kidney of carp (*Cyprinus carpio* L.) after simazine exposure. Haematoxylin and eosin, × 400. A – control group 1, B – group E4 (concentration 3000 μg.l^{-1}of simazine). The stars indicate destructed tubular epithelium.

opment: hatching, swim-up, first-feeding, early development of gills, and metamorphosis (McKim 1995). Studies of the embryonic development of fishes are important, not only to increase knowledge of the developmental processes, but also to understand species-specific adaptations and their ecological value in the course of speciation. Also, the economic relevance for aquaculture and fishery biology is generally accepted (Meijide & Guerrero 2000). Aquatic toxicologists have convincingly defended the usefulness of particularly the advantages of fish early life stage tests as a fairly rapid, lowcost and comparable tool to screen test chemicals and effluents (Woltering 1984). Although we have information on the toxicity of the simazine in adult stages of fish, we do not have too much information on the toxicity of this compound in early life stages of fish. The results of this study provide further data on chronic exposure to simazine for consideration in risk assessment. The findings contribute to improved knowledge of the toxic profile of simazine at actual concentrations in Czech river on early life stage of carp.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). The present study revealed no significant negative effects of simazine on hatching, embryo viability, morphology, mortality of embryos and larvae and early ontogeny at the concentrations tested (0.06–3000 μg.l^{-1}). In the present study, beginning on day 32 of exposure, fish exposed to the two highest tested groups simazine showed significantly lower mass compared with controls. Simazine in highest concentration (3000 μg.l^{-1}) caused decrease of total length of carp after 36 day exposure. Growth represents an integration of a variety of physiological and environmental factors. It provides a sensitive gauge of environmental conditions and is important for reviewing the success with which organisms adapt to their environment (Bengtsson 1974). Growth reductions might delay maturation and reproduction as well as increase the susceptibility of young fish to predation and disease. Their ability to obtain food and to compete for suitable habitats might also be reduced (Rosenthal & Alderdica 1976; Woltering 1984). Plhalova *et al.* (2009) and Velisek *et al.* (2012a) reported a significant decrease growth after terbutryn exposure in zebrafish (*Danio rerio*), respectively in carp.

Little is known about the histopathological changes in development stages of carp after triazine exposure. Generally, triazine pesticides have a direct effect on kidney structure and function in freshwater fish (Velisek *et al.* 2009; 2010; 2011; 2012a,b). The caudal kidney of carp exposed to simazine in the three highes concentrations showed alteration of tubular system of caudal kidney. The kidney is important for the maintenance of a stable internal environment with respect to water and sodium chloride, excretion, and, partially, for the metabolism of pesticides (Ortiz *et al.* 2003). It is evident that renal alteration was related to simazine exposition, while the degree of kidney alteration was not affected by liver. On the basis of our findings it is possible to describe simazine as a primary nephrotoxic substance. Histopathological tissue changes in cranial kidney were similar to the changes found in rainbow trout, sea bream and common carp by other authors (Fischer-Scherl *et al.* 1991; Arufe *et al.* 2004; Oropesa *et al.* 2009).

Histopathological changes were used for estimation of NOEC and LOEC. The values for NOEC and LOEC were estimated at 0.06 and 60 μg.l^{-1} simazine, respectively. It appears that simazine may not be a serious problem for early-life stages of carp in the wild in the Czech Republic. Because we found, that simazine in real concentration in Czech rivers has not influence on early-life stages of carp. But changes founded in caudal kidney were found in fish exposed to the second lowest concentration tested (i.e., 60 μg.l^{-1}), which is

about 10 μg.l^{-1} higher than reported in Colorado rivers in recent years. Concentrations of simazine in World rivers have been reported to generally vary in the range 0.0003–49.20 μg.l^{-1} (Beitz *et al.* 1994; U.S. EPA 1994).

Chronic simazine exposure of early-life stages of common carp affected their growth rate, and histology. Some of the changes were observed only at higher exposures (600, 3000 μg.l^{-1}), but change founded in caudal kidney was affected in fish exposed to the second lowest concentration tested (i.e., 60 μg.l^{-1}). Aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. For detailed elucidation of simazine effects further research is necessary. This research should be focused not only on the studies of effects of simazine alone, but in view of possible synergic or potentiation effect.

ACKNOWLEDGMENTS

This research was supported by the Czech Science Foundation Project No. 525/09/P218, the centre CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No.047/2010/Z, Project No. QH82117.

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CHAPTER 3

EFFECT OF TERBUTRYN AT ENVIRONMENTAL CONCENTRATIONS ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Prokes, M., Svobodova, Z., 2012. Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pesticide Biochemistry and Physiology 102, 102–108.

It was allowed by publisher on 9th January, 2014 to include the paper in this Ph.D. thesis.

Pesticide Biochemistry and Physiology 102 (2012) 102–108

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.)

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ARTICLE INFO

Article history:
Received 19 August 2011
Accepted 15 November 2011
Available online 23 November 2011

Keywords:
Triazine
Embryo–larval toxicity test
Lowest observed-effect concentration
No observed-effect concentration
Early development
Malformation

ABSTRACT

The aim of this study was to assess the toxicity of terbutryn to early developmental stages of common carp (*Cyprinus carpio*). Based on accumulated mortality in the experimental groups, lethal concentrations of terbutryn were estimated at 36 day LC50 = 3.06 mg l^{-1} terbutryn. Based on inhibition of growth in the experimental groups, lowest observed-effect concentration (LOEC) = 0.005 mg l^{-1} terbutryn; and no observed-effect concentration (NOEC) = 0.0007 mg l^{-1} terbutryn. Fulton's condition factors were significantly lower in fish exposed to 2 mg l^{-1} compared with controls. By day 30, fish exposed to 0.00002 mg l^{-1} – real environmental concentration in Czech rivers – 0.02, 0.2, and 2 mg l^{-1} terbutryn showed significantly lower mass and total length compared with controls. No significant negative effects on hatching or embryo viability were demonstrated at the concentrations tested, but significant differences in early ontogeny among groups were noted. Fish from the two highest tested concentrations (0.2 and 2 mg l^{-1}) showed a dose-related delay in development compared with the controls. At concentrations of 0.02, 0.2, and 2 mg l^{-1} damage to caudal kidney tubules when compared to control fish was found.

1. Introduction

The growing use of chemicals in agriculture, including fertilizers and pesticides, significantly contributes to environmental pollution. It is estimated that currently the contribution of pesticides to pollution is comparable to emissions from industrial sources. A number of studies confirmed the presence of pesticides in the atmosphere and in precipitation. They have been detected in even the most remote locations.

Symmetrical triazines (s-triazines) constitute a group of herbicides used primarily to control broadleaf and grassy weeds in agriculture and nonagricultural sites that have become ubiquitous contaminants of the environment [1]. Eight s-triazines (atrazine, cyanazine, prometryn, propazine, sebuthylzine, simazine, terbuthylazine, and terbutryn) have been identified as relevant [2], based on a compilation of monitoring data from freshwaters in the member states of the European Community.

Terbutryn (*N2-tert*-butyl-*N4*-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) is used as a selective pre- and early post-emergence control agent of most grasses and many annual broadleafed weeds for a variety of crops such as cereals and tree fruits and is also used as a herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs, and fish ponds [3]. Terbutryn is moderately toxic to fish. Bathe et al. [4] and Kidd and James [5] reported the mean lethal toxicity of terbutryn (96hLC50) as 4 mg l^{-1} for common carp. Plhova et al. [6] reported a no observed effect concentration (NOEC) of 0.2 mg l^{-1} and lowest observed effect concentration (LOEC) of 0.6 mg l^{-1} of terbutryn for zebrafish (*Danio rerio*).

A tendency of terbutryn to move from treated soils into water compartments through runoff and leaching has been demonstrated, and residual amounts of terbutryn and its metabolites have been found in drinking water long after application [7]. The application of terbutryn has been banned in many countries because it has the potential to bioaccumulate in organisms, but it has been still detected in water environment [8]. Preparations containing terbutryn have not been registered in the Czech Republic since 2005, but its presence can be still detected in the environment. The highest concentration reported in surface water in the Czech Republic is 0.02 μg l^{-1} [9]. In Europe simazine levels can reach values up to 5.6 μg l^{-1} [10].

Although the effects of acute and sub-chronic exposure of fish to atrazine, another s-triazine herbicide, have been well-documented, there is a dearth of data on the sub-chronic toxicity of terbutryn at environmentally realistic concentrations in early life stages of common carp. The aim of the present study was to

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0048-3575/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2011.11.005

describe lethal and sublethal effects of terbutryn on embryo and larvae of common carp using a 36 day embryo larval toxicity test. Toxicity tests with early life stages of aquatic organisms have been proposed as a faster and more cost-efficient way of testing chemicals and environmental samples. Moreover, experience shows that these developmental stages of fishes are often the most sensitive to toxic effects, although the various embryonic and larval stages differ in their susceptibility due to of physiological and biochemical differences [11,12]. Newly hatched larvae constitute a particularly critical and sensitive life stage, since at hatching the embryos lose their protective membrane and are fully exposed to potential toxicants. Common carp was selected because it is the most frequently bred fish in the Czech Republic. It is often used for the toxicity tests of chemicals and the contamination monitoring of surface waters [13,14]. The toxicity of terbutryn was assessed on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, and Fulton's condition factor (FCF) during and at the conclusion of the test.

2. Materials and methods

2.1. Experimental animals

Fertilized eggs of common carp (*Cyprinus carpio* L.) were obtained from the breeding station of the Research Institute of Fish Culture and Hydrobiology in Vodnany, University of South Bohemia (Czech Republic). Eggs were produced according to standard methods of artificial reproduction by mating 15 females with 25 males (full-factorial scheme of crossing) as described by Kocour et al. [15]. Sampling was done randomly from a homogenized batch of eggs, so possible genetic and/or maternal effects on the results of the trial were minimized.

2.2. Water parameters

Aerated tap water was used, with the following parameters: dissolved oxygen >85%, temperature 19.0–21.0 °C, pH 7.3–8.2, $ANC_{4.5}$ (acid neutralization capacity) 1.09 mmol l^{-1}, COD_{Mn} (chemical oxygen demand) 1.3 mg l^{-1}, total ammonia 0.02 mg l^{-1}, NO_3^- 8.23 mg l^{-1}, NO_2^- 0.01 mg l^{-1}, sum of Ca^{2+}+ Mg^{2+} 12.2 mg l^{-1}. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature were measured daily. Terbutryn concentrations were checked daily by high performance liquid chromatography (HPLC). The water was chromatographed on a reverse phase HPLC column (Lichrosphere 100 RP_{18}, Vertex column, pore size 100 μm, particle size 5 μm, 250 × 5 mm ID) using a solvent system of methanol:water:ammonium acetate 70:30:0.2 and 80:20:0.2 (v:v:v) isocratically, at a flow rate of 0.7 ml min^{-1}. Injection volumes of samples were 100 μl per injection. UV detection was recorded at 230 nm. Column eluents (1 min fractions) were collected in scintillation vials using a fraction collector (LKB 2212 HeliRac; Amersham Pharmacia Biotech, Freiburg), dissolved in a scintillation cocktail, and counted by LSC. Water samples were assayed using the method of Richter and Nagel [16]. Measured values did not differ from the value stated for test purposes by more than 2%.

2.3. Experimental protocol

The trial was carried out using the modified test design of the Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals 210 [17]. At 24 h post-fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into 15 crystallization basins containing the test solutions of terbutryn (Sigma–Aldrich, Czech Republic, chemical purity 99.2%) as well as into a control dish. Four ascending concentrations of test solutions and a control were used, each with 100 fertilized eggs in triplicate groups. The concentrations were as follows: 0.00002 mg l^{-1} (reported environmental concentration in Czech rivers, Group 1 – E1), 0.02 mg l^{-1} (Group 2 – E2), 0.2 mg l^{-1} (Group 3 – E3), and 2 mg l^{-1} (Group 4 – E4). Terbutryn concentrations of 0.02, 0.2, and 2 mg l^{-1} corresponded to the 0.5%, 5%, and 50% 96hLC50 for carp.

The basins were placed in a laboratory (open-air conditions) with the natural light exposure (16:8 h light:dark). The arrangement of basins was random. The water for each treatment was renewed twice daily by gentle draining each chamber and adding new solution slowly to prevent disturbing embryos and larvae. Observations of hatching, survival, and behavior were made twice daily and dead embryos and larvae were removed. When able to feed, larvae were given freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii *ad libitum* twice daily prior to water exchange. The nauplii were rinsed with tap water to avoid contaminating the exposure water with chloride.

During, and at the conclusion of, the trial samples of embryos and larvae were collected to monitor development, occurrence of morphological anomalies, rate of length and weight increase, FCF, and the length/weight relationship. Samples were collected on days 13, 20, 26, 30, and 36. Samples were fixed in 4% formalin, with 10 specimens per replicate (i.e., 30 per group).

Determination of developmental periods and stages followed Penaz et al. [18], who described nine embryonic (E1–E9), six larval (L1–L6), and two juvenile stages (J1–J2). Final measurements included accumulated mortality, basic length parameters for fish with no cranial/skeletal deformities (TL, total length; FL, fork length; SL, standard length), and mass.

The length parameters were measured under a stereomicroscope (Olympus SZ61/SZ51) using a micrometer (accuracy of 0.01 mm). Weight to 0.1 mg was measured by using a Mettler–Toledo balance.

2.4. Trial schedule

The trial schedule was as follows: day 1, trial beginning (1 day after fertilization of eggs); day 6, hatching completed; day 9, beginning of exogenous feeding (*A. salina*); day 36, end of the trial (at that time, the majority of fish in the control group had reached the first juvenile stage).

2.5. Growth rate evaluation

The mean specific growth rate (SGR) for fish in each of the experimental groups was calculated for the period beginning on day 6 (the first sampling time) and ending on day 36 (end of the trial). The following formula was used:

$$\mathrm{SGR} = \frac{\overline{\ln w_2} - \overline{\ln w_1}}{t_2 - t_1} \cdot 100$$

where SGR = mean specific growth rate in the group, w_1 = mass of one fish at time t_1 individually (μg), w_2 = mass of one fish at time t_2 individually (μg), $\overline{\ln w_1}$ = mean value of the $\ln w_1$ values, $\overline{\ln w_2}$ = mean value of the $\ln w_2$ values, t_1 = time (days) – first sampling time, t_2 = time (days) – end of exposure.

The inhibition of specific growth rate in each experimental group was calculated as follows

$$I_x\ [\%] = \frac{\mathrm{SGR}_x(\mathrm{control}) - \mathrm{SGR}_x(\mathrm{group})}{\mathrm{SGR}_x(\mathrm{control})} \cdot 100$$

where I_x = inhibition of specific growth in selected experimental group after x days of exposure, SGR_x (control) = mean specific

growth rate in the control group, SGR_x (group) = mean specific growth rate in selected experimental group.

2.6. Statistical analysis

The statistical software program Statistica (ver. 8.0 for Windows, StatSoft) 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 [19]. The differences in accumulated mortality among the test groups were checked by using contingency tables (χ^2).

2.7. Evaluation of 36 day LC50, LOEC, and NOEC

For the evaluation of 36 day LC50 values, a probit analysis was used based on mortality at different terbutryn concentrations. For the evaluation of LOEC and NOEC values, the probit analysis was based on inhibition of growth at different terbutryn concentrations; 36 day IC5 and 36 day IC10 values were used to express the NOEC and LOEC values, respectively. For evaluation, the EKOTOX 5.1 software (Ingeo Liberec) was used.

2.8. Histopathology

Histopathology was evaluated in all experimental groups at the end of the experiment (day 36). Six whole fish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, and stained with hematoxylin and eosin, examined by light microscopy, and photographed using a digital camera.

3. Results

3.1. Hatching

Hatching began 4 day after the onset of exposure, on days 4–5. The majority of eggs in all treatment groups hatched by day 6. No significantly negative effects of terbutryn on hatching and embryo viability were observed.

3.2. Accumulated mortality

Significant ($p < 0.01$) differences in total accumulated mortality were found in fish exposed to the highest terbutryn concentration, 2 mg l^{-1} terbutryn (50% 96hLC50), compared with controls (Fig. 1). Massive mortality in this group occurred on days 26 and 27. Based on accumulated mortality in the experimental groups, values of lethal concentrations of terbutryn was estimated at 36 day LC50 = 3.06 mg l^{-1} terbutryn.

3.3. Length and weight growth parameters

Mass and total length of fish as related to terbutryn concentration in water are shown in Figs. 2–4. Beginning on day 30 of exposure, fish exposed to all tested terbutryn concentrations showed significantly ($p < 0.01$) lower mass and total length compared with controls. The FCF values are given in Table 1. The FCF values were significantly ($p < 0.01$) lower into the highest terbutryn concentration, 2 mg l^{-1} after 30 and 36 days compared with controls.

Specific growth rates and inhibition of growth are shown in Table 2. Inhibition of growth in the group exposed to the highest terbutryn concentration (2 mg l^{-1}) was 39.83% compared to control. Based on inhibition of growth in the experimental groups, values of LOEC = 0.005 mg l^{-1} terbutryn, NOEC = 0.0007 mg l^{-1} terbutryn were estimated.

3.4. Early ontogeny

The developmental stages observed at the sampling times in all tested concentrations and controls are listed in Table 3. Significant differences in early ontogeny among test groups were observed for the duration of the trial. Fish from two highest tested

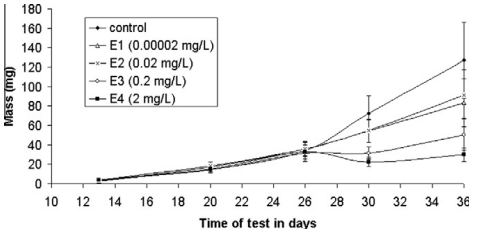

Fig. 2. Mean mass ± SD of common carp larvae (juveniles) after terbutryn exposure.

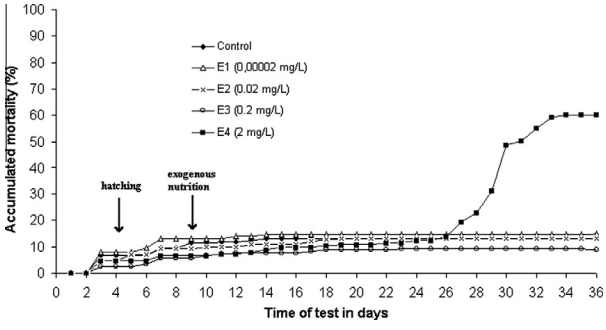

Fig. 1. Accumulated percent mortality of common carp embryos, larvae, and juveniles after terbutryn exposure.

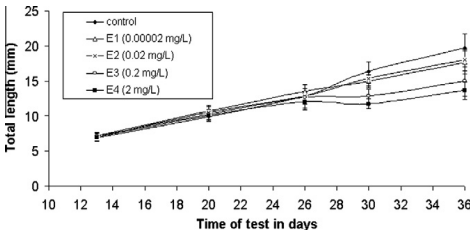

Fig. 3. Total length ± SD of common carp larvae (juveniles) after terbutryn exposure.

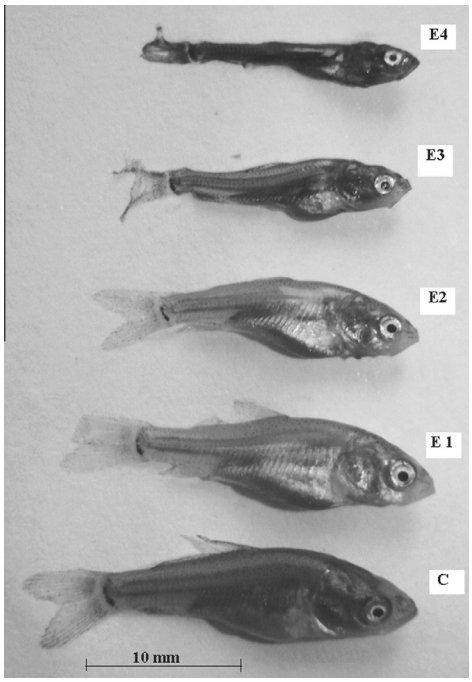

Fig. 4. Common carp after terbutryn exposure. Control (C), E1-0.00002 mg l^{-1} (reported environmental concentration in Czech rivers), E2-0.02, E3-0.2, and E4-2 mg l^{-1}.

concentrations (0.2 and 2 mg l^{-1}) were delayed in development compared with the control group. The percent of individuals in larval stages (L5 or L6) increased with higher concentrations of terbutryn, whereas the majority of control fish reached the juvenile stage.

3.5. Macroscopic morphological anomalies

Similar morphological anomalies were found in fish in both experimental and control groups. These included axial and/or lateral curvature of the spine (lordosis, and scoliosis), yolk sac deformity, and body shortening. The incidence of these anomalies was 0.1%, which could be considered a spontaneous appearance.

3.6. Histopathology

The majority of histological changes were observed in caudal kidney in groups E2, E3, and E4 compared to control fish and Group 1 fish. Fish exposed to higher levels of terbutryn showed alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomeruli (Fig. 5).

Histological examination of liver revealed diffused steatosis of the liver associated with the loss of cellular shape and the presence of lipid inclusions in hepatic cells. Described pathologies were found in fish within the experimental groups, and in the control group.

No histopathological changes were demonstrated in gills following exposure to terbutryn.

4. Discussion

Many studies have dealt with the effects on fish of acute toxic concentrations of terbutryn and triazine herbicides, e.g., atrazine, simazine, metribuzin, and terbutryn [1,20–22]. Toxicity tests in the first stages of a fish life cycle (embryo, larva, and juvenile) are commonly used to predict environmental damage. Early life stages are more sensitive to environmental impacts than are subsequent stages [23,24]. Studies of the embryonic development of fishes are important, not only to increase knowledge of the developmental processes, but also to understand species-specific adaptations and their ecological value in the course of speciation. Also, the economic relevance for aquaculture and fishery biology is generally accepted [25]. The results of this study provide further data on chronic exposure to terbutryn for consideration in risk assessment. The findings contribute to improved knowledge of the toxic profile of terbutryn at actual concentrations in Czech river on early life stage of common carp.

The present study revealed no significant negative effects of terbutryn on hatching or embryo viability at the concentrations tested (0.00002–0.2 mg l^{-1}). However, a marked influence was found on survival of larvae. Studies have reported that hatching can be affected by exposure to chemicals [26,27]. Larval mortality of 60% was observed at the highest terbutryn concentration

Table 1
Mean Fulton's condition factor values of common carp larvae and juveniles after terbutryn exposure.

Fish group	Control	E1	E2	E3	E4
Terbutryn (mg l^{-1})	–	0.00002	0.02	0.2	2
Times (day)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
13	1.00 ± 0.29	0.68 ± 0.18	0.99 ± 0.32	1.00 ± 0.19	1.05 ± 0.39
20	1.46 ± 0.32	1.39 ± 0.18	1.57 ± 0.42	1.34 ± 0.20	1.51 ± 0.23
26	1.62 ± 0.42	1.47 ± 0.25	1.80 ± 0.94	1.52 ± 0.28	1.80 ± 0.25
30	1.61 ± 0.17	1.60 ± 0.18	1.48 ± 0.15	1.52 ± 0.34	1.27 ± 0.34[a]
36	1.62 ± 0.20	1.46 ± 0.17	1.54 ± 0.21	1.46 ± 0.37	1.17 ± 0.20[a]

[a] Experimental groups significantly ($p < 0.01$) different from the control group.

Table 2
Growth rate and fish mortality results of the 36 day embryo–larva toxicity test on common carp after terbutryn exposure.

Fish Group	Control	E1	E2	E3	E4
Terbutryn (mg l^{-1})	–	0.00002	0.02	0.2	2
m_{13} (Mean ± SD, mg)	3.35 ± 0.94	2.76 ± 0.80	3.62 ± 1.33	3.78 ± 1.08	3.59 ± 1.42
m_{36} (Mean ± SD, mg)	127.64 ± 39.20	83.40 ± 24.54*	91.62 ± 25.76*	50.41 ± 16.04*	29.73 ± 6.63*
SGR	15.74	15.13	14.57	11.22	9.74
I (%)	–	3.88	7.43	28.72	39.83
Total mortality (%)	13	15	13	9	60*

[a] m_{13}, m_{36} = mean fish mass in selected group after 16 and 36 days exposure; SGR = mean specific growth rate in selected group after 20 days exposure; I = inhibition of specific growth in selected group after 24 days exposure; SD = standard deviation.
* Experimental groups significantly ($p < 0.01$) different from the control group.

Table 3
Developmental periods (DPS) during the 36 day embryo–larva toxicity test on common carp.

Fish group	Control	E1	E2	E3	E4
Terbutryn (mg l^{-1})	–	0.00002	0.02	0.2	2
Times (day)	DSP	DSP	DSP	DSP	DSP
13	L1–L4	L1–L3	L1–L4	L1–L4	L1–L4
20	L4–L5	L4–L5	L4–L5	L4–L5	L4–L5
26	L4–L6	L5–L6	L5–L6	L5–L6	L4–L5
30	L5–L6	L5–L6	L5–L6	L4–L5	L4–L5
36	L6–J1	L6–J1	L6–J1	L5–L6	L4–L5

(2 mg l^{-1}) and was higher on days 26–31 of the experiment. Most control larvae survived to 26 days post-hatching, at which time it is likely that all yolk sac nutrients were exhausted. This is in accordance with data concerning the so called "point of no return" – the moment when the larvae irreversibly lose ability to feed, and die even if provided with food. In common carp larvae the point of no return occurs about day 15 post-hatching [28], and starvation-induced mortality occurs after that time.

On the basis of accumulated mortality data, the 36-day lethal concentration of terbutryn (36 day LC50) was estimated at 3.06 mg l^{-1}. The values obtained in the present long-term trial on early-life stages of carp are similar to those reported in short-term tests on adult specimens, 4 mg l^{-1} terbutryn [4,5]. Kidd and James [5] reported a mean lethal toxicity of terbutryn (96hLC50) of 3 mg l^{-1} for rainbow trout (*Oncorhynchus mykiss*). Arufe et al. [1] stated the 72hLC50 of a commercial herbicide formulation containing terbutryn (59.4%) as 1.41 mg l^{-1} for seabream (*Sparus aurata* L.). Terbutryn 96 h LC50 values for the freshwater fish *Lepomis macrochirus* and rainbow trout are 4 and 3 mg l^{-1}, respectively [4]. In this respect, the present study confirms the previously published results of acute toxicity tests on fish of varying age categories, which showed that adult fish were less sensitive to pesticide than their younger counterparts [29,30].

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses [11,31]. Growth represents an integration of a variety of physiological and environmental factors. It provides a sensitive gauge of environmental conditions and is important for reviewing the success with which organisms adapt to their environment. Growth can be considered a more sensitive measure than mortality [32]. Growth reductions might delay maturation and reproduction as well as increase the susceptibility of young fish to predation and disease. Their ability to obtain food and to compete for suitable habitats might also be reduced [31,33]. A few authors have studied effects of sublethal concentrations of triazines on fish growth, histopathological changes, hematological indices, and biochemical indices [6,14,34].

In the present study, beginning on day 30 of exposure, fish exposed to all concentrations of terbutryn showed significantly lower mass and total length compared with controls. Alvarez and Fuiman [34] found that the growth of red drum fish larvae was significantly affected by atrazine. Plhalova et al. [6] reported a significant decrease in zebrafish growth associated with a terbutryn concentration of 0.6 mg l^{-1}. Avoaja and Oti [35] observed that *Heteroclarias* (hybrid) exposed to sublethal concentrations of thiodon, malathion, and carbaryl decreased the rate of feeding, food conversion ratio, and feeding efficiency in a dose dependent manner, resulting in drastic reduction in growth. Similar observations were made by Aquigwo [36] who reported that specific growth rate, food conversion efficiency, and protein ratio decreased as the concentration of cypermethrin increased.

Inhibition of growth was used for estimation of NOEC and LOEC. The values for NOEC and LOEC were estimated at 0.0007 and 0.005 mg l^{-1} terbutryn, respectively. These values are lower compared with the results on juvenile zebrafish, for which the NOEC value was estimated at 0.2 mg l^{-1} and LOEC at 0.6 mg l^{-1} terbutryn in a 28 day trial [6]. It appears that terbutryn may not be a serious problem for early-life stages of carp in the wild in the Czech Republic, since concentrations exceeding NOEC levels usually do not occur in rivers of the area [37]. However, fish can be subjected to similar or even higher concentrations than estimated LOEC. For example in Weschmitz River, Germany, a maximal concentration of 0.0056 mg l^{-1} of terbutryn was found [10]. Other results of terbutryn exposure also should be taken into an account, since, in the

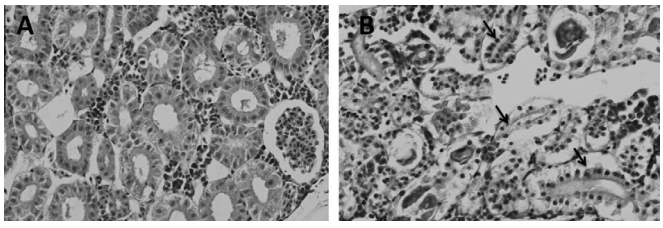

Fig. 5. Caudal kidney of carp (*Cyprinus carpio* L.) after terbutryn exposure. Hematoxylin and eosin, 400×. (A) Control group 1, (B) group E4 (concentration 2 mg l^{-1} of terbutryn). The arrows indicate damage tubular epithelium.

present study, terbutryn was related to a decrease of growth in fish at all tested concentrations and, at higher concentrations, was accompanied by delayed development compared to the control fish.

The developing fish embryo and early larval stages have been shown to be especially sensitive indicators of many types of aquatic pollution [38,39]. Chemically induced adverse effects on embryonic stages is based on developmental events, e.g., organogenesis. Although we have diverse information on the toxicity of terbutryn in adult stages of fish, little is known of effects of this compound on embryonic development in early life stages of common carp at environmental concentrations. Significant differences in early ontogeny among test groups were observed for the duration of this trial. Fish from two highest tested concentrations (0.2 and 2 mg l^{-1}) were delayed in development compared with the control group.

In fish, developmental malformations have been linked to the presence of environmental pollutants such as persistent organochlorines and pesticides [23]. Occurrence of body malformation was not observed after terbutryn exposure in the present study. In other studies, pesticide effects that have been observed on fish embryonic development have included malformations in myoskeletal development (such as notochord abnormalities of degeneration), defects along the rostral–caudal body axis, curvature of the vertebral column, and edemas in the pericardial area or yolk sac [40–44].

Triazine pesticides have a direct effect on kidney structure and function in freshwater fish [22,45,46]. The caudal kidney and liver of carp exposed to terbutryn showed cellular alterations such as destruction of the tubular system and glomeruli in caudal kidney and loss of cellular shape and lipid inclusions in hepatocytes. We observed massive diffused steatosis in liver of all examined fish. There were no differences in intensity of damage between control and experimental groups, we attribute these pathologies to inadequate diet and test condition. Although Arufe et al. [1] described similar alteration of liver in gilthead seabream (*S. aurata*) after mixture terbutryn–triasulfuron exposure. The kidney is important for the maintenance of a stable internal environment with respect to water and sodium chloride, excretion, and, partially, for the metabolism of pesticides [47]. The caudal kidney parenchyma was intact in the control group, while the fish in the experimental group show extensive alteration of renal epithelium. Fischer-Scherl et al. [48] observed changes in renal corpuscles and tubules of rainbow trout after chronic exposure to atrazine at a concentration of 5–40 μg l^{-1}. Biagianti-Risbourg and Bastide [49] reported that atrazine affects other tissues in fish, particularly liver, which shows a substantial increase in the size of lipid inclusions, followed by lipoid degeneration, enlargement of the secondary lysosomes, mitochondrial malformation and vacuolization, and a reduction in glycogen content. It is evident that renal alteration was related to terbutryn exposition, while the degree of kidney alteration was not affected by liver dystrophy. On the basis of our findings it is possible to describe terbutryn as a primary nephrotoxic substance.

5. Conclusions

Chronic terbutryn exposure of early-life stages of common carp affected their survival, growth rate, early ontogeny, and histology. Some of the changes were observed only at higher exposures (0.02, 0.2, and 2 mg l^{-1}), but growth rate was affected in fish exposed to the lowest concentration tested (i.e., 0.00002 mg l^{-1}), which is that reported in Czech rivers in recent years. Concentrations of terbutryn in Czech and European rivers have been reported to generally vary in the range $\leqslant$5.6 μg l^{-1}. However, the aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. For detailed elucidation of terbutryn effects further research is necessary. This research should be focused not only on the studies of effects of terbutryn alone, but potentiation may occur antagonism with other pollutants.

Acknowledgments

This research was supported by the Czech Science Foundation Project No. 525/09/P218, the centre CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No.047/2010/Z.

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CHAPTER 4

EFFECT OF CHRONIC EXPOSURE TO PROMETRYNE ON OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

Stara, A., Machova, J., Velisek, J., 2012. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinology Letters 33, 130–135.

It was allowed by publisher on 24th January, 2014 to include the paper in this Ph.D. thesis

ORIGINAL ARTICLE

Neuroendocrinology Letters *Volume 33 Suppl. 3 2012*

Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in early life stages of common carp (*Cyprinus carpio* L.)

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Submitted: 2012-09-01 Accepted: 2012-11-15 Published online: 2012-12-26

Key words: **triazine; fish; oxidative stress; antioxidant indices**

Neuroendocrinol Lett 2012; **33**(Suppl.3):130–135 **PMID:** 23353856 NEL330912A19 • **www.nel.edu**

Abstract

OBJECTIVES: The aim of the study was to investigate effects of the triazine herbicide prometryne on early life stages of common carp Cyprinus carpio as indicated by oxidative stress and antioxidant indices.

DESIGN: Toxicity tests were performed according to OECD 210 methodologies. Common carp larvae and embryos exposed for 35 days to prometryne at three concentrations, 0.51 (reported concentration in Czech rivers), 80 (1% 96 h LC50), and 1200 (15% 96 h LC50) $\mu g l^{-1}$, were compared to carp in a non-treated control group. Activity of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), as well as levels of thiobarbituric acid reactive substances (TBARS) were assessed.

RESULTS: Chronic exposure of early life stages of carp to prometryne showed no effect on growth and mortality rates. Levels of oxidative damage in fish test groups showed no significant differences from the controls. Glutathione reductase activity at exposure 0.51 $\mu g l^{-1}$ was significantly increase ($p<0.01$) compared with controls and other exposures.

CONCLUSION: The chronic exposure to prometryne showed no influence on oxidative stress. Differences from control fish was observed in GR activity in exposure prometryne 0.51 $\mu g l^{-1}$.

Abbreviations

CAT	- catalase
GR	- glutathione reductase
ROS	- reactive oxygen species
SOD	- superoxide dismutase
TBARS	- levels of thiobarbituric acid reactive substances

To cite this article: **Neuroendocrinol Lett** 2012; **33**(Supp.3):130–135

INTRODUCTION

Environmental pollution, an increasing worldwide concern (Abrantes *et al.* 2010), can be attributed to a variety of sources resulting from industrial and agricultural technologies (Figueiredo-Fernandes *et al.* 2006). Agricultural development has led to growth in the use of chemical agents for pest control. These can disperse in the environmental media, and directly or indirectly threatening human health and the environment (Bermudez-Saldana *et al.* 2005; Abrantes *et al.* 2010). Specifically rivers, lakes, ponds, and reservoirs are predisposed to receiving and accumulating contaminants discharged in industrial sewage, as well as agriculture runoff (Ceyhun *et al.* 2010).

Triazine herbicides are among pollutants frequently monitored in the aquatic environment. They are relatively persistent, with detectable levels in drinking water, foods, and fish (Plhalova *et al.* 2011). Some evidence has linked the mode of triazine compound toxicity in mammals to a disruption of the vitamin metabolism (Kamrin 1997). Triazine herbicides have the ability to disrupt energy metabolism leading to symptoms such as difficulty walking, tremors, convulsions, paralysis, slowed breathing, and diarrhea (Hayes & Laws 1991).

Prometryne, 2,4-bis (isopropylamino)-6-methylthio-s-triazine, a selective herbicide of the s-triazine family, has been utilized as a pre- or post-emergence controller of annual grasses and broadleaf weeds in a variety of crops, including cotton, celery, pigeon peas, and dill (U.S. EPA 1996a; Kamrin 1997). Prometryne was first registered in 1964 by Ciba Crop Protection (U.S. EPA 1996a). Prometryne has a soil half-life of 60 days and persists for up to three months. Following multiple annual applications of the herbicide, prometryne activity can persist for 12–18 months after the final application (U.S. EPA 1996b). The maximum concentration of prometryne in Czech rivers is 0.51 $\mu g l^{-1}$ (Czech Hydrometeorological Institute). In Europe, concentrations of 0.190 to 4.40 $\mu g l^{-1}$ have been reported in surface waters (Vryzas *et al.* 2011) and exceeding 1 $\mu g l^{-1}$ in ground water (Papadopoulou-Mourkidou *et al.* 2004). Studies indicate that prometryne poses an acute risk to non-target terrestrial and aquatic plants. Five-day EC50 of prometryne for algae (*Selenastrum capricornutum*) is 0.023 $mg l^{-1}$ (U.S. EPA 1996a). Prometryne is slightly to moderately toxic to fish. Acute toxicity (96 h LC50) in rainbow trout (*Oncorhynchus mykiss*) is 2.9 $mg l^{-1}$ (U.S. EPA 1996b); for sheepshead minnow (*Cyprinodon variegatus*), 5.1 $mg l^{-1}$ (Kegley *et al.* 2010); for bluegill sunfish (*Lepomis macrochirus*), 7.9 $mg l^{-1}$; and for common carp (*Cyprinus carpio*), 8 $mg l^{-1}$ (Popova 1976). Chronic prometryne toxicity in freshwater fish has not been investigated to a significant extent. Adverse effects on growth have been reported in fathead minnows (*Pimephales promelas*) exposed to 1 to 2 $mg l^{-1}$ prometryne for 32 days (Erickson & Turner 2002). Triazine herbicides are among the most commonly used pesticides in the world. Prometryne is widely used in China, Australia, Canada, New Zealand, South Africa, and the United States, but is banned in Europe (Kegley *et al.* 2010).

In recent years, concerns about the persistence, mobility, and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in water and in various environmental compartments (Chapadense *et al.* 2009). It is necessary to study the long-term effects of these substances on non-target organisms, especially fish, which are an important component of the aquatic environment and the human diet. Oxidative stress, along with antioxidant systems, may be a good indicator of prometryne accumulation in fish tissue. Although the effects of acute and sub-chronic exposure of fish to triazine herbicides have been well documented, there is a scarcity of data on the chronic exposure to these compounds at environmentally realistic concentrations with respect to oxidative stress and antioxidant responses in the fish species typical of Central Europe. The aim of the present study was to investigate effects of long-term exposure of common carp to low prometryne concentrations with respect to oxidative stress and the antioxidant defense system.

MATERIALS AND METHODS

Experimental animals

Fertilized eggs of common carp were obtained from the breeding facility of the Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in Vodnany, Czech Republic. Eggs were produced according to standard methods of artificial reproduction by mating 15 females with 25 males (full-factorial crossing) as described by Kocour *et al.* (2005).

Experimental design

The trial was carried out using the modified test design of the Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals 210 (Fish, Early-life stage toxicity test). At 24 h post-fertilization, unfertilized eggs were discarded, and 100 randomly selected fertile eggs were transferred to crystallization basins for each containing one of three concentrations of prometryne (Sigma Aldrich, Czech Republic, chemical purity 99.3%) and to a control dish. The trial was done in triplicate groups. The concentrations were: Group 1, 0.51 $\mu g l^{-1}$ (reported environmental concentration in Czech rivers); Group 2, 80 $\mu g l^{-1}$ (1% 96 h LC50); and Group 3, 1200 $\mu g l^{-1}$ (15% 96 h LC50). The test baths consisted of continuously gently aerated tap water with the following parameters: dissolved oxygen >93%, temperature 19.3–21.1 °C, pH 7.56, $ANC_{4.5}$ 3.3 mmol l^{-1}, COD_{Mn} 1.1 $mg l^{-1}$, total ammonia < 0.02 $mg l^{-1}$, NO_3^- 5.55 $mg l^{-1}$, NO_2^- < 0.01 $mg l^{-1}$, PO_4 0.228 $mg l^{-1}$, Ca_2^+ 49.10 $mg l^{-1}$, and Mg_2^+ 13.60 $mg l^{-1}$. Oxygen saturation, pH, and temperature were mea-

sured daily. Prometryne concentrations were checked daily by high performance liquid chromatography. Water samples were assayed following Katsumata *et al.* (2005). The values measured did not differ from the value stated for test purposes by more than 7%.

The basins were placed in a laboratory (open-air conditions) with ambient light exposure (16:8 h light:dark). The arrangement of basins was random. The exposure water for each treatment was renewed twice daily by gently draining each chamber and adding new solution slowly to prevent disturbing the embryos and larvae. Observations of hatching, survival, and behavior were made twice daily. Dead embryos and larvae were collected, and the numbers recorded for each concentration. When able to feed, larvae were given freshly hatched brine shrimp (*Artemia salina*) nauplii *ad libitum* twice daily prior to water exchange. The nauplia were rinsed with tap water to avoid contaminating the exposure water with chlorine.

Samples of carp early life stage and preparation of post-mitochondrial supernatant

The trial ended at 35 days when fish were weighed and measured for total length. Samples were immediately frozen and stored at –80 °C for analysis. Frozen tissue samples were weighed and homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and a second centrifuged at 4 °C to obtain the post-mitochondrial supernatant for other antioxidant analyses.

Indices of oxidative stress

The TBARS method described by Lushchak *et al.* (2005) was used to evaluate lipid peroxidation.

Antioxidant parameters

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically by the method of Marklund & Marklund (1974). The catalase (CAT; EC 1.11.1.6) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers & Sizer (1952). Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg & Mannervik 1975). One unit of CAT or GR activity is defined as the amount of the enzyme that consumes 1 mol l^{-1} of substrate or generates 1 mol l^{-1} of product per min. Activity was expressed in international units (or milliunits) per mg of protein.

Protein estimation

Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The software program STATISTICA (version 8.0 for Windows, StatSoft) was used to compare differences among the test groups. Prior to analysis, all measured variables were checked for normality (Kolmogorov-Smirnov test) and homoskedasticity of variance (Bartlett's test). If these conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. When a significant difference was detected ($p<0.05$), Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a non-parametric test (Kruskal-Wallis) was used.

RESULTS

Length, weight, and mortality

Length and weight growth parameters and total mortality of carp early life stages after 35 days exposure to prometryne are shoved in Table 1. Fish groups exposed to the selected prometryne concentrations did not differ in weight, total length, or mortality rate compared with controls.

Oxidative stress

Effects of chronic exposure to prometryne on TBARS in the homogenate carp early life stages are given in Table 2. The test groups were not significantly different from the control group in TBARS in homogenates.

Antioxidant response

Effect of chronic exposure to prometryne on antioxidants responses (SOD, CAT, GR) in homogenate of carp early life stages are given in Table 3. Exposure Group 1, showed significantly higher GR activity than the controls and other exposures tested to prometryne ($p<0.01$). No differences from controls were observed in SOD and CAT activity of exposed groups.

Tab. 1. Total length, weight, and mortality of early life stages carp after 35 day exposure to prometryne.

Fish Group Prometryne (µg l^{-1})	Control –	1 0.51	2 80	3 1 200
Total length (mm)	19.93±1.92	19.31±2.09	18.35±1.61	18.77±1.45
Weight (mg)	106.73±32.52	90.98±29.88	80.01±19.83	84.77±20.79
Total mortality (%)	14	17	13	29

Tab. 2. Effects of chronic exposure to prometryne on level of thiobarbituric acid reactive substances (TBARS, nmol/mg protein) activity in homogenates of carp early life stages.

Fish Group Prometryne (µg l^{-1})	Control –	1 0.51	2 80	3 1 200
TBARS	0.40±0.06	0.43±0.04	0.39±0.03	0.38±0.01

Tab. 3. Effect of chronic exposure to prometryne on superoxide dismutase (SOD, nmol NBT/min/mg protein), catalase (CAT, μmol H_2O_2/min/mg protein) and glutathione reductase (GR, nmol NADPH/min/mg protein) activity in homogenate of carp early life stages.

Fish Group Prometryne (μg l^{-1})	Control –	1 0.51	2 80	3 1 200
SOD	0.28±0.03	0.24±0.03	0.27±0.03	0.26±0.06
CAT	0.61±0.13	0.51±0.16	0.50±0.16	0.49±0.12
GR	0.34±0.10	0.90±0.19**	0.43±0.38	0.26±0.15

** Experimental groups significantly (p<0.01) different from the control group.

DISCUSSION

Studying the toxicity of pesticides to early life stages of fish is useful as embryos and larvae are often the most sensitive to toxic effects, although they may differ in susceptibility due to physiological and biochemical differences (McKim 1995). We observed chronic exposure to prometryne at concentrations commonly occurring in the aquatic environment on the early life stages of carp. No significant effect was observed on total length, weight, and mortality the fish. Effects of the triazine herbicide terbutryne at environmental concentrations on early life stages of carp were studied by Velisek *et al.* (2012). The fish exposed to terbutryne at 0.02 μg l^{-1} showed significantly lower weight and overall length, and at 2 mg l^{-1}, high mortality compared to controls was found. Terbutryne at 0.02 μg l^{-1} had no affect growth on *Danio rerio* (Plhalova *et al.* 2009). Mikulikova *et al.* (2011) found the environmental concentration of terbuthylazin (380 ng l^{-1}) affected biometric indices of carp experimentally exposed for 91 days.

The main objective of this study was to determine the influence of prometryne on carp oxidative stress and antioxidant parameters. This knowledge has a great importance for environmental and aquatic toxicology. Oxidative stress can be evoked by numerous chemicals including some pesticides. Pro-oxidant action on fish can be used to assess water pollution (Slaninova *et al.* 2009). Contaminant-stimulated reactive oxygen species (ROS) production, and resulting oxidative damage, may be a significant mechanism of toxicity in aquatic organisms exposed to pollution (Livingstone 2003). Oxidative stress occurs when there is an imbalance between pro-oxidant and antioxidant production, leading to the generation of ROS (Nwani *et al.* 2010). Environmental xenobiotics are known to alter antioxidant defense systems and to cause oxidative damage in aquatic organisms by ROS production (Monteiro *et al.* 2006). Among the ROS produced, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (OH·) at high concentrations can react with biological macromolecules potentially leading to lipid peroxidation, enzyme inactivation, and possibly cell death (Kevin *et al.* 2005). At low concentrations effects are less pronounced. Lipid peroxidation determined by the formation of TBARS is one of the commonly used markers of oxidative stress (Nwani *et al.* 2010). In the present study, no elevated levels of TBARS in response to the exposure to prometryne were observed. Toni *et al.* (2010) described increased TBARS levels in brain of carp exposed to the herbicide bispyribac-sodium at 20.87 μg l^{-1} after 7, 21, and 72 days. Changes were observed in muscle after 21 days. Levels were increased in liver after 72 days. Moreas *et al.* (2007) observed effects of the herbicides clamazone (0.5 mg l^{-1}) and propanil (3.6 mg l^{-1}) on *Leporinus obtusidens*. The TBARS levels were lower in brain and muscle, and higher in liver, after 30 days exposure. Responses to oxidative stress may differ with respect to species, age, habitat, feeding, behavior, duration of exposure, particular tissues, and concentration of the herbicide tested.

Virtually in all tissues of vertebrates contain ROS continuously generated during metabolism. Living cells have developed a variety of defense mechanisms to neutralize the damaging effects of free radicals of ROS (Zhang *et al.* 2004). The antioxidant defense system includes enzymes such as superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase (Menezes *et al.* 2011a). These antioxidants scavenge free radicals to prevent oxidative damage. SOD and CAT systems provide a first line of defense against ROS (Nwani *et al.* 2010). Superoxide dismutase catalyses the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by CAT. This enzyme removes the hydrogen peroxide, which is metabolized to oxygen and water (Menezes *et al.* 2011b). Glutathione reductase also provides a first line of defense against oxidative damage by maintaining cytosolic concentrations of gluthatione (Stephensen *et al.* 2002). In this study, change was observed only in GR activity. At tested concentration 0.51 μg l^{-1} levels of GR was high compared to the control group and other tested concentrations. Regulatory mechanisms of GR activity in fish have not been fully elucidated. It has been reported that oxidized glutathione content is a critical factor in GR induction (Stephensen *et al.* 2002). Generally, elevated GR activity reflects the oxidation of reduced glutathione, which is converted to glutathione, the substrate of GR activity (Elia *et al.* 2006). Santos & Martinez (2012) evaluated the effects of atrazine on *Prochilodus lineatus* as indicated by antioxidant biomarkers in liver. Antioxidant enzymes SOD and CAT were significantly reduced in fish exposed to atrazine concentrations of 2 and 10 μg l^{-1} for 24 and 48 h. On the other hand, activity of GR significantly increased in fish exposed to these concentrations, in both experimental periods. Nwani *et al.* (2010) exposed *Channa punctatus* to atrazine at concentrations of 4.238, 5.3, and 10.6 mg l^{-1} for 15 days. Activity of SOD, CAT, and

GR were increased in liver in a concentration dependent pattern, suggesting the use of these antioxidants as potential biomarkers of contamination exposure in freshwater fish. Numerous studies have demonstrated that exposure to triazine herbicides affects the antioxidant defense system in fish, causing an imbalance between reactive oxidative system production and elimination and resulting in oxidative stress and organism damage (Stara *et al.* 2012). These studies provide evidence that the biochemical responses are dependent on stressor type, species, and duration of exposure (Nwani *et al.* 2010).

This study focused on the effect of chronic exposure to prometryne on early life stages of carp, using biomarkers of oxidative stress and antioxidant enzymes. Chronic exposure to prometryne significantly increased GR activity in fish. The use of these biomarkers to determine damage in fish and pollution of the aquatic environment were shown to be an important tool for detecting adverse effects of pesticides such as triazine herbicides, even at low concentrations.

ACKNOWLEDGMENTS

This research was supported by the center CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No.047/2010/Z, Project No. GAJU 022/2012 Z.

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CHAPTER 5

BIOCHEMICAL, PHYSIOLOGICAL AND MORFOLOGICAL RESPONSES IN COMMON CARP (*CYPRINUS CARPIO* L.) AFTER LONG-TERM EXPOSURE TO TERBUTRYN IN REAL ENVIRONMENTAL CONCENTRATION

Velisek, J., Stara, A., Kolarova, J., Svobodova, Z., 2011. Biochemical, physiological and morfological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryn in real environmental concentration. Pesticide Biochemistry and Physiology 100, 305–313.

It was allowed by publisher on 9th January, 2014 to include the paper in this Ph.D. thesis.

Pesticide Biochemistry and Physiology 100 (2011) 305–313

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Biochemical, physiological and morfological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryn in real environmental concentration

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ARTICLE INFO

Article history:
Received 20 April 2011
Accepted 20 May 2011
Available online 30 May 2011

Keywords:
Triazine
Hematology
Blood biochemistry
Histology
Biomarkers
Oxidative stress

ABSTRACT

The effects of terbutryn at concentrations of 0.02 (reported concentration in Czech rivers), 0.2, and 2.0 μg l^{-1} were assessed in one-year-old common carp (*Cyprinus carpio* L.) exposed for 90 days. Influence on biometric parameters, hematology, biochemistry, histology, and oxidative stress was investigated. Exposure to 0.02, 0.2 and 2.0 μg l^{-1} showed significant differences oxidative stress biomarkers compared to controls but exposure to 0.2 and 2.0 μg l^{-1} significantly affected biochemical and hematological profiles. Long-term exposure of terbutryn in carp resulted in slight alterations in internal organs and increased reactive oxygen species formation, resulting in oxidative damage to lipids and proteins and inhibition of antioxidant capacities.

1. Introduction

Environmental pollution caused by pesticides, especially in aquatic ecosystems, has become a serious problem. Due to their extensive use in agriculture and their persistence, many of these compounds are present in surface and ground waters and must be considered a potential risk for aquatic organisms as well as for drinking water quality [1,2]. Contamination of surface waters has been well-documented worldwide and constitutes a major issue at local, regional, national, and global levels [3–5].

Triazine herbicides are categorized in two groups, the asymmetrical triazines or triazinones, such as metribuzin, and the symmetrical triazines. The major commercially used symmetrical triazines are simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon, and terbutryn. Terbutryn (*N2-tert*-butyl-*N4*-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) is a selective herbicide for grasses and many annual broadleaf weeds used in culture of a variety of crops, legumes, and tree fruits. It is also used as an herbicide for control of submerged and free-floating weeds and algae in water [6]. Terbutryn is a selective systemic herbicide which acts as a photosythesis inhibitor in the xylem and accumulates in the apical meristems. Large quantities of terbutryn have been used since the mid-1980s [7].

Terbutryn degrades slowly, with a half-life of 240 and 180 days in pond and river sediments, respectively [8]. Its tendency to move from treated soils into water compartments through runoff and leaching has been demonstrated, and residual amounts of terbutryn and its metabolites have been found in drinking water and industrial food products long after application [9]. The application of terbutryn has been banned in many countries because of its potential to accumulate in organisms, but is still being detected in water [10]. Preparations containing terbutryn have not been registered in the Czech Republic since 2005, but its presence can be still detected in the environment. The highest concentration of terbutryn detected in river water in the Czech Republic in the past five years was 0.02 μg l^{-1} [2]. Concentrations of this substance, both in surface and ground water, are variable, since they are influenced by season and by the extent of terbutryn-based herbicide use in the location investigated (Table 1).

The mean lethal toxicity of terbutryn (96hLC50) to be 4 mg l^{-1} for common carp [19]. Little information is available on the mutagenic and/or genotoxic potential of terbutryn [20,21], and no effect was observed in several reviews [19,22].

Although the effects of acute and sub-chronic exposure of fish to atrazine, another s-triazine herbicide, have been well-documented, there is a dearth of data on the chronic toxicity of terbutryn to common carp at environmentally realistic concentrations. The aim of the present study was to evaluate, through biometric, biochemical, hematological, and histopathological examination and oxidative stress biomarkers, the effects of

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doi:10.1016/j.pestbp.2011.05.004

Table 1
Reported concentration of terbutryn in surface water ($\mu g\ l^{-1}$).

Country (region)	Concentration ($\mu g\ l^{-1}$)	Collection	Reference
Germany (Weschmitz River)	5.60	2003–2006	Quednow and Putmann [11]
Germany (Elbe River)	0.02	1996–1997	Agre Elbe [12]
Germany (Brandbach River)	0.12–0.48	1987–1997	LUA [13]
Switzerland (Lake Geneva)	0.0024	1988	Buser [14]
Hungary (Lake Balaton)	0.10–0.4	2003–2004	Maloschik et al. [15]
Spain (Ebro River Bosin)	0.50	2006	Ormad et al. [16]
Monaco (Costed'Azur)	0.184	1994	Tolosa et al. [17]
UK (South Yorkshire)	0.13–0.077	2002–2004	Lapworth et al. [18]

long-term exposure of common carp (*Cyprinus carpio* L.) to terbutryn in real environmental concentration.

2. Materials and methods

2.1. Experimental design

Eighty common carp (mean body length 25.84 ± 1.96 cm, mean weight 353.24 ± 81.67 g), were obtain from a local commercial hatchery (Vodnany, Czech Republic). They were held in aquaria containing 200 l of freshwater. The aquaria were provided with continuous aeration, a constant temperature (18.0 ± 1.9 °C) and a 12:12 h light–dark cycle. Dissolved oxygen values were maintained at 90–99% and pH was 7.5 ± 0.4. Fish were fed with commercial fish food and were acclimatized for 14 days before the beginning of the experiment.

The experiment was a semi-static assay conducted over a 90-days period. One-year-old common carp were allocated, in groups of 10, to one of three experimental regimes or to an untreated control group. The conditions were duplicated for a total of eight groups, each held in an aquarium containing 200 l of water. The physicochemical parameters of the water in all aquaria were acid neutralization capacity, $ANC_{4.5}$ 1.02 mM l^{-1} total ammonia 0.02 mg l^{-1}; NO_3^- 7.92 mg l^{-1}; NO_2^- 0.001 mg l^{-1}, PO_4^{3-} 0.015 mg l^{-1} and chemical oxygen demand, COD_{Mn} 1.2 mg l^{-1}. Water temperature was 16.3–20.8 °C, and oxygen saturation 90–99%.

The experimental fish were exposed to terbutryn (Sigma Aldrich, Czech Republic, chemical purity 99.2%) at: 0.02 $\mu g\ l^{-1}$ water (reported environmental concentration in Czech rivers – group 1), 0.2 $\mu g\ l^{-1}$ water (group 2), and 2 $\mu g\ l^{-1}$ water (group 3). The terbutryn concentration of 0.2 and 2 $\mu g\ l^{-1}$ corresponded to the 0.0005% 96hLC50 and 0.005% 96hLC50 for carp.

Aquaria for all treated fish and controls were replicated, and fish were transferred daily to the replicate aquarium containing freshly diluted terbutryn at the appropriate concentration or into freshwater for the controls. Fish were fed commercial fish pellets at about 1% body weight per day in two feedings. During the experiment, fish behavior, their willingness to feed, and the number of dead fish were recorded.

Terbutryn concentrations were checked daily by high performance liquid chromatography (HPLC) using the method of Richter and Nagel [23]. Measured values did not differ from the value stated for test purposes by more than 8%.

2.2. Hematological, biochemical and histopathological examination

After 90 days exposure, the fish were individually sampled and weighed. Eight fish from each replicate of each group were examined to determine biometric parameters, and hematological, biochemical, and histopathological profiles, as well as for indicators of oxidative stress.

Blood was drawn from the *vena caudalis* and samples stabilized with 40 IU sodium heparin 1 ml blood. Erythrocyte count (RBC), hematocrit (PCV), leucocrit (Bc), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), leukocyte count (Leuko), and the differential leukocyte count (Leukogram), were determined by Svobodova et al. [24].

Blood was separated by centrifugation at 12,000*g* for 10 min at 4 °C. Plasma samples were held at −80 °C until analysis. Biochemical indices evaluated included glucose (GLU), total protein (TP), albumin (ALB), total globulins (GLOB), ammonia (NH_3), triacylglycerols (TAG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gama-glutamyl-transferase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK), creatine (CREA), lactate (LACT), amylase (AMYL), lipase (LIPA), alkaline phosphatase (ALP), calcium (Ca^{2+}), magnesium (Mg), and inorganic phosphate (PHOS). For the biochemical analysis of plasma, the VETTEST 8008 analyzer (IDEXX Laboratories Inc. USA) manufactured by Medisoft was used.

Vitellogenin (VTG) and 11-ketotestosterone (KT) in plasma was measured using pre-coated ELISA kits (Biosense laboratoriess Norway) according to the manufacturer's instructions. The use of carp vitellogenin ELISA for determination of vitellogenin in carp was validated by Flammarion et al. [25]. Absorbance was measured using an SLT Spectra (A5082) set at 492 nm for VTG and at 420 nm for KT detection. The limits of detection (LOD) of the procedure for VTG and KT were 10 ng ml^{-1} and 13 pg ml^{-1}, respectively.

For histological studies, the liver, spleen, and cranial and caudal kidney were immediately fixed in formalin (10% formaldehyde), dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin, examined by light microscopy, and photographed using a digital camera.

2.3. Determination of biometric parameters

After blood sampling, body weight (BW) and standard length (SL) were recorded. Condition factor (CF) of each fish was calculated according to the formula CF = BW (g)/SL $(cm)^3 \times 100$. Livers (LW) and spleens (SW) were removed and weighed, and a hepatosomatic index (HSI) for each fish was calculated (HSI = liver weight/BW × 100).

2.4. Liver biomarkers

After blood sampling, samples of liver was taken for liver biomarker assessment. The tissue was quickly removed, immediately frozen, and stored at −80 °C until analysis. Liver samples were homogenized in buffer (0.25 M saccharose, 0.01 M TRIS and 0.1 mM EDTA) and centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was transferred to ultracentrifugation tubes and centrifuged again at 100,000*g* for 1 h at 4 °C. The supernatant was drained, and pellets were washed with buffer and resuspended in buffer. This suspension was put into individual Eppendorf tubes.

Before the enzymes were assayed, microsomal protein concentrations were determined by the Lowry method [26].

The hepatic ethoxyresorufin-O-deethylase (EROD) activity was determined spectrofluorometrically (LOD 2 pmol mg^{-1} min^{-1}). In the presence of NADPH, EROD activity converts the substrate ethoxyresorufin, which is a fluorescent product. Standard phosphate buffer, NADPH, and suspension adequate for 0.2 mg ml^{-1} protein were put into a cell. Ethoxyresorufin was added, and the increase in fluorescence was monitored for 5 min (excitation/emission wavelengths were 535/585 nm). The EROD activity was subsequently calculated based on comparison with fluorescence of the standard (resorufin) of known concentration [27,28]. Total cytochrome P450 (Cyt P450) was determined by visible light spectrophotometry at 400–490 nm on the basis of the difference between absorbance readings at 450 and 490 nm, and the values were transformed to final concentrations. Measurements were made after cytochrome reduction by sodium dithionite and after the complex with carbon oxide was formed. The method is described in detail in Siroka et al. [28].

2.5. Oxidative stress biomarkers

Frozen tissue (brain, gill, muscle, liver, and intestine) samples were weighed and homogenized (1:10 w/v) (Ultra Turrax Ika, Germany) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring TBARS and CP and a second centrifuged at 12,000g for 30 min at 4 °C to obtain the post-mitochondrial supernatant for antioxidant enzymes analyses.

2.5.1. Indices of oxidative stress

A 500 μl aliquot of homogenate was mixed with 1 ml of 30% (w/v) TCA and centrifuged for 10 min at 5000g. The supernatant was used for peroxidation of lipids (LPO) assays and the pellet used for carbonyl protein (CP) assay. The thiobarbituric acid (TBARS) method described by Lushchak et al. [29] was used to evaluate branchial LPO in fish tissues. The concentration of TBARS was calculated from the absorption at 535 nm and a molar extinction coefficient of 156 mM cm^{-1}. Carbonyl derivatives of proteins were detected by reaction with tritiated sodium borohydride according to the method described by Lenz et al. [30]. The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM cm^{-1}.

2.5.2. Antioxidant enzymes assays

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund [31]. This assay depends on the autoxidation of pyrogallol. Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme per milligram of protein. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed from the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase. The specific activity was determined using the extinction coefficient 6.22 mM cm^{-1} [32]. Glutathione reductase (GR; EC 1.6.4.2) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [33].

2.5.3. Protein estimation

Protein levels were estimated spectrophotometrically by the method of Bradford [34] using bovine serum albumin as a standard.

2.6. Statistical analysis

The statistical software program STATISTICA (version 8.0 for Windows, StatSoft) was used to compare differences among the test groups. Prior to analysis, all measured variables were checked for normality (Kolmogorov–Smirnov test) and homoskedasticity of variance (Bartlett's test). If those conditions were satisfied, a one-way analysis of variance (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.

3. Results

3.1. Fish behavior

During the experiment, both control and exposed carp showed normal feeding behavior. There were no signs of respiratory distress such as rapid ventilation, increased rate of gill opercular movements, or fish floating at the water surface. There were no mortalities during the experiment.

3.2. Biometric parameters

No differences in parameters (standard length, body weight, liver weight spleen weight, condition factor and hepatosomatic index) investigated were found among any groups (Kruskal–Wallis test in all comparisons $P > 0.05$).

3.3. Hematological profile after long-term exposure to terbutryn

Results of hematological profiling are given in Table 2. Hematological profiles of common carp exposed to terbutryn at the recorded environmental concentration of 0.02 μg l^{-1} (group 1) showed no differences from untreated fish. In groups 2 and 3, fish erythrocyte count, mean corpuscular hemoglobin concentration, and neutrophil granulocyte bands increased significantly ($P < 0.01$), and leukocyte count, lymphocyte count, and mean corpuscular volume decreased significantly ($P < 0.01$) relative to controls. The values for Hb, PCV, MCH, and Bc were similar among all groups.

3.4. Biochemical blood plasma profiles after long-term exposure to terbutryn

Results of carp plasma biochemical profiling are given in Table 3. Biochemical profiles of group 1 carp showed no significant differences from controls. In the group 2 and 3 fish, significant ($P < 0.01$) decreases in the level of creatine and magnesium and a significant ($P < 0.01$) increase in glucose, aspartate aminotransferase, lactate dehydrogenase, and lactate levels in plasma were observed compared with controls. The remaining indices: TP, ALB, GLOB, NH_3, TAG, ALT, GGT, CK, AMYL, LIPA, ALP, Ca^{+2}, PHOS, VTG, and KT were similar in all groups.

3.5. Liver biomarkers

The activity of the first phase of detoxication enzymes (CYP 450, EROD) in liver exposed fish showed no significant differences compared to control.

3.6. Histology

No histopathological changes were demonstrated in liver, spleen or cranial and caudal kidney of carp following long-term exposure to terbutryn at concentrations of 0.02, 0.2, and 2 μg l^{-1}.

Table 2
Derived hematological parameters in common carp following long-term exposure to terbutryn ($n = 16$).

Fish group	Control	1	2	3
Terbutryn ($\mu g\ l^{-1}$)	–	0.02	0.2	2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RBC ($T\ l^{-1}$)	1.36 ± 0.27	1.25 ± 0.16	1.89 ± 0.11*	1.84 ± 0.10*
Hb ($g\ l^{-1}$)	56.00 ± 13.79	60.70 ± 6.50	68.97 ± 11.53	74.48 ± 20.14
PCV ($l\ l^{-1}$)	0.203 ± 0.045	0.233 ± 0.032	0.241 ± 0.041	0.280 ± 0.036
Bc ($l\ l^{-1}$)	0.037 ± 0.004	0.041 ± 0.006	0.037 ± 0.004	0.043 ± 0.009
MCV (fl)	187.38 ± 17.67	188.45 ± 23.84	126.63 ± 21.70*	132.30 ± 16.22*
MCH (pg)	41.05 ± 5.40	45.07 ± 7.78	37.81 ± 5.83	40.84 ± 5.24
MCHC ($g\ l^{-1}$)	254.82 ± 13.24	261.49 ± 12.30	298.19 ± 17.83*	294.11 ± 10.37*
Leuko ($G\ l^{-1}$)	97.00 ± 42.16	112.19 ± 57.88	31.06 ± 13.43*	28.32 ± 10.15*
Lymphocytes ($G\ l^{-1}$)	86.99 ± 6.86	104.06 ± 3.29	12.59 ± 4.16*	15.86 ± 3.26*
Monocytes ($G\ l^{-1}$)	1.76 ± 1.51	2.31 ± 1.44	1.75 ± 1.40	1.25 ± 0.89
Neutrophil granulocytes segments ($G\ l^{-1}$)	2.12 ± 2.08	0.84 ± 0.78	0.21 ± 0.17	0.31 ± 0.28
Neutrophil granulocytes bands ($G\ l^{-1}$)	6.12 ± 2.44	4.98 ± 2.62	16.50 ± 4.62*	10.97 ± 2.21*

* Experimental groups are significantly ($P < 0.01$) different from the control.

Table 3
Derived biochemical indices of blood plasma in common carp following long-term exposure to terbutryn ($n = 16$).

Fish group	Control	1	2	3
Terbutryn ($\mu g\ l^{-1}$)	–	0.02	0.2	2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
GLU ($mmol\ l^{-1}$)	4.35 ± 0.76	4.48 ± 0.43	9.62 ± 1.15*	8.54 ± 1.35*
TP ($g\ l^{-1}$)	31.05 ± 2.45	29.98 ± 3.14	31.21 ± 1.87	30.58 ± 1.47
ALB ($g\ l^{-1}$)	5.13 ± 1.62	5.38 ± 2.06	6.00 ± 2.00	5.38 ± 1.78
GLOB ($g\ l^{-1}$)	25.60 ± 2.15	24.31 ± 2.89	25.44 ± 2.15	25.01 ± 3.12
NH_3 ($\mu mol\ l^{-1}$)	286.26 ± 12.14	273.17 ± 25.33	270.22 ± 21.39	268.39 ± 36.15
TAG ($mmol\ l^{-1}$)	0.61 ± 0.15	0.65 ± 0.17	0.65 ± 0.12	0.68 ± 0.14
AST ($\mu kat\ l^{-1}$)	1.12 ± 0.26	1.36 ± 0.19	3.11 ± 0.25*	3.36 ± 0.20*
ALT ($\mu kat\ l^{-1}$)	0.45 ± 0.15	0.52 ± 0.13	0.41 ± 0.18	0.40 ± 0.16
GGT ($\mu kat\ l^{-1}$)	0.09 ± 0.03	0.05 ± 0.04	0.06 ± 0.03	0.08 ± 0.02
LDH ($\mu kat\ l^{-1}$)	13.25 ± 1.28	14.11 ± 2.35	20.65 ± 3.62*	21.08 ± 3.01*
CK ($\mu kat\ l^{-1}$)	12.74 ± 2.46	13.12 ± 1.92	12.08 ± 2.14	12.65 ± 2.01
CREA ($mmol\ l^{-1}$)	50.95 ± 5.67	48.63 ± 4.12	30.15 ± 2.54*	29.78 ± 3.14*
LACT ($mmol\ l^{-1}$)	1.28 ± 0.28	1.02 ± 0.14	2.63 ± 0.58*	2.80 ± 0.36*
AMYL ($\mu kat\ l^{-1}$)	0.104 ± 0.03	0.136 ± 0.07	0.246 ± 0.12	0.167 ± 0.14
LIPA ($\mu kat\ l^{-1}$)	0.78 ± 0.26	0.64 ± 0.31	0.70 ± 0.28	0.87 ± 0.38
ALP ($\mu kat\ l^{-1}$)	0.21 ± 0.09	0.29 ± 0.13	0.31 ± 0.18	0.25 ± 0.13
Ca^{2+} ($mmol\ l^{-1}$)	2.11 ± 0.45	2.36 ± 0.63	2.14 ± 0.51	2.28 ± 0.66
Mg ($mmol\ l^{-1}$)	1.12 ± 0.11	1.18 ± 0.12	0.57 ± 0.10*	0.48 ± 0.13*
PHOS ($mmol\ l^{-1}$)	1.12 ± 0.21	1.32 ± 0.32	1.22 ± 0.28	1.18 ± 0.46
VTG ($\mu g\ ml^{-1}$)	14.50 ± 6.40	16.23 ± 10.12	25.60 ± 16.23	26.11 ± 16.10
KT ($pg\ ml^{-1}$)	31.01 ± 14.80	24.13 ± 20.60	43.41 ± 18.40	50.10 ± 36.28

* Experimental groups are significantly ($P < 0.01$) different from the control.

3.7. Oxidative stress biomarkers

3.7.1. Oxidative stress indices

Levels of LPO (measured by tissue TBARS level) in brain, gill, muscle, liver, and intestine of all groups are summarized in Fig. 1. The levels of LPO in brain and liver of all experimental groups were significantly increased ($P < 0.01$) compared to the control group. The levels of LPO in gill, muscle, and intestine of experimental group were not significantly increased ($P > 0.05$) compared to the control group.

The levels of CP in liver, muscle, and intestine of experimental groups were not significantly increased ($P > 0.05$) compared to the control group. There was a significant increase ($P < 0.01$) in gill and brain in groups 2 and 3 compared to the control group. Levels of CP in brain, gill, muscle, liver and intestine of all groups are summarized in Fig. 2.

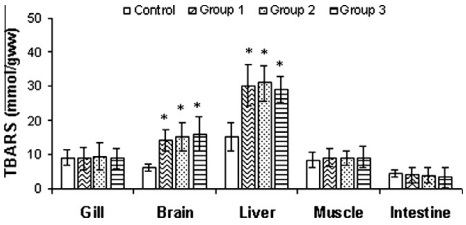

Fig. 1. Effects of long-term terbutryn exposure on level of thiobarbituric acid reactive substances (TBARS) in common carp tissues. All values are means ± SD, $n = 16$. *Significance levels observed are ($P < 0.01$) in comparison to the control group.

3.7.2. Antioxidant enzymes

The activity of SOD in brain, gill, muscle, liver, and intestine of all groups is summarized in Fig. 3. The activity of SOD in gill, muscle, and intestine of experimental groups was not significantly higher ($P > 0.05$) than that seen in the control group. But a significant increase ($P < 0.01$) was observed in liver in all experimental groups, and in brain in groups 2 and 3, compared to the control group.

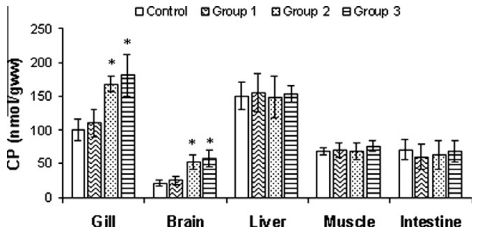

Fig. 2. Effects of long-term terbutryn exposure on level of carbonyl proteins (CP) in common carp tissues. All values are means ± SD, $n = 16$. *Significance levels observed are ($P < 0.01$) in comparison to the control group.

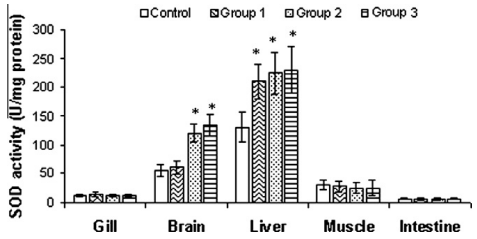

Fig. 3. Effects of long-term terbutryn exposure on superoxide dismutase (SOD) activity in common carp tissues. All values are means ± SD, $n = 16$. *Significance levels observed are ($P < 0.01$) in comparison to the control group.

The activity of GR in liver was significantly lower ($P > 0.01$) in all experimental groups compared to the control group. The activity of GR in intestine was significantly lower in groups 2 and 3 and significantly greater ($P < 0.01$) in muscle in group 3 compared to the controls. No differences were found in activity of GR in gill between the experimental groups and control group. Activity of GR in brain, gill, muscle, liver, and intestine of all groups is summarized in Fig. 4.

The activity of GPx in brain, gill, muscle, liver, and intestine of all groups is summarized in Fig. 5. The activity of GPx in gill was significantly decreased ($P > 0.01$) in group 3 compared to the control, 1 and 2 groups. No differences were found in activity of GPx in brain, liver, muscle, and intestine between the experimental groups and control group.

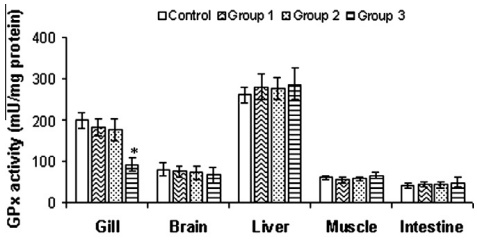

Fig. 4. Effects of long-term terbutryn exposure on glutathione peroxidase (GPx) activity in common carp tissues. All values are means ± SD, $n = 16$. *Significance levels observed are ($P < 0.01$) in comparison to the control group.

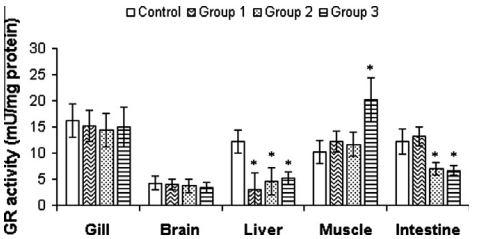

Fig. 5. Effects of long-term terbutryn exposure on glutathione reductase (GR) activity in common carp tissues. All values are means ± SD, $n = 16$. *Significance levels observed are ($P < 0.01$) in comparison to the control group.

4. Discussion

Laboratory studies of biochemical, physiological and morfological responses in tissues of fish exposed to herbicides can provide information on, and help to elucidate the mechanisms of, the impact of residual herbicides on fish. The results of this study provide further data on long-term exposure to terbutryn for consideration in risk assessment. The findings contribute to knowledge of the toxic potential of terbutryn to common carp at actual concentrations in Czech rivers.

In order to make an accurate assessment of the hazards that a contaminant may pose in a natural system, behavioral indices selected for monitoring must reflect the organism's behavior in the field [35]. These findings are in accord with results of Velisek et al. [2,36] who found no change in carp behavior following subchronic exposure to terbutryn and simazine. On the other hand, Velisek et al. [37,38] reported accelerated respiration and loss of movement coordination in rainbow trout and carp following acute poisoning with metribuzin. These characteristics have also been reported in *Oreochromis niloticus,* and *Chrysichthyes auratus* [39] and by Saglio and Trijasse [40] in *Carassius auratus* following acute poisoning with atrazine. Movement imbalance in freshwater fish (*Labeo rohita, Mystus vittatus*, and *Cirrhinus mrigala*) acutely exposed to simazine and cyanazine has been reported by Dad and Tripathi [41]. Oropesa et al. [42] reported respiratory distress such as rapid ventilation, increased rate of gill cover movements, or floating at the surface of water in common carp after exposure to simazine. The exposure of zebrafish, *Brachydanio rerio*, to atrazine caused changes in behavior, such as significant preference for habitat with a dark substratum, even at concentrations lower than 6 $\mu g\,l^{-1}$ [43]. Our results differ from these, as, during the assay, both control and exposed carp behaved normally. However, different exposure regimes as well as different fish species were used in our study.

Biometric parameters are regarded as a general indicator of fish health and the quality of the aquatic environment. The HSI is a non-specific biomarker, influenced by factors such as sex, season, disease, and nutritional level [44]. The findings in the present study are in accord with results of Velisek et al. [2,36] who also found no change in carp HSI, CF, and LW following subchronic exposure to triazines. Dewey [45] reported reduction in body weight and length and decrease of condition in brook trout (*Salvelinus fontinalis*) following 306 days exposure to low atrazine concentrations (120 $\mu g\,l^{-1}$). Davies et al. [46] observed growth rate reduction in *Galaxias maculatus* after exposure to low concentrations of atrazine. Atrazine, at doses of 100 and 1000 $\mu g\,l^{-1}$, showed no dose- or time-related effects on gonad growth (GSI) in either males or females over a 21-days study period [47]. Our results differ in that long-term exposure of carp to terbutryn did not affect

biometric parameters. However, a different exposure period, regime, and different fish species were used in our study.

The evaluation of hematological and biochemical characteristics in fish has become an important means of understanding normal and pathological processes and toxicological impacts. In our study, the highest RBC value was found in fish exposed to higher concentrations (groups 2 and 3), when stress-induced RBC release from spleen to blood circulation was reported. Hematological changes may result from the release of immature erythrocytes from the spleen, and could be an immediate response to acute stress mediated by catecholamines [48]. Acute stress can cause significant changes in the white blood cell count. The response to environmental challenges often leads to leucopenia with lymphopenia and sometimes neutrophilia, which is similar to the classic leukocytic response to stress in mammals. In the present study, the decrease in leukocyte count and the lymphopenia in carp exposed to terbutryn indicate a reduction in non-specific immunity. Prolonged stress may have caused disruption of leukopoiesis, resulting in reduction in the total leukocyte count. Velisek et al. [37] also reported lower values of RBC and PVC after metribuzin exposure. Significant decreases in PCV have been reported by Horn and Hanke [49] in common carp exposed to atrazine. Changes in the hematocrit value, lymphocytes, erythrocytes, and neutrophil granulocytes were reported by Svobodova and Pecena [50] in carp following acute poisoning with atrazine. Oropesa et al. [42] reported no effect on the hematological profiles of common carp exposed to 45 $\mu g\,l^{-1}$ simazine. Our results differ from these, as, different tested substances, different concentrations of tested substances, and exposure period were used in our study.

The increase in blood glucose concentration demonstrated the response of exposed fish to metabolic stress. LDH is the terminal enzyme of anaerobic glycolysis, and therefore of crucial importance to muscle physiology, particularly in conditions of chemical stress when high levels of energy may be required for a short period of time [51]. The increase in LDH level indicated metabolic changes, i.e. glycogen catabolism and a glucose shift towards the formation of lactate, primarily in muscle. The activity of plasma enzymes LDH and the transaminase AST are also relevant stress indicators. A significant increase in the activity of these enzymes indicates stress-based tissue impairment. Increased activity of transaminase indicates amplified transamination processes. An increase in transamination occurs with amino acid input into the TCA cycle to cope with the energy crisis during pesticide stress [52]. It has been suggested that, in general, stress induces elevation of the transamination pathway, and it is likely to have contributed to toxic effects induced by terbutryn and increased transaminase activity observed in the present study. Velisek et al. [36] found a decrease in the activity of AST and an increase in GLU, NH_3, LDH, CK, and CREA levels in common carp after subchronic exposure to simazine. Mekkawy et al. [53] observed increases in GLU and decreased TP levels in *Oreochromis niloticus* and *Chrysichthyes auratus* after atrazine exposure at 3 $mg\,l^{-1}$. Increases in glucose and cortisol and decreased serum protein and cholesterol were found by Gluth and Hanke [54] in carp with subchronic exposure to atrazine (100 $\mu g\,l^{-1}$). Davies et al. [46] also observed a decrease in TP in rainbow trout after acute exposure to atrazine at a concentration of 50 $\mu g\,l^{-1}$, whereas Oropesa et al. [42] reported no effects of simazine on levels of TP and activity of LDH in common carp. Neskovic et al. [55] found an increase in ALT activity in rainbow trout after 14 days exposure to atrazine at concentrations of 1.5–6.0 $mg\,l^{-1}$, and Waring and Moore [56] found an increase in cortisol levels in Atlantic salmon (*Salmo salar*) after exposure to atrazine. Prasad and Reddy [57] observed a decrease in serum calcium in Mozambique tilapia, *Tilapia mossambica*, exposed to atrazine. The values of biochemical profiles determined in the present study suggest that liver and muscle of common carp were altered with exposure to concentrations of 0.2 and 2 $\mu g\,l^{-1}$ terbutryn.

The present study attempted to evaluate xenoestrogenic potency of terbutryn using vitellogenin and 11-ketotestosterone as a biomarker of exposure to (xeno)estrogens. The monitoring of vitellogenin and 11-ketotestosterone are proving to be useful tools for study of the effects of endocrine disrupting chemicals in fish [58]. Synthesis of VTG, a lipophosphoprotein, is induced by estradiol in the liver of female fish. In the presence of substances with estrogenic effects, synthesis of VTG is carried out in the liver of male fish, which may lead to degenerative alterations of male gonads, reproductive breakdown, and, in extreme cases, sex reversal [59]. In our study, long-term terbutryn exposure showed no effect on vitellogenin and 11-ketotestosterone. Moore and Waring [60] observed that atrazine concentration of 3.6 $\mu g\,l^{-1}$ altered plasma testosterone and, at 6.0 $\mu g\,l^{-1}$, affected 11-ketotestosterone in Atlantic salmon. No changes were observed in VTG in carp hepatocytes following atrazine exposure [61]. No change in VTG in goldfish exposed to high doses (100 and 1000 $\mu g\,l^{-1}$) of atrazine were found by Spano et al. [47]. Nadzialek et al. [62] observed a significant decrease of plasma 11-KT in goldfish with atrazine exposure at a concentration of 1000 $\mu g\,l^{-1}$. Tennant et al. [63], working with rats, concluded that, while the chloro-s-triazine herbicides atrazine and simazine did not possess any intrinsic estrogenic activity, these two compounds were capable of weak inhibition of estrogen-stimulated responses in the rat uterus (i.e. effect on progesterone receptor binding and thymidine incorporation into uterine DNA). Crain et al. [64] showed that chloro-s-triazine herbicides have the ability to stimulate production of the enzyme aromatase, which converts androgens to estrogens, and presumably could interfere with sexual differentiation and development.

Cytochromes P450, members of a large family of heme proteins, are membrane-bound proteins which are predominantly located in the endoplasmic reticulum of the liver. The enzyme system of CYP450 is composed of a large number of isoforms. The CYP450 reactions can be grouped according to the type of substrate and separated into the synthesis and degradation of endogenous substrates and the metabolism of xenobiotic substrates [65]. The presence of the CYP450 1A isoform is expressed as EROD activity. EROD induction is commonly observed in fish and other vertebrates exposed to Ah-receptor (AhR) agonists [i.e. dioxins, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pesticides] (reviewed in van der Oost et al. [44]). The EROD activity, however, appeared to be the most sensitive catalytic probe for determining the inductive response of the CYP450 system in fish [65]. It has been stated that terbutryn is able to induce EROD activity in rainbow trout [66]. Our results suggest that a role for CYP450 1A in the metabolism of terbutryn in common carp can be ruled out, since no changes in total CYP450 concentrations and EROD activity were demonstrated. However, in rodents, pigs and mouse the dominant phase I metabolic reaction for triazine pesticides is cytochrome P450-mediated N-dealkylation, as demonstrated by urine analysis and liver fraction studies [67,68].

Neskovic et al. [55] found hyperplasia of gill epithelial cells in common carp (*Cyprinus carpio*) exposed to atrazine at a concentration of 1500 $\mu g\,l^{-1}$. Hyperplasia of gill epithelial cells is also reported by Oropesa et al. [69] in carp following acute poisoning with simazine. Velisek et al. [2] found cell shape changes and lipid inclusions in hepatocytes and destruction of caudal kidney tubules in common carp with sub-chronic terbutryn exposure in concetration 4, 20, and 40 $\mu g\,l^{-1}$. Similar alterations in liver were observed by Arufe et al. [70], who exposed the larvae of gilthead seabream (*Sparus aurata*) to terbutryn-triasulfuron at a concentration of 2.5 $mg\,l^{-1}$ for 72 h. On the other hand, Biagianti-Risbourg and Bastide [71] reported that atrazine affects other tissues in fish, particularly liver, which shows a substantial increase in the size

of lipid inclusions, followed by lipoid degeneration, enlargement of the secondary lysosomes, mitochondrial malformation and vacuolization, and a reduction in glycogen content. Triazine pesticides have a direct effect on kidney structure and function in freshwater fish [37,38].

Oxidative stress results from an increase in reactive oxygen species or an impairment of antioxidant defense systems. Antioxidants may be induced or inhibited by exposure to environmental pollutants, and inhibition of antioxidants impairs the capacity to prevent ROS formation and cell damage [72]. Chronic exposure to triazines may affect endocrine and metabolic functions as well as the activity of enzymes involved in oxidative stress [73]. Exposure to pesticides is known to induce lipid peroxidation in various tissues, which is responsible for adverse biological effects. The mechanism of pesticide toxicity is presumed to be associated with the increase of lipid peroxidation in liver [74]. Reactive oxygen species such as superoxide anions, hydroxyl radicals, and hydrogen peroxide enhance the oxidative process and induce peroxidative damage to membrane lipids. Induction of LPO in brain and liver with long-term terbutryn exposure may be one of the molecular mechanisms involved in terbutryn-induced toxicity. Protein carbonylation is a result of protein oxidation. Reactive oxygen species directly attack protein and lead to the formation of carbonyl. The formation of CP is non-reversible, causing conformational changes and decreased catalytic activity in enzymes and ultimately resulting in, owing to increased susceptibility to protease action, breakdown of proteins by proteases [75]. We found that CP levels in the higher terbutryn concentration treatment groups were significantly higher than in controls, indicating that long-term terbutryn-exposure induced cellular protein metabolism disruption.

Cells have a wide array of enzymatic and non-enzymatic antioxidant defense systems. Defense systems that tend to inhibit oxyradical formation include the antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [76]. These enzymes play an important role in countering the oxidative stress induced by the formation of ROS. Superoxide dismutase is an enzyme that catalyzes the dismutation of superoxide to hydrogen peroxide, which is detoxified by CAT and GPx [77]. Induction of SOD in brain and liver after long-term terbutryn exposure may be an adaptive response to the stress which neutralizes the impact of ROS, demonstrating that SOD is the first enzyme to break down oxyradicals. It has been reported that exposure of animals to pesticides increases SOD activity in various tissues [74]. Glutathione reductase plays an important role in cellular antioxidant protection and adjustment processes of metabolic pathways [78]. Although perhaps not involved in antioxidant defense in the same way as the enzymes previously described, GR merits attention because of its importance in maintaining GSH/GSSG homeostasis under oxidative stress [79]. Glutathione reductase catalyses the reduction of glutathione disulfide to reduced glutathione in an NADPH-dependent reaction [80]. Glutathione reductase activity in the present study was inhibited in liver and intestine but increased in muscle, probably due to a difference in the availability of NADPH. Glutathione peroxidase is considered to play an especially important role in protecting membranes from damage due to LPOX. In this study, decrease in activity of GPx in gill at the highest concentration of terbutyn may indicate that its antioxidant capacity was exceeded by the level of hydroperoxide products and reflects a possible failure of the antioxidant system of fish. However, Elia et al. [81] have reported significant increase in the activity of hepatic GPx but no change in the activity of GPx in gills of fish following atrazine exposure. The long-term exposure of carp to terbutryn increased ROS formation, resulting in oxidative damage to lipids and proteins and inhibition of antioxidant capacities. In short, a low level of oxidative stress may induce the adaptive responses of antioxidant enzymes.

5. Conclusions

The results of our study suggest that the long-term exposure of common carp to terbutryn can affect the biochemical and hematological profiles and oxidative stress biomarkers. Some changes were observed only with the higher exposures (0.2, and 2 $\mu g\, l^{-1}$), but in fish exposed to the lowest concentration tested (i.e. 0.02 $\mu g\, l^{-1}$), similar to that reported in Czech rivers, only changes in oxidative stress biomarkers were observed. It has been reported that, in most cases, concentrations of terbutryn in Czech and European rivers is maximum 5.60 or less micrograms per liter. However, the aquatic environment may be polluted by many different substances, the effects of which can be potentiated with concurrent exposures. For detailed elucidation of terbutryn's effects, further research is necessary. This research should be focused not only on the effects of terbutryn alone, but also on interactions of terbutryn with other pollutants.

Acknowledgments

This research was supported by the Czech Science Foundation Project No. 525/09/P218, the centre CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No.047/2010/Z.

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CHAPTER 6

EFFECT OF CHRONIC EXPOSURE TO PROMETRYNE ON OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN COMMON CARP (*CYPRINUS CARPIO* L.)

Stara, A., Kristan, J., Zuskova, E., Velisek, J., 2013. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.). Pesticide Biochemistry and Physiology 105, 18–23.

It was allowed by publisher on 9th January, 2014 to include the paper in this Ph.D. thesis.

Pesticide Biochemistry and Physiology 105 (2013) 18–23

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.)

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ARTICLE INFO

Article history:
Received 20 June 2012
Accepted 7 November 2012
Available online 29 November 2012

Keywords:
Triazine
Fish
Oxidative stress
Antioxidant enzyme
Tissue

ABSTRACT

The effects of the triazine herbicide prometryne, commonly present in surface and ground waters, on oxidative stress and antioxidant status of common carp (*Cyprinus carpio*) were investigated. Fish were exposed to sublethal concentrations of prometryne (0.51, 8, and 80 μg/l) for 14, 30, and 60 days. Activity of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), as well as levels of thiobarbituric acid reactive substances (TBARSs) were assessed in brain, gill, intestine, liver, and muscle. After 14 days exposure, decreased GR activity in brain was observed for all prometryne-exposed groups compared with the controls. Changes were observed in SOD activity in brain and gill after 30 days in all exposure groups. Changes in CAT activity were observed only at the highest concentration (80 μg/l) in liver and intestine after 60 days. The observed effects on carp antioxidant systems may be a defense against oxidative damage. The study demonstrated changes in antioxidant parameters and the importance of evaluating the potential long-term risk to fish of prometryne, at environmentally realistic concentrations (0.51 μg/l). The results suggest that antioxidant responses may have potential as biomarkers for monitoring residual triazine herbicides in aquatic environments.

1. Introduction

Worldwide pesticide use has increased dramatically over the past two decades [1]. Pesticides have become some of the most frequently occurring organic pollutants of agricultural soils [2] and ground and surface waters [3], causing ecological imbalances [4] that may have toxicological effects on natural ecosystems, especially aquatic systems [5]. They cause damage to non-target organisms, including fish. In aquatic toxicology studies, fish are important indicators of the impact of toxic substances [4]. The potential effects of pesticide use on human health, ecosystems, and the environment is of great interest [2,6].

Herbicides have the potential to introduce reactive oxygen species into biological systems, leading to oxidative stress in non-target organisms [7]. Oxidative stress can also occur as damage to biological systems or by impairing antioxidant defense systems [8]. One of the commonly used markers of oxidative stress is lipid peroxidation indicated by the formation of thiobarbituric acid reactive substances (TBARSs) [9]. An antioxidant defense pathway in animals comprises antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) [10], and glutathione reductase (GR) [11]. Changes in the activity of these enzymes can indicate disorders indicative of redox alterations related to oxidative stress [12]. TBARS and antioxidant levels (SOD, CAT, GR) in fish tissue have been frequently used to assess environmental contamination [8,10,12–14].

Prometryne [2,4-bis(isopropylamino)-6-methylthio-s-triazine], a selective herbicide of the s-triazine chemical family [15], is used for the control of annual broadleaf and grass weeds in terrestrial crops; major uses are for cotton and celery [16]. Prometryne is applied before weeds emerge or to emerged weeds. It affects plant photosynthesis by inhibiting electron transport [17]. The major metabolic process of prometryne in animal is N-dealkylation along with hydrolysis and/or amino acid conjugation [6]. Prometryne application is not permitted in Europe, but is widely used in China [18], Australia, Canada, New Zealand, South Africa, and the United States [19].

Significant traces of prometryne are documented in the environment, mainly in water, soil, and plants used for human and domestic animal consumption [20]. The maximal environmental concentration of prometryne in Czech rivers is 0.51 μg/l [21]. In surface waters of Greece, prometryne has been recorded at concentrations from 0.190 to 4.40 μg/l [22] and reported in ground water at concentrations exceeding 1 μg/l [23]. Prometryne is slightly to moderately toxic to fish and invertebrates. Acute toxicity 96-h LC50 for rainbow trout (*Oncorhynchus mykiss*) is 2.9 mg/l [16]; for bluegill sunfish (*Lepomis macrochirus*), 7.9 mg/l; for sheepshead minnow (*Cyprinodon variegatus*), 5.1 mg/l [19]; and for common carp (*Cyprinus carpio*), 8 mg/l [24]. Chronic prometryne toxicity in

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http://dx.doi.org/10.1016/j.pestbp.2012.11.002

freshwater fish has not been investigated to a significant extent. Adverse effects on growth of fish have been reported at test concentrations of 1–2 mg/l for fathead minnows (*Pimephales promelas*) exposed to prometryne for 32 days [17]. Oxidative stress and antioxidant levels may be good indicators of prometryne contamination in fish tissue. Although the effects of acute and sub-chronic exposure of fish to triazine herbicides have been well documented, there is a dearth of data on chronic exposure to these compounds at environmentally realistic concentrations with respect to oxidative stress and antioxidant responses in the fish species typical of Central Europe. It is necessary to assess the environmental risks of prometryne, including its mobility and persistence in the aquatic environment and effects on non-target organisms, mainly freshwater fish [18]. The aim of the present study was to investigate effects of long-term exposure to low concentrations of prometryne on oxidative stress and the antioxidant defense in organs of common carp.

2. Materials and methods

2.1. Fish

One-year-old common carp (mean body length 24.18 ± 1.62 cm, mean weight 343.64 ± 80.25 g) were obtained from a local commercial hatchery (Vodnany, Czech Republic). They were held in aquaria containing 200 l of freshwater with continuous aeration, at 20.1 ± 1.4 °C and a 12L:12D cycle. Dissolved oxygen concentration was 90–98%, and pH was 7.5 ± 0.3. Fish were fed commercial food (Tetra Pond Sticks) and were acclimatized for 10 days before the beginning of the experiment.

2.2. Experimental design

The experiment was a semi-static assay conducted over 60 days. Fish were allocated, in groups of 10, to one of three experimental regimes or to an untreated control group, each held in an aquarium containing 200 l of water. The physicochemical parameters of the water in all aquaria were: acid neutralization capacity, $ANC_{4.5}$ 0.75 mM/l total ammonia, 0.010 mg/l; NO_3^-, 5.13 mg/l NO_2^-, 0.011 mg/l; PO_4^{3-}, 0.01 mg/l and chemical oxygen demand COD_{Mn}, 0.8 mg/l. Water temperature was 18.6–21.1 °C and oxygen saturation 90–98%.

The experimental fish were exposed to prometryne (Sigma Aldrich, Czech Republic, chemical purity 99.3%) at the following concentrations: 0.51 μg/l (the reported environmental concentration in Czech rivers, Group 1), 8 μg/l (Group 2), and 80 μg/l (Group 3). Concentrations of 8 μg/l and 80 μg/l corresponded to 0.1% of the 96-h LC50 and 1% of 96-h LC50 for carp. The experimental conditions were duplicated for a total of eight groups ($n = 96$).

Fish were fed commercial pellets at about 1% body weight per day in two feedings. Eighty percent of the exposed solution was renewed each day 2 h after feeding to maintain water quality and the appropriate concentration of prometryne. During the experiment, fish behavior and the number of dead fish were recorded.

To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by LC–MS/MS [25]. Water samples were collected from the aquaria 1 h and 24 h after renewing the test solutions. The mean concentration of prometryne in the water samples was always within 5% of the intended concentration.

2.3. Tissue samples and preparation of post-mitochondrial supernatant

At the completion of each exposure period, four fish from each aquarium were randomly selected and killed. The brain, gill, muscle, liver, and intestine were quickly removed, immediately frozen, and stored at −80 °C for analysis. Frozen tissue samples were weighed and homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS), and a second centrifuged at 12,000*g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for other antioxidant parameter analyses.

2.3.1. Indices of oxidative stress

The TBARS method described by Lushchak et al. [26] was used to evaluate lipid peroxidation.

2.3.2. Antioxidant parameters

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund [27]. This assay depends on the autoxidation of pyrogallol. Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme per milligram of protein. The catalase (CAT; EC 1.11.1.6) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer [28]. Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [29]. One unit of CAT or GR activity is defined as the amount of the enzyme that consumes 1 mol/l of substrate or generates 1 mol/l of product per min. Activity was expressed in international units (or milliunits) per mg of protein.

2.3.3. Protein estimation

Protein levels were estimated spectrophotometrically by the method of Bradford [30] using bovine serum albumin as a standard.

2.4. Statistical analysis

The statistical software program STATISTICA (version 8.0 for Windows, StatSoft) 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 these conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. When a significant difference was detected ($p < 0.05$), Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a non-parametric test (Kruskal–Wallis) was used [31].

The study was conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology Vodnany, based on the EU-harmonized animal welfare act of Czech Republic. The principles of laboratory animal care and the national laws 246/1992, and regulations on animal welfare were followed (Ref. No. 22761/2009-17210).

3. Results

3.1. Fish behavior during the experiment

During the experiment, both control and exposed common carp showed normal feeding behavior. There were no signs of respiratory distress, such as rapid ventilation, increased rate of gill opercular movements, or floating at the surface of water. There were no mortalities during the experiment.

3.2. Oxidative stress

Effects of chronic exposure to prometryne on TBARS in tissue of common carp are presented in Table 1. In the test groups none showed significant differences from the control group in brain, gill, muscle, liver, or intestine.

3.3. Antioxidant response

Significant differences from the control value ($p < 0.01$) were seen in Groups 1, 2, and 3 in brain and gill. In the brains of fish from Groups 2 and 3 higher SOD activity was observed after 14 days exposure, but at 30 days the level was significantly reduced in all test groups relative to controls. In gill, SOD activity was decreased in Group 1 after 30 days, in Group 2 after 14 and 30 days, and in Group 3 SOD activity was reduced for the duration of the experiment compared to controls. In intestine, SOD activity showed a significant decrease ($p < 0.05$) after 14 days in Group 3 when compared with control group (Table 2).

Significant differences from controls ($p < 0.05$) in tissue CAT activity were found for Group 3 in liver and intestine after 60 days exposure to prometryne. CAT activity was reduced in liver and was increased in intestine compared with control (Table 3).

GR activity was significant lower ($p < 0.05$) in brain in all groups tested prometryne with compared the control group after 14 days. In muscle GR activity was higher in Group 3 after 14 days compared to the control group (Table 4).

4. Discussion

Awareness of residual pesticides, especially triazines, in the aquatic environment is growing as investigations of these pollutants increase and detection techniques improve. Fish exposed to environmental pollutants exhibit a variety of physiological responses, including oxidative metabolism imbalances [32].

In the present study SOD, CAT, and GR activity differed from that of the control group at all experimental exposure levels. Results indicated the presence of antioxidants that serve as a defense against oxidative stress along with elevated TBARS levels, and cell damage. The evaluation of antioxidant biomarkers is critical to the investigation of oxidative stress in organisms [33]. Antioxidant systems regulate reactive oxygen species in the cells to maintain a

Table 1
Effect of chronic exposure to prometryne on level of thiobarbituric acid reactive substances (TBARS, nmol/mg protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.51 μg/l)	2 (8 μg/l)	3 (80 μg/l)
Brain	14	0.52 ± 0.10	0.47 ± 0.06	0.48 ± 0.05	0.48 ± 0.06
	30	0.55 ± 0.06	0.53 ± 0.06	0.54 ± 0.06	0.51 ± 0.03
	60	0.45 ± 0.09	0.54 ± 0.06	0.49 ± 0.03	0.54 ± 0.06
Gill	14	0.39 ± 0.08	0.43 ± 0.07	0.40 ± 0.03	0.42 ± 0.08
	30	0.32 ± 0.08	0.35 ± 0.08	0.32 ± 0.06	0.31 ± 0.08
	60	0.38 ± 0.09	0.39 ± 0.04	0.38 ± 0.08	0.32 ± 0.05
Muscle	14	0.91 ± 0.19	0.74 ± 0.27	0.76 ± 0.04	0.67 ± 0.17
	30	0.91 ± 0.09	0.84 ± 0.11	0.73 ± 0.07	0.90 ± 0.10
	60	0.80 ± 0.09	0.92 ± 0.13	0.81 ± 0.15	1.01 ± 0.28
Liver	14	0.56 ± 0.16	0.49 ± 0.07	0.38 ± 0.06	0.49 ± 0.07
	30	0.57 ± 0.12	0.55 ± 0.10	0.49 ± 0.07	0.57 ± 0.14
	60	0.70 ± 0.12	0.63 ± 0.13	0.62 ± 0.11	0.59 ± 0.09
Intestine	14	0.51 ± 0.08	0.47 ± 0.10	0.45 ± 0.07	0.51 ± 0.04
	30	0.37 ± 0.09	0.39 ± 0.03	0.37 ± 0.05	0.39 ± 0.06
	60	0.47 ± 0.12	0.40 ± 0.08	0.48 ± 0.15	0.55 ± 0.09

Data are means ± SD, $n = 8$. Significant differences compared with control value, *<0.05.

Table 2
Effect of chronic exposure to prometryne on superoxide dismutase (SOD, nmol NBT/min/mg protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.51 μg/l)	2 (8 μg/l)	3 (80 μg/l)
Brain	14	0.09 ± 0.06	0.06 ± 0.02	0.29 ± 0.11**	0.31 ± 0.09**
	30	0.13 ± 0.08	0.01 ± 0.01**	0.01 ± 0.03**	0.02 ± 0.03**
	60	0.11 ± 0.09	0.22 ± 0.12	0.20 ± 0.08	0.15 ± 0.05
Gill	14	0.43 ± 0.10	0.32 ± 0.07	0.02 ± 0.01**	0.14 ± 0.16**
	30	0.44 ± 0.18	0.15 ± 0.03**	0.17 ± 0.05**	0.14 ± 0.05**
	60	0.19 ± 0.04	0.16 ± 0.02	0.14 ± 0.06	0.05 ± 0.05**
Muscle	14	0.13 ± 0.06	0.14 ± 0.10	0.09 ± 0.02	0.09 ± 0.03
	30	0.09 ± 0.04	0.07 ± 0.05	0.04 ± 0.02	0.05 ± 0.02
	60	0.04 ± 0.04	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Liver	14	0.31 ± 0.11	0.26 ± 0.12	0.16 ± 0.11	0.22 ± 0.05
	30	0.20 ± 0.02	0.22 ± 0.10	0.14 ± 0.04	0.13 ± 0.03
	60	0.10 ± 0.03	0.10 ± 0.05	0.15 ± 0.05	0.17 ± 0.05
Intestine	14	0.28 ± 0.07	0.23 ± 0.07	0.23 ±.0.06	0.16 ± 0.03*
	30	0.24 ± 0.06	0.23 ± 0.08	0.17 ± 0.05	0.19 ± 0.09
	60	0.17 ± 0.05	0.15 ± 0.05	0.17 ± 0.06	0.22 ± 0.02

Data are means ± SD, $n = 8$. Significant differences compared with control value.
* <0.05
** <0.01.

Table 3
Effect of chronic exposure to prometryne on catalase (CAT, μmol H_2O_2/min/mg protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.51 μg/l)	2 (8 μg/l)	3 (80 μg/l)
Brain	14	0.07 ± 0.02	0.09 ± 0.03	0.09 ± 0.03	0.10 ± 0.01
	30	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.04	0.12 ± 0.02
	60	0.10 ± 0.03	0.07 ± 0.03	0.11 ± 0.04	0.11 ± 0.04
Gill	14	0.12 ± 0.04	0.13 ± 0.06	0.11 ± 0.03	0.10 ± 0.02
	30	0.12 ± 0.03	0.11 ± 0.02	0.12 ± 0.04	0.10 ± 0.03
	60	0.11 ± 0.06	0.09 ± 0.03	0.17 ± 0.06	0.11 ± 0.04
Muscle	14	0.14 ± 0.05	0.13 ± 0.09	0.11 ± 0.04	0.12 ± 0.05
	30	0.16 ± 0.06	0.21 ± 0.06	0.14 ± 0.04	0.14 ± 0.08
	60	0.11 ± 0.02	0.12 ± 0.04	0.13 ± 0.06	0.15 ± 0.03
Liver	14	2.34 ± 0.73	1.95 ± 0.25	1.62 ± 0.28	1.63 ± 1.47
	30	1.76 ± 0.49	1.43 ± 0.46	1.78 ± 0.35	2.09 ± 0.68
	60	2.25 ± 0.51	3.20 ± 0.73	2.86 ± 0.49	1.90 ± 0.42*
Intestine	14	0.27 ± 0.05	0.29 ± 0.25	0.36 ± 0.05	0.40 ± 0.11
	30	0.47 ± 0.14	0.33 ± 0.06	0.46 ± 0.07	0.44 ± 0.05
	60	0.54 ± 0.23	0.42 ± 0.20	0.49 ± 0.14	0.74 ± 0.15*

Data are means ± SD, $n = 8$. Significant differences compared with control value.
* <0.05.

Table 4
Effect of chronic exposure to prometryne on glutathione reductase (GR, nmol NADPH/min/mg protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.51 μg/l)	2 (8 μg/l)	3 (80 μg/l)
Brain	14	0.35 ± 0.14	0.18 ± 0.05*	0.22 ± 0.08*	0.19 ± 0.05*
	30	0.18 ± 0.06	0.25 ± 0.06	0.25 ± 0.15	0.19 ± 0.04
	60	0.21 ± 0.16	0.17 ± 0.06	0.20 ± 0.09	0.30 ± 0.17
Gill	14	0.31 ± 0.15	0.47 ± 0.24	0.26 ± 0.15	0.32 ± 0.08
	30	0.28 ± 0.15	0.36 ± 0.17	0.25 ± 0.11	0.22 ± 0.08
	60	0.24 ± 0.16	0.18 ± 0.06	0.26 ± 0.19	0.26 ± 0.11
Muscle	14	0.01 ± 0.01	0.07 ± 0.06	0.07 ± 0.12	0.15 ± 0.08*
	30	0.24 ± 0.06	0.23 ± 0.05	0.19 ± 0.08	0.28 ± 0.15
	60	0.24 ± 0.18	0.10 ± 0.07	0.14 ± 0.12	0.07 ± 0.08
Liver	14	0.18 ± 0.08	0.16 ± 0.08	0.16 ± 0.04	0.08 ± 0.05
	30	0.11 ± 0.06	0.09 ± 0.04	0.14 ± 0.02	0.14 ± 0.04
	60	0.06 ± 0.01	0.09 ± 0.03	0.10 ± 0.02	0.52 ± 0.67
Intestine	14	0.30 ± 0.15	0.17 ± 0.08	0.16 ± 0.06	0.16 ± 0.05
	30	0.41 ± 0.23	0.36 ± 0.15	0.66 ± 0.34	0.54 ± 0.52
	60	0.38 ± 0.21	0.43 ± 0.24	0.28 ± 0.24	0.34 ± 0.07

Data are means ± SD, $n = 8$. Significant differences compared with control value.
* <0.05.

steady-state balance of ROS production and elimination. Disturbance of this dynamic equilibrium can lead to enhanced lipid peroxidation processes and damage to cell constituents linked to changes in the antioxidant systems [33,34]. In this study, we observed no statistically significant differences in TBARS levels in experimental fish from that of the control group. Lipid peroxidation as determined by TBARS levels was found in tissues of common carp by Cattaneo et al. [35] after long-term exposure to the herbicide penoxsulam (23 μg/l). They observed increased levels of TBARS in brain, liver, and muscle after 7 days exposure, and decreased TBARS levels in brain after 21 days, followed by increased TBARS levels in brain and liver compared to control fish after 72 days. Toni et al. found increased levels of TBARS in the brain, liver, and muscle of common carp exposed to the commercial herbicide bispyribac-sodium at a concentration of 20.87 μg/l for 72 days [11]. Ninety days exposure to the herbicide clamazone at a concentration 0.5 mg/l was reported to be associated with changes in common carp brain, muscle, and liver TBARS levels [8]. Changes in TBARS levels in brain, liver, and muscle of *Leporinus obtusidens* exposed for 90 days to commercial herbicide formulations containing quinclorac and metsulfuron-methyl were observed Pretto et al. [36]. These alterations in TBARS levels may indicate a compensatory response of the fish to herbicide-induced oxidative stress. The levels of lipid peroxidation may differ among fish species. Differences observed in TBARS levels could reflect variation in the antioxidant mechanisms of fish species, duration of exposure, and the pesticide tested [35]. Previous studies have shown that oxidative stress indices in fish may vary depending on the tissue and environmental pollutant assessed.

Organisms are equipped with interdependent cascades of enzymes to alleviate oxidative stress and repair macromolecules damaged during normal metabolism or by exposure to xenobiotics [37]. The changes in levels of antioxidant enzymes demonstrate a pollutant-induced adaptive response in fish, and an attempt to neutralize the generated reactive oxygen species [38]. An important feature of antioxidant enzymes is their altered activity under conditions of oxidative stress, an important adaptation to address pollutant-induced stress. Superoxide dismutase and CAT are the major enzymes in eliminating reactive oxygen species formed during bioactivation of xenobiotics in the tissues [37] and the induction of SOD and CAT systems provides a first line of defense against reactive oxygen species. Superoxide dismutase catalyzes

the dismutation of superoxide radical O_2^- to oxygen and hydrogen peroxide (H_2O_2). Catalase activity in organ tissues may be in response to H_2O_2 produced by SOD activity, since CAT is responsible for the detoxification of H_2O_2 to water [9]. We found many changes in gill, with decreased SOD activity during the experiment in all prometryne exposed groups. Changes in brain tissue were also observed, with an increase in SOD activity at the beginning of the test, followed by decrease, and finally a subsequent rise in SOD activity. A decrease in SOD activity in intestine was found only at the highest concentration (80 μg/l) after 14 days.

An increase in SOD activity indicates an increase in O_2^- production [37]. Stara et al. [39] found increased SOD activity in common carp following exposure to simazine at 2 and 4 mg/l, especially in muscle after 28 days exposure. Superoxide dismutase activity in these groups decreased after 60 days. Exposure to atrazine was reported to be associated with increased SOD activity, especially in the liver of the zebrafish (*Danio rerio*) [40]. Fifteen days exposure to atrazine at 10.6 mg/l caused increase of SOD activity in *Channa punctatus* [9]. Paulino et al. [41] observed the increased SOD activity in gill of *Prochilodus lineatus* after 14 days exposure to 10 μg/l atrazine. Oruc and Usta [42] reported an increase in SOD activity in gill, muscle, and kidney of common carp after 15 days exposure to diazinon, and after 60 days exposure, observed a reduction in SOD activity.

Tissue antioxidant response to oxidative stress shows species differences due to differences in tissue antioxidant potential [43]. The increase in SOD activity may be due to increased production of reactive oxygen species, or SOD activity may be reduced due to direct damage to the structure of proteins exposed to herbicides and the resulting increase in amounts of hydrogen peroxide [44].

While a significant increase in CAT activity has been observed in some studies after fish exposure to pesticides [4,7,8,14,39], other studies have reported reduced CAT activity in fish organ tissues [5,11,36]. Overall, results indicate disruption of the normal oxidation process, suggesting a failure of antioxidant defense systems represented by SOD and CAT [11].

Glutathione reductase is another enzyme providing a first line of defense against oxidative damage [45]. In our study we found reduced GR activity in brain after 14 days exposure to all tested concentrations of prometryne. Muscle tissue showed increased GR activity in fish exposed to the highest concentration prometryne (80 μg/l) after 14 days. Velisek et al. [46] reported reduced GR activity in liver and intestine and increased GR activity in muscle of common carp after 90-days exposure to terbutryn at environmentally realistic concentrations (0.02, 0.2 and 2.0 μg/l). Exposure to sublethal concentrations of atrazine for 15 days caused elevated GR in liver of *Channa punctatus* [9]. It appears that dose and exposure time affects the GR activity in fish exposed to triazine herbicides. Glutathione reductase activity can effectively regulate reactive oxygen species formation during the exposure period. This compensatory response accompanied by the induction of other antioxidants (SOD, CAT) may help to prevent accumulation of free radicals and their products in stressed organisms. The herbicide may interact with the tissue, resulting in the alteration of enzyme activity mediated by reactive oxygen radicals resulting from stress in the fish [47]. Enzymatic antioxidants are essential to maintain the redox status of fish cells and serve as an important defense against oxidative stress [48]. Oxidative stress in fish is represented by damage to biological systems or failure of the antioxidant defense system [49].

5. Conclusions

Chronic exposure to prometryne was shown to alter the antioxidant status of carp. Significant changes in enzyme activity (SOD, CAT, GR) in tissue of common carp were seen with chronic exposure to prometryne, with no observed oxidative damage to the cells. Changes in CAT activity were observed only at the highest concentration of prometryne (80 μg/l) after 60 days. Changes in SOD and GR activity were observed at commonly occurring environmental concentrations of prometryne (0.51 μg/l) after 14 and 30 days of exposure. This study provided important results for the evaluation of long-term impact and influence of prometryne, even in concentrations commonly measured in surface waters. Antioxidant responses could provide useful parameters for evaluating physiological effects of herbicides on common carp, but the application of these findings will need more detailed laboratory study before they can be established as biomarkers for monitoring the aquatic environment. Fish brain, gill, and liver could potentially be used as indicators for monitoring residual prometryne present in the aquatic environment.

Acknowledgments

This research was supported by the center CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No. 047/2010/Z and GA JU 022/2012 Z.

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CHAPTER 7

EFFECT OF CHRONIC EXPOSURE TO SIMAZINE ON OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN COMMON CARP (*CYPRINUS CARPIO* L.)

Stara, A., Machova, J., Velisek, J., 2012. Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.). Environmental Toxicology and Pharmacology 33, 334–343.

It was allowed by publisher on 9th January, 2014 to include the paper in this Ph.D. thesis.

ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY 33 (2012) 334–343

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/etap

Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.)

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ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form
8 December 2011
Accepted 19 December 2011
Available online 26 December 2011

Keywords:
Triazine
Fish
Oxidative stress
Antioxidant enzyme
Tissue

ABSTRACT

We investigated the chronic effect of simazine, an s-triazine herbicide commonly present in aquatic environments, on the antioxidant system and oxidative stress indices in common carp (*Cyprinus carpio* L.). Fish were exposed to sub-lethal concentrations of 0.06 $\mu g\,l^{-1}$ (environmental concentration in Czech rivers), 2 $mg\,l^{-1}$, and 4 $mg\,l^{-1}$ for 14, 28 and 60 days. Indices of oxidative stress [reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS)], and antioxidant parameters [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH)] in fish brain, gill, muscle, liver, and intestine were measured. Chronic exposure to simazine showed the impact of the increased production of ROS leading to oxidative damage to lipids, proteins, and inhibition of antioxidant capacity. Activity of the antioxidant enzymes SOD, CAT, GPx and GSH in groups with high concentrations (2 $mg\,l^{-1}$, 4 $mg\,l^{-1}$) increased at 14 and 28 days, but decreased after 60 days exposure ($p < 0.01$) as compared with the control group. Changes in enzyme activity were mainly in the liver, but also in gills and brain. Prolonged exposure to simazine resulted in excess ROS formation finally resulting in oxidative damage to cell lipids and proteins and also inhibited antioxidant capacities in common carp tissue.

1. Introduction

Increased agricultural and industrial activities contribute to environmental pollution affecting water systems, which are the end points of contamination (Fu et al., 2003; Faigueiredo-Fernandes et al., 2006). In the aquatic environment pesticides such as herbicides, insecticides, and their mixtures often negatively affect non-target organisms (Zhang et al., 2010).

Symmetrical triazines (s-triazines) have been used as selective herbicides in agriculture in the United States and other parts of the world for more than 50 years (Breckenridge et al., 2008). They include simazine, atrazine, ametryn, propazine, terbutryn, and prometron. In North America and Europe they are most often used to treat maize crops. A widely used asymmetrical triazine, metribuzin, is used primarily in forestry and on potatoes (Sanderson et al., 2001).

Triazine herbicides are absorbed through leaves and roots of plants (Bowmer et al., 1995) and inhibit photosynthesis (Lin et al., 2008). They are relatively stable, and demonstrable in drinking water, food, and fish. Triazines induce liver microsomal enzymes and converted to N-dealkyl derivatives. S-chlortriazines affect fish estrogen levels (Sanderson et al., 2001).

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doi:10.1016/j.etap.2011.12.019

Simazine, 6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine, was one of the first compound triazines (Kamrin, 1997), registered in 1957. From 1990 to 1993 it was among the most widely used herbicides in the US for pre and post-emergence weed control. It is a white crystalline solid with a melting point of 226 °C, slightly soluble in water (5 mg l^{-1}) and highly soluble in organic solvents. When applied to the soil it is absorbed by leaves and roots, causing inhibition of photosynthesis in the entire plant (Manahan, 2005; Edwards, 2006). It is biodegradable, metabolized in plants and soil through both chemical and microbiological processes. Prior to 1992, simazine was used to kill submerged weeds and algae in large aquariums and ponds (US EPA, 1994). In the Czech Republic, simazine, distributed under the trade mark Simazine 50WP, is used for potatoes, alfalfa, orchards, vineyards, hop gardens, gooseberries, raspberries, blackberries, strawberry, trees, and ornamental plants in combination with the product Gesetop 90 WG(H). The registration has expired, but existing stock may be used. In 2000 (the last year of application) consumption was 200 kg $year^{-1}$ in the Czech Republic. Currently simazine is considered dangerous for the environment. According to Directive 196/2010 simazine use is banned in the countries of the European Union (Commission Regulation, 2010).

To strengthen the effect of simazine it is often combined with other pesticides such as diuron, ametryn, or atrazine. Simazine is moderately persistent, remaining in the soil 28–149 days. Residual activity may remain in the soil up to one year after application.

Simazine is highly toxic to Two-spot barb (*Barbus ticto*) (Turner, 2003), and may cause long-term adverse effects in the aquatic environment (Velisek et al., 2012). Excessive use of simazine for weed control in field crops has affected the aquatic environment, and it has long been used to control submerged aquatic vegetation in stagnant and slowly flowing waters (Turner, 2003; Oropesa et al., 2009). Residues of simazine have been detected in drinking water (Oropesa et al., 2008). It was reported to be the most frequently detected pesticide in California with concentrations of 0.02–49.2 μg l^{-1} (US EPA, 1994). In some European countries (Greece, Spain, Czech Republic) are reported simazine concentration from 0.06 to 4.5 mg l^{-1} in surface and ground waters (Papadopoulou-Mourkidou et al., 2004; Oropesa et al., 2008; Czech Hydrometeorological Institute, 2011).

Tests of acute toxicity have shown simazine toxicity to daphnia (*Daphnia magna*) and stonefly (*Plecoptera*) with 96 h $LC_{50} > 3.7$ mg l^{-1}; rainbow fish (*Poecilia reticulata*), 48 h $LC_{50} = 3.5$ mg l^{-1}; common carp (*Cyprinus carpio* L.), 48 h $LC_{50} > 4$ mg l^{-1}; crucian carp (*Carassius Carassius*), 96 h $LC_{50} > 100$ mg l^{-1}; and rainbow trout (*Oncorhynchus mykiss*), 96 h $LC_{50} > 100$ mg l^{-1} (Kamrin, 1997).

Simazine and seven other s-triazines have been identified as relevant in the prioritization of substances dangerous to the aquatic environment in the member states of the European Community (European Commission, 1999). Simazine is included in the EU Priority Pollutants List and the US Environmental Protection Agency's list. The presence of simazine in the soil–water system in considered an environmental hazard, and, because of its estrogenic effect on various cell lines in laboratory experiments, it has recently become subject to control (Sanderson et al., 2001; Zorrilla et al., 2010).

Information on oxidative stress in aquatic organisms, principally fish, has great importance for environmental and aquatic toxicology. Because oxidative stress is induced by many chemicals, including some pesticides, these contaminants may stimulate reactive oxygen species and alteration in antioxidant systems. Pro-oxidant factor actions in fish can be used to assess pollution of specific areas or worldwide marine pollution (Monteiro et al., 2006; Uner et al., 2006; Slaninova et al., 2009). One of the most important early events in cell degeneration leading to necrosis is lipid peroxidative damage, which occurs mainly in the cell membrane. In addition, lipid peroxidation represents a frequent reaction to free radical attack on biological structures (Tagliari et al., 2004). The genesis of oxidative stress in fish is also affected by pesticide biodegradation products (Slaninova et al., 2009). This study addresses whether, and how, low concentrations of simazine affect the physiology of common carp. Although the effects of acute and sub-chronic exposure of fish to atrazine, another s-triazine herbicide, have been well-documented, there is a dearth of data on the chronic exposure to simazine at environmentally realistic concentrations with respect to oxidative stress and antioxidant responses in common carp. The aim of this study was to evaluate oxidative stress and antioxidant response of common carp exposed to simazine.

2. Materials and methods

2.1. *Experimental design*

Common carp (mean body length 21.31 ± 2.05 cm, mean weight 282.05 ± 38.08 g) were obtained from a commercial hatchery (Vodnany, Czech Republic). They were held in aquaria containing 200 l of freshwater with continuous aeration, temperature of 19.1 ± 1.3 °C, and a 12:12 L:D cycle. Dissolved oxygen values were maintained at 90–95%, and pH was 7.5 ± 0.4. Fish were fed commercial fish food and were acclimatized for 10 days before experimentation.

The experiment was a semi-static assay conducted over a 60 day period. One hundred and four one-year-old common carp were allocated, in groups of 13, to one of three experimental regimes or to an untreated control group. The experimental fish were exposed to simazine (Sigma–Aldrich, Czech Republic, chemical purity 99.5%) at concentrations in water of: 0.06 μg l^{-1} (reported environmental concentration in Czech rivers) (Group 1), 2 mg l^{-1} (Group 2), and 4 mg l^{-1} (Group 3). The simazine concentration of 2 mg l^{-1} and 4 mg l^{-1} corresponded to the 48 h LC_{10}, and 48 h LC_{30} for common carp, respectively. Each experimental condition was duplicated. The test fish were exposed to simazine for 14, 28, and 60 days. Fish were fed commercial fish pellets at about 1% body weight per day in two feedings and were starved for 24 h prior to sampling to avoid prandial effects during the assay. Eighty percent of the exposure solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of simazine and to maintain water quality. The physicochemical parameters of the water in all aquaria were acid neutralization capacity, $ANC_{4.5}$ 0.76 mM l^{-1}; total ammonia, 0.011 mg l^{-1}; NO_3^-, 6.11 mg l^{-1}(NO_2^-, 0.010 mg l^{-1};

PO_4^{3-}, 0.03 mg l^{-1}(and chemical oxygen demand, COD_{Mn} 0.8 mg l^{-1}. Water temperature was 18.4–21.2 °C, and oxygen saturation 90–96%.

To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by high performance liquid chromatography (HPLC) using the method of Katsumata et al. (2005). Water samples were collected from the test aquaria 1 h and 24 h after renewing the test solutions. The values measured did not differ from the value stated for test purposes by more than 2%.

2.2. Tissue samples and preparation of post-mitochondrial supernatant

At the completion of each exposure period, four fish from each aquarium were randomly selected and killed. The brain, gill, muscle, liver, and intestine were quickly removed, immediately frozen, and stored at −80 °C for analysis. Frozen tissue samples were weighed and homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS), and a second centrifuged at 12,000 × *g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for other antioxidant parameter analyses.

2.2.1. *Indices of oxidative stress*

Reactive oxygen species (ROS) were measured using 2,7-dichlorofluorescin diacetate (DCFH-DA) following Driver et al. (2000) with modifications by Zhang et al. (2008). The TBARS method described by Lushchak et al. (2005) was used to evaluate lipid peroxidation.

2.2.2. *Antioxidant parameters*

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund (1974). This assay depends on the autoxidation of pyrogallol. Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme per milligram of protein. The catalase (CAT; EC 1.11.1.6) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer (1952). Glutathione peroxidase (GPx; EC 1.11.1.9) activity was derived from the rate of NADPH oxidation at 340 nm by the reaction with glutathione reductase (GR; EC 1.6.4.2). The specific activity was determined using the extinction coefficient 6.22 mM cm^{-1} (Lawrence and Burk, 1976). Glutathione reductase activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). Reduced glutathione (GSH) level was assayed using the methods of Sedlak and Lindsay (1968) with a modification by Ferrari et al. (2007). One unit of CAT, GPx, or GR activity is defined as the amount of the enzyme that consumes 1 mol l^{-1} of substrate or generates 1 mol l^{-1} of product per min. Activity was expressed in international units (or milliunits) per mg of protein.

2.2.3. *Protein estimation*

Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

2.3. Statistical analysis

The statistical software program STATISTICA (version 8.0 for Windows, StatSoft) was used to compare differences among the test groups. Prior to analysis, all measured variables were checked for normality (Kolmogorov–Smirnov test) and homoskedasticity of variance (Bartlett's test). If those conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. When a significant difference was detected ($p < 0.05$), Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a non-parametric test (Kruskal–Wallis) was used (Zar, 1996).

3. Results

3.1. Fish behavior during the experiment

During the experiment, both control and exposed common carp showed normal feeding behavior. There were no signs of respiratory distress such as rapid ventilation, increased rate of gill opercular movements, or floating at the surface of water. There were no mortalities during the experiment.

3.2. Oxidative stress

Effects of chronic exposure to simazine on ROS in tissue of common carp are presented in Table 1. Group 1 showed no significant differences from the control value. Group 3 showed a significant difference from controls in liver at 28 days. After 60 days exposure, significant differences ($p < 0.01$) from controls were observed in Group 2 in liver and Group 3 in brain, muscle, and liver. Activity was higher in these group compared to the control group. Table 2 shows the effect of chronic exposure to simazine on TBARS. No test group showed significant differences from the control group in brain, gill, muscle, liver, or intestine.

3.3. Antioxidant response

Effect of chronic exposure to simazine on the activity of superoxide dismutase (SOD) in tissue of common carp is shown in Table 3. Significant differences from the control value ($p < 0.01$) were seen in Group 2 and Group 3 in muscle at all exposure durations. With 14 and 28 days exposure, these groups showed increased activity of SOD, but at 60 days the level was significantly reduced relative to controls.

Table 4 shows the effect of the chronic exposure to simazine on the activity of CAT in tissue of common carp. Significant differences from controls were found for Group 3 in brain, muscle, and liver ($p < 0.01$) at all exposure durations. At 14 and 28 days of simazine exposure, CAT activity increased and at 60 days was reduced. In Group 2, CAT activity was increased in liver after 14 and 28 days, while after 60 days activity was

Table 1 – Effect of chronic exposure to simazine on reactive oxygen species (ROS, %) values in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	100.11 ± 18.54	100.08 ± 19.51	106.75 ± 8.54	111.26 ± 8.63
	28	100.91 ± 12.29	105.22 ± 6.98	111.31 ± 10.02	113.17 ± 8.04
	60	100.91 ± 12.29	107.87 ± 9.43	112.17 ± 7.92	144.76 ± 17.19*
Gill	14	100.62 ± 2.09	100.13 ± 6.17	105.80 ± 5.07	111.24 ± 5.89
	28	100.96 ± 9.65	108.13 ± 7.69	116.10 ± 25.25	117.70 ± 12.96
	60	100.81 ± 11.14	111.37 ± 11.50	127.98 ± 21.47	128.58 ± 24.80
Muscle	14	100.77 ± 10.19	103.73 ± 9.74	110.90 ± 7.65	112.05 ± 9.19
	28	100.98 ± 13.01	116.32 ± 32.03	128.03 ± 31.69	136.48 ± 32.5
	60	100.63 ± 9.73	112.46 ± 17.50	118.54 ± 17.54	141.80 ± 20.73*
Liver	14	100.63 ± 9.38	105.99 ± 5.59	110.53 ± 8.58	116.68 ± 9.05
	28	100.35 ± 12.35	110.56 ± 23.99	115.05 ± 23.67	149.15 ± 21.67*
	60	100.83 ± 7.16	108.82 ± 8.07	133.83 ± 11.64*	145.07 ± 17.47*
Intestine	14	100.08 ± 10.80	109.39 ± 9.22	114.91 ± 11.24	119.05 ± 8.64
	28	100.82 ± 19.86	109.39 ± 11.11	117.54 ± 24.13	119.21 ± 18.45
	60	101.00 ± 7.69	109.37 ± 7.72	113.06 ± 13.79	116.64 ± 10.79

Data are means ± S.D., $N = 8$.
* Significant differences compared with control value, <0.01.

reduced. Increased CAT activity was found in muscle after 28 days, after 60 days exposure time this was again reduced. In brain, the activity of CAT after 60 days exposure time increased. Group 1 showed significant increased CAT activity in muscle tissue after 60 days exposure relative to controls.

Chronic effects of simazine on GPx activity in tissue of common carp are shown in Table 5. Activity GPx in liver was lower compared to the control group in Group 3 for all exposure durations and in Group 2 after 28 days and 60 days.

No significant differences from control values were found in any group for effects of chronic exposure to simazine on activity of GR in tissue of common carp (Table 6).

Chronic exposure to simazine on levels of GSH in tissue of common carp (Table 7). Elevated levels of GSH compared to the control group were found in brain tissue in Group 3 at all exposure durations and in Group 2 after 60 days exposure. Significant differences from controls in GSH values were detected in liver of all groups. In Group 3, after 14 days exposure increased the concentration of GSH compared with the controls, and GSH was increased at 28 days exposure. After 60 days, the GSH concentration decreased significantly. Group 2 showed increased GSH levels in all of exposure time. After 28 and 60 days exposure was observed increasing of concentrations of GSH in Group 1 compared to the Control group.

Table 2 – Effect of chronic exposure to simazine on level of thiobarbituric acid reactive substances (TBARS, nmol/gww) in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	4.65 ± 2.63	4.82 ± 2.36	3.04 ± 1.99	4.30 ± 1.56
	28	5.70 ± 2.18	6.08 ± 1.63	6.34 ± 1.59	6.53 ± 1.87
	60	5.04 ± 2.17	5.42 ± 1.89	4.75 ± 2.11	5.31 ± 1.94
Gill	14	10.76 ± 3.98	12.94 ± 3.49	12.17 ± 4.95	12.24 ± 3.66
	28	10.78 ± 4.61	13.67 ± 5.60	11.72 ± 5.50	10.24 ± 4.67
	60	10.93 ± 2.58	11.94 ± 4.34	12.17 ± 2.72	11.02 ± 4.02
Muscle	14	6.91 ± 3.22	7.72 ± 3.04	7.64 ± 2.19	7.88 ± 2.45
	28	7.69 ± 2.00	9.40 ± 3.76	8.43 ± 2.52	8.74 ± 3.30
	60	9.92 ± 3.67	7.94 ± 2.92	7.41 ± 2.08	7.82 ± 3.02
Liver	14	30.21 ± 6.53	30.55 ± 5.96	24.95 ± 6.07	25.40 ± 7.72
	28	28.83 ± 6.77	28.92 ± 7.07	29.60 ± 6.45	27.68 ± 7.20
	60	25.96 ± 3.01	25.91 ± 3.55	24.23 ± 3.82	27.78 ± 4.81
Intestine	14	3.06 ± 1.29	3.41 ± 1.71	3.93 ± 1.14	3.74 ± 2.18
	28	2.70 ± 1.28	2.85 ± 1.04	2.55 ± 0.84	2.60 ± 0.60
	60	2.14 ± 0.78	2.25 ± 0.91	2.51 ± 0.93	2.64 ± 0.62

Data are means ± S.D., $N = 8$. *Significant differences compared with control value, <0.05.

Table 3 – Effect of chronic exposure to simazine on superoxide dismutase (SOD, U mg^{-1} protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	14.36 ± 4.53	15.86 ± 3.22	13.84 ± 4.30	15.84 ± 2.95
	28	13.70 ± 4.33	15.60 ± 4.44	15.43 ± 8.53	15.06 ± 4.09
	60	14.31 ± 3.18	16.21 ± 2.36	15.47 ± 2.60	15.16 ± 2.85
Gill	14	9.22 ± 4.81	10.46 ± 4.55	9.87 ± 2.79	10.57 ± 3.12
	28	11.77 ± 3.00	13.49 ± 5.58	14.70 ± 4.47	15.30 ± 3.16
	60	11.49 ± 2.89	13.58 ± 4.32	13.70 ± 2.18	13.81 ± 3.61
Muscle	14	33.25 ± 12.27	35.02 ± 5.89	43.70 ± 7.98*	49.30 ± 9.08*
	28	36.99 ± 10.69	40.76 ± 8.07	60.91 ± 8.30*	71.85 ± 8.81*
	60	32.54 ± 6.10	33.92 ± 5.71	20.99 ± 6.17*	16.94 ± 4.77*
Liver	14	35.91 ± 7.80	32.10 ± 7.25	31.22 ± 8.09	28.77 ± 6.10
	28	33.77 ± 9.61	32.73 ± 7.71	31.56 ± 7.92	28.41 ± 5.16
	60	36.01 ± 5.55	34.19 ± 4.46	35.80 ± 9.74	33.48 ± 6.04
Intestine	14	36.88 ± 13.08	37.05 ± 8.64	38.53 ± 8.43	35.82 ± 9.47
	28	33.85 ± 7.10	31.73 ± 5.45	32.24 ± 6.03	33.38 ± 5.65
	60	29.22 ± 7.25	27.38 ± 4.97	26.39 ± 6.53	27.11 ± 5.65

Data are means ± S.D., $N = 8$.
* Significant differences compared with control value, <0.01.

Table 4 – Effect of chronic exposure to simazine on catalase (CAT, U mg^{-1} protein) activity in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	2.48 ± 0.99	2.90 ± 1.20	2.27 ± 0.59	7.04 ± 1.55*
	28	2.75 ± 0.93	2.88 ± 0.85	3.91 ± 1.08	12.60 ± 3.77*
	60	4.67 ± 1.87	5.19 ± 1.452	14.24 ± 3.01*	0.85 ± 0.62*
Gill	14	1.97 ± 0.89	2.18 ± 0.60	2.23 ± 0.81	2.35 ± 0.67
	28	2.71 ± 0.87	3.13 ± 1.38	3.58 ± 1.18	3.70 ± 1.84
	60	2.85 ± 0.75	2.93 ± 1.02	2.86 ± 0.86	3.03 ± 1.17
Muscle	14	6.37 ± 2.96	6.51 ± 2.07	10.29 ± 2.41	13.05 ± 2.67*
	28	10.35 ± 2.48	14.61 ± 3.58	17.91 ± 4.39*	22.77 ± 4.22*
	60	12.87 ± 2.71	30.48 ± 5.67*	7.61 ± 3.94*	4.69 ± 2.14*
Liver	14	37.87 ± 6.60	39.30 ± 5.61	49.77 ± 8.96*	54.18 ± 9.65*
	28	37.44 ± 8.55	40.60 ± 5.65	56.77 ± 8.13*	60.72 ± 7.71*
	60	39.69 ± 6.33	38.10 ± 6.49	22.88 ± 4.16*	19.06 ± 5.29*
Intestine	14	2.32 ± 0.83	2.50 ± 1.24	2.88 ± 1.01	2.92 ± 1.20
	28	2.39 ± 0.86	2.28 ± 0.86	2.58 ± 0.64	2.77 ± 0.74
	60	2.44 ± 1.47	2.27 ± 0.72	2.79 ± 1.09	2.81 ± 0.86

Data are means ± S.D., $N = 8$.
* Significant differences compared with control value, <0.01.

4. Discussion

Many classes of environmental pollutants or their metabolites may exert toxicity related to oxidative stress and can cause oxidative damage in fish (Faigueiredo-Fernandes et al., 2006; Velisek et al., 2011). The present study evaluated effects of chronic exposure to simazine on oxidative stress and antioxidant activity in organ tissue of common carp. The evaluation of oxidative stress markers is critical to the investigation of oxidative stress in organisms. Precautions should be taken in the assessment of oxidative stress (Lushchak, 2011). The steady-state concentration of the markers of oxidative stress is a balance between production and elimination producing a steady-state ROS level. The dynamic equilibrium can be disturbed, leading to enhanced peroxidative processes (ROS) and damage to cellular constituents linked to low antioxidant concentrations (Lushchak, 2011), resulting in oxidative stress. Under normal conditions, antioxidant systems within the cell minimize the perturbations caused by ROS (Kavitha and Rao, 2009). Our study showed increased ROS, mainly in the liver, following exposure to simazine at concentrations of 4 mg l^{-1} for 28 and 60 days. To neutralize ROS, animals possess an antioxidant defense pathway comprising antioxidant enzymes such as SOD, CAT, GPx, and GR as well as non-enzymatic

Table 5 – Effect of chronic exposure to simazine on glutathione peroxidase activity (GPx, mU/mg protein) in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	18.87 ± 5.69	15.62 ± 4.53	14.90 ± 3.37	15.49 ± 1.42
	28	18.75 ± 6.92	16.51 ± 5.92	16.87 ± 7.99	17.84 ± 7.29
	60	24.62 ± 9.36	23.05 ± 7.41	25.54 ± 8.62	25.12 ± 6.54
Gill	14	25.77 ± 9.05	28.21 ± 7.55	28.48 ± 10.52	28.75 ± 8.66
	28	23.06 ± 9.76	27.16 ± 24.33	26.65 ± 5.65	23.92 ± 6.34
	60	26.12 ± 6.68	27.14 ± 5.05	25.17 ± 3.83	27.27 ± 4.93
Muscle	14	28.13 ± 7.16	28.98 ± 7.66	29.59 ± 6.36	30.31 ± 6.17
	28	30.4 ± 9.02	30.66 ± 8.79	29.08 ± 6.01	28.15 ± 8.67
	60	31.88 ± 10.90	28.44 ± 6.44	32.52 ± 6.00	33.62 ± 7.71
Liver	14	44.36 ± 8.27	41.39 ± 9.81	38.82 ± 10.76	25.788 ± 8.578*
	28	47.64 ± 8.08	42.98 ± 4.07	34.45 ± 6.43*	22.92 ± 4.97*
	60	47.86 ± 8.18	44.49 ± 4.71	33.29 ± 6.69*	28.83 ± 6.38*
Intestine	14	28.05 ± 8.23	25.55 ± 6.54	23.73 ± 7.59	25.60 ± 12.54
	28	26.24 ± 5.21	26.00 ± 4.70	24.31 ± 3.82	23.76 ± 4.05
	60	23.17 ± 5.64	23.08 ± 5.06	26.56 ± 5.68	27.71 ± 6.32

Data are means ± S.D., N = 8.
* Significant differences compared with control value, <0.01.

antioxidants such as GSH, which prevent oxidative damage (Modesto and Martinez, 2010).

The TBARS assay quantifies oxidative stress by measuring the lipid peroxidation that occurs with free radical generation and is used to quantify oxidative damage in fish tissue (Oakes and Van der Kraak, 2003). Our data suggested that chronic exposure to simazine did not affect TBARS measures in tissue of common carp. Almroth et al. (2005) did not observe significant seasonal differences in TBARS levels in liver of eelpout (*Zoarces viviparus*). Acute toxicity has been reported to increase levels of TBARS in the organs of fish exposed to pesticides. Oropesa et al. (2009) found increased GSH levels in common carp after a 90 day exposure to simazine at 45 μg l^{-1}. Toni et al. (2011) exposed common carp to varying concentrations of fungicide (tebuconazol) and found a significant increase of TBARS levels in liver, brain, and muscle. Miron et al. (2008) observed effects of the herbicide clomazone at concentrations of 0.5 mg l^{-1} on piava (*Leporinus obtusidens*). They found increased TBARS levels in brain but decreases in liver and muscle. It is often difficult to predict antioxidant effects on oxidative stress, since various factors affect the activity of antioxidant enzymes (Li et al., 2011).

The first line of defense against oxidative stress consists of the antioxidant enzymes SOD, CAT, and GPx, which convert

Table 6 – Effect of chronic exposure to simazine on glutathione reductase activity (GR, mU mg^{-1} protein) in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 (0.06 μg l^{-1})	2 (2 mg l^{-1})	3 (4 mg l^{-1})
Brain	14	3.53 ± 1.10	3.24 ± 1.93	3.37 ± 1.53	3.37 ± 1.06
	28	3.64 ± 0.91	3.45 ± 1.22	3.87 ± 2.48	4.22 ± 1.48
	60	5.56 ± 2.79	5.25 ± 3.89	5.21 ± 1.50	5.45 ± 1.93
Gill	14	7.14 ± 2.06	8.72 ± 3.55	9.05 ± 2.72	8.80 ± 2.44
	28	6.67 ± 2.26	7.46 ± 2.60	7.57 ± 2.53	8.05 ± 2.56
	60	5.60 ± 1.91	5.89 ± 1.79	5.65 ± 2.38	5.98 ± 1.43
Muscle	14	3.09 ± 1.21	2.94 ± 1.37	3.04 ± 1.24	3.09 ± 0.08
	28	1.76 ± 0.68	1.88 ± 0.84	1.97 ± 0.86	1.77 ± 0.64
	60	2.93 ± 1.18	2.78 ± 1.21	2.87 ± 0.83	2.77 ± 0.91
Liver	14	25.13 ± 3.66	25.69 ± 6.71	25.53 ± 6.29	27.14 ± 4.06
	28	23.91 ± 6.64	24.09 ± 6.20	26.02 ± 6.58	24.92 ± 7.06
	60	25.96 ± 5.79	25.83 ± 5.38	24.93 ± 6.53	27.95 ± 5.91
Intestine	14	3.27 ± 0.99	3.33 ± 2.34	3.65 ± 1.00	3.99 ± 2.17
	28	2.87 ± 0.89	3.00 ± 1.04	2.77 ± 1.00	2.68 ± 0.79
	60	2.71 ± 0.92	2.90 ± 1.14	2.90 ± 0.99	3.23 ± 0.70

Data are means ± S.D., N = 8. *Significant differences compared with control value, <0.05.

Table 7 – Effect of chronic exposure to simazine on level of reduced glutathione (GSH, $\mu g\,mg^{-1}$ protein) in common carp tissue.

Indices	Exposure time (days)	Test groups			
		Control	1 ($0.06\,\mu g\,l^{-1}$)	2 ($2\,mg\,l^{-1}$)	3 ($4\,mg\,l^{-1}$)
Brain	14	23.06 ± 6.53	22.31 ± 10.96	21.69 ± 5.04	32.05 ± 6.14*
	28	24.21 ± 8.13	22.53 ± 7.86	25.25 ± 10.15	42.16 ± 12.92*
	60	23.60 ± 7.15	25.73 ± 4.89	34.67 ± 4.88*	50.23 ± 12.76*
Gill	14	5.99 ± 2.91	6.50 ± 2.28	6.78 ± 1.92	7.38 ± 1.92
	28	6.84 ± 4.37	6.30 ± 2.44	7.03 ± 2.15	8.12 ± 3.39
	60	8.15 ± 1.51	8.85 ± 2.54	9.31 ± 2.46	10.74 ± 3.29
Muscle	14	26.37 ± 6.22	27.06 ± 5.20	29.22 ± 7.50	30.61 ± 5.55
	28	32.82 ± 7.62	30.52 ± 7.01	29.08 ± 7.17	28.58 ± 5.52
	60	33.91 ± 8.87	31.38 ± 8.04	32.10 ± 6.04	31.42 ± 9.75
Liver	14	38.46 ± 11.51	40.30 ± 7.43	51.19 ± 6.11*	54.94 ± 9.27*
	28	34.67 ± 7.77	45.26 ± 9.11*	49.11 ± 10.50*	64.97 ± 7.20*
	60	34.58 ± 3.73	49.71 ± 8.04*	45.65 ± 10.62*	19.71 ± 9.16*
Intestine	14	6.27 ± 2.79	6.35 ± 3.22	6.83 ± 2.70	7.36 ± 2.25
	28	7.59 ± 2.85	7.41 ± 2.86	8.37 ± 2.40	8.43 ± 3.32
	60	10.13 ± 3.16	9.01 ± 2.91	10.57 ± 3.37	10.64 ± 2.82

Data are means ± S.D., N = 8.
* Significant differences compared with control value, <0.01.

superoxide anions (O_2^-) into H_2O_2 and then into H_2O and O_2. Decrease in the activity of these enzymes changes the redox status of the cells (Ojha et al., 2011). Thus, it is possible that an increase in the activity of these enzymes contributes to the elimination from the cell of ROS induced by pesticide exposure (Jin et al., 2010).

Superoxide dismutase is an antioxidant enzyme important in inhibiting oxyradical formation and is used as a biomarker to indicate oxidative stress (Zhang et al., 2004). Similar changed activities of SOD in common carp have also been reported by other authors. In a study of common carp, Oruc et al. (2004) reported the greatest change in SOD activity in gill after exposure to pesticides (2,4-dichlorophenoxyacetic acid and azinphosmethyl). The gill is in direct contact with aquatic-borne pesticides, whereas its effects are more limited in other tissues, during an acute test exposure of 96 h. Jin et al. (2010) observed increased SOD activity, especially in liver, of zebrafish (*Danio rerio*), after 14 day atrazine exposure. The reduction of superoxide radicals to H_2O_2 is catalyzed by SOD. Oruc and Usta (2007) reported an increase in SOD activity in gill, muscle, and kidney of common carp after a 15 day exposure to varying concentrations of diazinon. An increase in SOD activity indicates an increase in O_2^- production (Zhang et al., 2004). After an exposure time of 60 days, Oruc and Usta (2007) observed a reduction in SOD activity. Velisek et al. (2011) observed in liver and in brain increased SOD activity in common carp after long-term exposure to terbutryn. The tissue response of the antioxidant system to oxidative stress shows species differences due to the differences in antioxidant potential of the tissues (Ahmad et al., 2004). Increase in SOD activity may be due to increased generation of ROS, while, on the other hand, SOD activity may be decreased due to direct damage of its protein structure by herbicides and by increasing amounts of hydrogen peroxide (Puerto et al., 2010).

CAT is an active enzyme and the first to show alterations following induction of oxidative stress (Jin et al., 2010). Pesticide-induced inhibition and induction of CAT activity has been reported in studies of various fish species. Oruc and Usta (2007) observed CAT activity increase in gill and a trend toward reduction in CAT activity in muscle of common carp. Jin et al. (2010) reported increased CAT activity, especially in liver of zebrafish, after 14 day atrazine exposure at a concentration of $1000\,\mu g\,l^{-1}$. Ahmad et al. (2004) demonstrated decreases in CAT activity in gill and kidney and increases in liver of eel (*Anguilla anguilla* L.). Ballesteros et al. (2009) states that the activity of CAT was significantly decreased in liver of the onesided livebearer (*Jenynsia multidentata*) exposed to endosulfan. Moraes et al. (2009) reported a decrease in liver catalase activity in teleost fish (*Leporinus obtusidens*) and silver catfish (*Schilbe intermedius*) after exposure to herbicides. These authors pointed out that the observed decrease could be explained by the flux of superoxide radicals due to the oxidative stress caused by pollutant exposure. Decrease in CAT activity could therefore be caused by excessive production of O_2 (Bainy et al., 1996). Ballesteros et al. (2009) states that the activity of antioxidant enzymes may be increased or inhibited under chemical stress depending on the intensity and the duration of the stress applied as well as on the susceptibility of the exposed species.

Glutathione peroxidase activity catalyses the reduction of H_2O_2 and lipid peroxides and is considered an efficient protective enzyme against lipid peroxidation at the expense of GSH (Moreno et al., 2005). Enzyme activity can decrease as negative feedback either from excess of substrate or damage induced by oxidative modification (Monteiro et al., 2006). Reduction in GPx prevents the formation of radical intermediates by oxygen reduction mechanisms (Cheung et al., 2001). Inhibition of GPx activity might reflect a failure of the antioxidant system in tissue of fish exposed to pesticides (Ballesteros et al., 2009). The exposure to pesticides can elicit pro-oxidant conditions that trigger adaptive responses such as increases in the activity of antioxidant enzymes. GPx activity in the

tissue was a significantly lower than controls in the present study. The reduction could be related to O_2^- production or to the direct action of pesticides on enzyme synthesis (Bainy et al., 1993). Li et al. (2011) observed decreasing of GPx activity in brain of rainbow trout (*Oncorhynchus mykiss*) after exposure of fungicide propiconazol. Ballesteros et al. (2009) observed the inhibition of GPx activity in gill, intestine, liver, and muscle of fish exposed to 1.4 mg l^{-1} endosulfan for 24 h. GPx activity is likely to be influenced by GSH level and GR activity, which regulates the level of GSH (Cheung et al., 2004).

Glutathione reductase activity functions to maintain the cytosolic concentration of reduced glutathione. The induction of GR activity is a potential biochemical marker of oxidative stress (Cazenave et al., 2006). In the current study, throughout the experimental period no alteration in GR activity was observed. Velisek et al. (2011) found change activity of GR in tissuess of common carp after long-term exposure to terbutryn. Generally, elevated GR activity could reflect the oxidation of reduced glutathione, which is converted to glutathione, the substrate of GR activity (Elia et al., 2006). Decreased GR activity may lead to GSH depletion if its loss cannot be compensated for by the synthesis of new glutathione molecules (Zhang et al., 2004).

Reduced glutathione is not an enzymatic scavenger of ROS, but acts as a substrate or cofactor for some enzyme reactions of the glutathione-dependent enzymes (Elia et al., 2006). Reduced glutathione also acts as a reactant in conjunction with electrophilic substances. Thus, a change in GSH levels may be an important indicator of the detoxification ability of an organism (Cheung et al., 2001). Reduced glutathione is a tripeptide g-L-glutamyl-L-cysteinyl-L-glycine which represents the major non-protein thiol in the body. This molecule is found in large quantities in organs exposed to toxins such as the kidney, liver, lung, and intestine. In the cell, GSH plays a role in protein synthesis, amino acid transport, DNA synthesis, and, more generally, in cellular detoxification. It is involved in the conversion of H_2O_2 to water and in the reduction of lipid hydroperoxides (Gate et al., 1999). Thiol content offers a first line of defense against oxidative stress in living organisms (Elia et al., 2006). Reduced glutathione serves as a cofactor for glutathione transferase, which facilitates the removal of certain chemicals and other reactive molecules from the cells. It can also interact directly with certain ROS (e.g., hydroxyl radical) for their detoxification as well as perform other critical activities in the cell. Its depletion can result in cell degeneration due to oxidative stress caused by pollutants (Zhang et al., 2008). A low level of oxidative stress may induce increase of GSH synthesis and detoxifying enzyme activity, while severe oxidative stress may cause the oxidation of reduced glutathione to glutathione and a reduction in antioxidant enzyme levels (Elia et al., 2006). Similar changed activity of GSH in liver and brain has also been reported by other authors. Zhang et al. (2004) studied GSH activity in goldfish (*Carassius auratus*) exposed to 2,4-dichlorophenol for 40 days. The study found high GSH levels in liver, indicating an adaptive and protective role of GSH against oxidative stress induced by chemical contaminants. They reported that during moderate oxidative stress, GSH levels in fish liver can increase through increased synthesis as an adaptive mechanism. Yonar and Sakin (2011) observed decrease GSH in liver and gill, and increase GSH they in kidney in carp after exposure daltametrin. Elia et al. (2002) observed increased antioxidant enzyme activity of GSH and decreased GPx activity in liver of bluegill sunfish after exposure to atrazine in concentrations of 6 and 9 mg l^{-1}. Oropesa et al. (2009) reported increased GSH content in fish after acute exposure to simazine. These results support the hypothesis that triazines induce oxidative stress in the tissue of fish.

Fish oxidative stress, as well as it is antioxidant potential, differs in relation to species, habitat, and feeding behavior (Ahmad et al., 2004). These differences indicate tissue specificity, which is a consequence of metabolic and antioxidative differences (Ognjanivic et al., 2008). Alterations in activity of antioxidants can be induced as a compensatory response by slight oxidative stress; however, severe oxidative stress suppresses activity of these enzymes due to oxidative damage and a loss in compensatory mechanisms (Zhang et al., 2004).

5. Conclusions

The present work demonstrated changes in oxidative stress indices, antioxidant defense systems, and enzyme activity in tissues of common carp after chronic exposure to simazine. During the first 14 days, all parameters measured remained at control values with low concentration exposures. After 28 and 60 days, ROS production overwhelmed the antioxidant defense balance, resulting in the accumulation of oxidants and severe inhibition of the antioxidant system. The parameters measured could provide useful information for evaluating the toxicological effects of simazine on fish, but needs more detailed investigation before these findings can be used to monitor the aquatic environment for pesticide pollution. Responses varied among organs, with liver being the most sensitive tissue. Potentially could be fish liver used as indicators for monitoring residual simazine present in the aquatic environment. Mechanisms of these physiological responses in fish are not clear, and need to be further studied.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was supported by the centre CENAQUA No. CZ.1.05/2.1.00/01.0024, Project No. USB (GAJU) No. 047/2010/Z.

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CHAPTER 8

EFFECT OF CHRONIC EXPOSURE TO PROMETRYNE ON OXIDATIVE STRESS AND ANTI-OXIDANT RESPONSE IN RED SWAMP CRAYFISH (*PROCAMBARUS CLARKII*)

Stara, A., Kouba, A., Velisek, J., 2014. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in red swamp crayfish (*Procambarus clarkii*). BioMed Research International, Article ID 680131.

It was allowed by publisher on 27th April, 2014 to include the paper in this Ph.D. thesis.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 680131, 6 pages
http://dx.doi.org/10.1155/2014/680131

Research Article

Effect of Chronic Exposure to Prometryne on Oxidative Stress and Antioxidant Response in Red Swamp Crayfish (*Procambarus clarkii*)

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Received 5 December 2013; Revised 14 February 2014; Accepted 14 February 2014; Published 18 March 2014

Academic Editor: Zdenka Svobodova

The aim of the study was to investigate effects of the triazine herbicide prometryne on red swamp crayfish on the basis of oxidative stress, antioxidant indices in hepatopancreas and muscle, and histopathology of hepatopancreas. Crayfish were exposed to prometryne concentrations of 0.51 μg L^{-1}, 0.144 mg L^{-1}, and 1.144 mg L^{-1} for 11 and 25 days. Indices of oxidative stress (thiobarbituric acid reactive substances (TBARS)), and antioxidant parameters (superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR)) in crayfish muscle and hepatopancreas were measured. Chronic exposure to prometryne did not showed the impact of oxidative damage to cells. Changes activity of the antioxidant enzymes SOD, CAT, and GR were observed in all tested concentrations to prometryne for 11 and 25 days ($P < 0.01$) as compared with the control group. We did not see any differences in histopatological examination to hepatopancreas. Prolonged exposure of prometryne did not result in oxidative damage to cell lipids and proteins, but it led to changes in antioxidant activity in crayfish tissues. Changes in antioxidant systems were also observed in the environmental prometryne concentration of 0.51 μg L^{-1}. The results suggest that antioxidant responses may have potential as biomarkers for monitoring residual triazine herbicides in aquatic environments.

1. Introduction

The increasing worldwide contamination of surface and groundwater systems with thousands of industrial and natural chemical compounds is one of the key environmental problems today [1]. Pesticides are one of the main components contributing to the pollution of aquatic ecosystems. Pesticides are used to control the live organisms in agriculture. However, there is compelling evidence that agricultural pesticide use has a major impact on water quality as it can cause extensive pollution of drinking water, such as rivers, lakes, and estuaries, and together they are affecting also nontarget aquatic organisms [2–4].

Prometryne, 2,4-bis(isopropylamino)-6-methylthio-s-triazine, a selective herbicide of the s-triazine family, has been used as a pre- or postemergence controller of annual grasses and broadleaf weeds in a variety of crops [5, 6]. Prometryne is an inhibitor of photosynthesis by inhibiting electron transport in targeted broad-leaved plants and grasses [7]. Because of its poor absorption in the soil, it is easily breaking down through this matrix and contaminates the groundwater [8].

Although prometryne has been banned in Europe since 2004 [9], it still can be found in surface and ground waters. Prometryne was reported in groundwater of Greece at concentration exceeding 1 μg L^{-1} [10] and in surface water it has been reported at concentrations from 0.190 to 4.40 μg L^{-1} [11]. In surface water of Western France, remains of prometryne were detected at concentrations from 0.1 to 0.44 μg L^{-1} [12]. In case of the Czech rivers, the maximum environmental concentration of prometryne 0.51 μg L^{-1} was detected [13]. But nevertheless prometryne is still being widely used in

China [9], Australia, Canada, New Zealand, South Africa, and the United States [14].

Prometryne poses an acute risk to nontarget aquatic organisms. Based on acute toxicity tests, it is slightly to moderately toxic to aquatic plants, zooplankton, and fish. Five-day EC50 of prometryne is 0.023 mg L^{-1} for algae *Selenastrum capricornutum* [5], 24hEC50 is 0.083 μmol L^{-1} for common duckweed (*Lemna minor*), and 24hLC50 is 0.071 μmol L^{-1} for bearded stonewort (*Chara canescens*) [15]. For water flea (*Daphnia pulex*), the average toxicity to prometryne is 96hLC50 40 μg L^{-1} [5]. Acute toxicity (96hLC50) in rainbow trout (*Oncorhynchus mykiss*) is 2.9 mg L^{-1} [16] and for common carp (*Cyprinus carpio*) is 8 mg L^{-1} [17]. Effect of chronic toxicity to prometryne on nontarget aquatic organisms has not been investigated to a significant extent. Some studies are dealing with fish, for example, fathead minnows (*Pimephales promelas*) [7] and various early live stages of common carp [18–20].

The main objective of this study was to evaluate the effects of chronic toxicity to prometryne on nontarget organisms. In this study, red swamp crayfish (*Procambarus clarkii*) was selected. Crayfish are an ecologically important benthic invertebrates in the environment, and they are considered an appropriate model organisms [21, 22]. The main aim was observing antioxidant activity changes and oxidative damage in the hepatopancreas and abdominal muscle of crayfish after long-term exposure to differing prometryne concentrations. Oxidative stress and antioxidant systems are considered as good indicators of xenobiotic accumulation in fish tissue [18, 19], and on crayfish these studies were not yet performed. Also, we observed the behavior of crayfish and examinated hepatopancreas histopathologically. Furthermore, the effects of acute, subchronic, and chronic exposure of fish to triazine herbicides have been documented. But on the other hand, there is a scarcity of data on the chronic exposure to these compounds at real environmental concentrations with respect to oxidative stress and antioxidant responses in the macroinvertebrates.

2. Materials and Methods

2.1. Chemical. High-purity-certified standard of prometryne (2,4-bis(isopropylamino)-6-methylthio-s-triazine) (99.3%) was purchased from Sigma Aldrich, Czech Republic.

2.2. Experimental Animals. European native species of crayfish are endangered and thus they should be protected. For our study, we have chosen the invasive species red swamp crayfish (*Procambarus clarkii*) as a model species. Crayfish (mean carapace length 28.53 ± 1.61 mm, postorbital carapace length 23.61 ± 2.31 mm, and weight 5.9 ± 1.08 g) were held in aquaria containing 100 L of freshwater with continuous aeration, at 19–23°C and a 12L:12D cycle. Dissolved oxygen concentration measured daily was 90–98%, and pH was 7.5 ± 0.3. Aquariums were equipped with plastic shelters to deter cannibalism [23]. Crayfish were acclimatized for 10 days before the beginning of the experiment.

2.3. Experimental Protocol. The experiment was a semistatic method conducted over 25 days. Red swamp crayfish were allocated, in groups of 12, to one of three experimental regimes or to an untreated control group. The experimental fish were exposed to prometryne at the following concentrations: 0.51 μg L^{-1} (the reported environmental concentration in Czech rivers [13]), 0.144 mg L^{-1}, and 1.144 mg L^{-1}. Concentrations of 0.144 mg L^{-1} and 1.144 mg L^{-1} corresponded to 1% of the 96hLC50 and 10% of 96hLC50 for signal crayfish (*Pacifastacus leniusculus*) (the only one crayfish species examined in acute toxicity test on prometryne [24]). The experimental conditions were duplicated for a total of eight groups (n = 96). Crayfish were fed experimental diet containing fish meal 40%, wheat flour 38%, soybean flour 10%, cornstarch 10%, vitamin and mineral premix 1%, fish oil 0.5%, and rapeseed oil 0.5% at 1% body weight per day in two feeding doses. Eighty percent of the exposed solution was renewed each day 2 h after feeding to maintain water quality and the appropriate concentration of prometryne.

During the experiment, crayfish behavior and survival rate were recorded. To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by LC-MS/MS [25]. Water samples were collected from the aquaria 1 h and 24 h after renewing the test solutions. The mean concentration of prometryne in the water samples was always within 5% of the intended concentration.

2.4. Tissue Samples and Preparation of Postmitochondrial Supernatant. At the completion of each exposure period (11 and 25 days), three crayfish from each aquarium were randomly selected and killed. The hepatopancreas and abdominal muscle were quickly removed, immediately frozen, and stored at −80°C for analysis. Frozen tissue samples were weighed and homogenized (1 : 10, w/v) with an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one to measure thiobarbituric acid reactive substances (TBARS) and the other, centrifuged at 12 000 g for 30 min at 4°C, to obtain the postmitochondrial supernatant for other antioxidant parameter analyses.

2.5. Indices of Oxidative Stress and Antioxidant Parameters. The TBARS method described by Lushchak et al. [26] was used to evaluate lipid peroxidation. The superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of S. Marklund and G. Marklund [27]. This assay depends on the autoxidation of pyrogallol. Superoxide dismutase activity was assessed spectrophotometrically with a spectrophotometer Tecan (Infinite M200 Tecan, Switzerland) at 420 nm and expressed as the amount nmoles NBT per milligram of protein. The catalase (CAT; EC 1.11.1.6) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer [28]. Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [29].

Table 1: Effect of chronic exposure to prometryne on level of thiobarbituric acid reactive substances (TBARS, nmol mg^{-1} protein) in red swamp crayfish tissues.

Indices	Exposure time (days)	Test groups			
		Control	Prometryne 0.51 $\mu g\,L^{-1}$	Prometryne 144 $mg\,L^{-1}$	Prometryne 1.44 $mg\,L^{-1}$
Hepatopancreas	11	0.535 ± 0.04	0.515 ± 0.03	0.614 ± 0.09	0.614 ± 0.07
	25	0.521 ± 0.09	0.538 ± 0.06	0.479 ± 0.04	0.495 ± 0.06
Muscle	11	0.594 ± 0.08	0.576 ± 0.10	0.619 ± 0.12	0.712 ± 0.19
	25	0.669 ± 0.11	0.650 ± 0.13	0.704 ± 0.06	0.681 ± 0.06

Data are means ± SD, $n = 6$.

2.6. Protein Content Determination. Total protein content in each sample was determined spectrophotometrically according to the method of Bradford [30] using bovine serum albumin as a standard.

2.7. Histopathology. Histopathological examination of crayfish hepatopancreas was performed at the end of toxicity test, after 25 days. The taken samples of hepatopancreas were immediately fixed in 10% formalin, drained, and embedded in paraffin. Sections were made of the paraffin blocks, stained with hematoxylin-eosin, examined by light microscopy, and photographed using a digital camera.

2.8. Statistical Analysis. The statistical software program STATISTICA (version 8.0 for Windows, StatSoft) 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 these conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. When a significant difference was detected ($P < 0.01$), Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal-Wallis) was used [31].

3. Results and Discussion

Laboratory studies of oxidative stress, antioxidant enzymes, and histopathological responses in tissues of crayfish exposed to herbicides can provide information on and help to elucidate the mechanisms of the impact of residual herbicides on crayfish. The results of this study provide further data on long-term exposure to prometryne for consideration in risk assessment. The findings contribute to knowledge of the toxic potential of prometryne to red swamp crayfish at actual concentrations in Czech rivers.

3.1. Crayfish Behavior during the Experiment. During the experiment, both control and exposed to prometryne crayfish showed normal feeding behavior. There were no signs of respiratory distress, increased rate of movement activities, or floating. To our knowledge, no other data on behavior of crayfish after triazine exposure are available in the literature. Buric et al. [22] reported excited behavior (fast and chaotic movement outside the shelters, specimens attempting to "escape") in crayfish following acute poisoning with diazinon (0.3–0.5 $\mu g\,L^{-1}$). In the acute course of triazines poisoning in fish, the following clinical symptoms are typical: accelerated respiration, loss of movement coordination, and alternating the resting phase to the excitation phase, and at the end, they fall into damp and move mainly on their flanks, their respiration slowed down, and the damp phase and subsequent agony are very long [32]. In chronic toxicity tests, any changes in fish behavior have not been shown [33], and also in the test on prometryne [18, 19], like in our tests on crayfish.

3.2. Indices of Oxidative Stress and Antioxidant Parameters. The damage of tissues and cellular components is the result of oxidative stress, due to the oxygen-free radicals and other reactive oxygen species [34]. The use of antioxidant defense enzyme activities before oxidative damage in aquatic organisms as molecular biomarkers of environmental pollution has been proved to be of great diagnostic value [35]. The effect of chronic exposure to prometryne on TBARS level in red swamp crayfish tissues is shown in Table 1. In this study, any significant differences in lipid peroxidation level among exposed groups were not observed.

Oxidative substances in cells may lead to an elevation of antioxidant enzymes as a defense mechanism. Induction of antioxidant enzymes is an important line of defense against oxidative stress in biological systems [36]. The organisms have their own cellular antioxidative defense system, composed of both enzymatic and nonenzymatic components [37]. Antioxidant defense system is mainly constituted of superoxide dismutase, catalase, and glutathione dependent enzymes (glutathione peroxidase, glutathione reductase, and glutathione-S-transferase) [38]. The SOD is a group of metalloenzymes that play a crucial role as antioxidants and constitute the primary defense system against the toxic effects of superoxide radicals (O_2^-) in organisms [39, 40]. Significant differences from the control value ($P < 0.01$) in the SOD activities were seen at all exposure durations to prometryne in hepatopancreas after exposure of 11 days (Table 2). In hepatopancreas, we observed significantly decreased SOD activities in concentrations to prometryne 0.144 and 1.44 $mg\,L^{-1}$ compared to control group at the end of the test. The SOD activity was observed significantly to be lower in muscle in concentration of prometryne 0.144 and 1.44 $mg\,L^{-1}$ compared with control group after 25 days. The CAT is an enzyme

Table 2: Effect of chronic exposure to prometryne on superoxide dismutase (SOD, nmol NBT min^{-1} mg^{-1} protein) activity in red swamp crayfish tissues.

Indices	Exposure time (days)	Test groups			
		Control	Prometryne 0.51 $\mu g\,L^{-1}$	Prometryne 0.144 $mg\,L^{-1}$	Prometryne 1.44 $mg\,L^{-1}$
Hepatopancreas	11	0.216 ± 0.01	0.111 ± 0.05*	0.126 ± 0.02*	0.132 ± 0.04*
	25	0.164 ± 0.04	0.155 ± 0.04	0.086 ± 0.02*	0.076 ± 0.02*
Muscle	11	0.075 ± 0.04	0.057 ± 0.03	0.093 ± 0.06	0.098 ± 0.17
	25	0.330 ± 0.01	0.043 ± 0.02	0.008 ± 0.00*	0.010 ± 0.00*

Data are means ± SD, $n = 6$. Significant differences compared with control value, * <0.01.

Table 3: Effect of chronic exposure to prometryne on catalase (CAT, μmol H_2O_2 min^{-1} mg^{-1} protein) activity in red swamp crayfish tissues.

Indices	Exposure time (days)	Test groups			
		Control	Prometryne 0.51 $\mu g\,L^{-1}$	Prometryne 0.144 $mg\,L^{-1}$	Prometryne 1.44 $mg\,L^{-1}$
Hepatopancreas	11	4.018 ± 0.78	4.098 ± 0.59	1.695 ± 1.10*	1.065 ± 0.84*
	25	3.953 ± 1.06	2.073 ± 0.69*	1.515 ± 0.60*	0.834 ± 0.40*
Muscle	11	0.121 ± 0.06	0.105 ± 0.04	0.106 ± 0.04	0.112 ± 0.07
	25	0.150 ± 0.03	0.149 ± 0.02	0.116 ± 0.05	0.146 ± 0.04

Data are means ± SD, $n = 6$. Significant differences compared with control value, * <0.01.

which facilitates the removal of hydrogen peroxide (H_2O_2), onto molecular oxygen and water [41]. The CAT activity in our test was significantly ($P < 0.01$) decreased in the groups tested, 0.144 and 1.44 $mg\,L^{-1}$ prometryne at 11 days, and in all tested groups after 25 days of exposure (Table 3). Glutathione reductase (GR) plays an essential role in cell defense against reactive oxygen metabolites. GR maintains the reduced status of glutathione, whose reducing power is also necessary for gluthatione peroxidase activity and so GR keeps of overall homeostatic oxido-reductive balance in any living cell [42]. The GR activity was observed significantly ($P < 0.01$) to be lower in hepatopancreas and higher in muscle after 25 days of all exposed groups compared to the control group (Table 4).

These oxidative stress and antioxidant enzymes changes have not yet been well documented in crayfish, but there are a number of studies on the effect of triazine herbicides on fish. Stara et al. [19] investigated changes in antioxidant systems against oxidative damage of tissues (muscle, brain, intestine, liver, and gills) in common carp after 60-day exposure to prometryne, also in the environmental concentration of 0.51 $\mu g\,L^{-1}$. Jin et al. [43] examine the effects of atrazine on zebra fish (*Danio rerio*) for 14 days. Fish have increased SOD activity (at the concentrations of 0.1 and 1 $mg\,L^{-1}$ atrazine) and CAT (in the concentration of 1 $mg\,L^{-1}$ of atrazine) in the liver. A similar result was demonstrated in an experiment to terbutryn in tissues common carp after 90 days. Velisek et al. [44] observed significant higher SOD activities in brain and liver, and the GR activity was lower in liver and intestine, and higher in muscle.

In summary, in this study, it has been found that the antioxidant enzymes (SOD, CAT, and GR) maintain a balance in cells and protect them from oxidative damage tissues (hepatopancreas, muscle) of crayfish after chronic exposure of the triazine herbicide prometryne after 11 and 25 days, it also in environmental concentration 0.51 $\mu g\,L^{-1}$. Thus, we could suggest that some antioxidants changes in crayfish tissues, which were contaminated by prometryne, could occur as an adaptive mechanism to this stressful situation. At the same time, we must not forget that the antioxidant defense is involved a number of other enzymes, and oxidative stress may influence other factors such as species, habitat, and the feeding behavior.

3.3. Histopathological Examination. No histopathological changes were demonstrated in hepatopancreas of red swamp crayfish following chronic exposure to prometryne at all tested concentrations. For the crayfish, the effect of chronic exposure to pesticides at low concentrations on histopathological changes in tissues has not yet been studied (monitored). Some studies describe histopathological changes in crayfish tissues after acute exposure to some pesticides. For example, Desouky et al. [45] reported extensive ultrastructural alterations to hepatopancreas epithelial cells, the most notable pathological features including vacuolation, degradation, and distinct cell lysis in red swamp crayfish following acute exposure with insecticide ethion (0.36 $mg\,L^{-1}$). However, the long-term effect of triazine herbicides is well documented on fish. Study on the common carp was most prominent in prometryne concentrations of 8 and 80 $\mu g\,L^{-1}$ after 60 days of treatment, when were observed histopathological changes severe hyaline degeneration of the epithelial cells of caudal kidney tubules in fish [24].

4. Conclusion

To our knowledge, this is the first report of the effects of triazine herbicide study, respectively prometryne with using oxidative parameter and antioxidants biomarkers on the

Table 4: Effect of chronic exposure to prometryne on glutathione reductase (GR, nmol NADPH min^{-1} mg^{-1} protein) activity in red swamp crayfish tissues.

Indices	Exposure time (days)	Test groups			
		Control	Prometryne 0.51 $\mu g\ L^{-1}$	Prometryne 0.144 $mg\ L^{-1}$	Prometryne 1.44 $mg\ L^{-1}$
Hepatopancreas	11	1.250 ± 1.47	0.300 ± 0.19	0.272 ± 0.17	0.249 ± 0.11
	25	1.221 ± 0.58	0.489 ± 0.31*	0.418 ± 0.11*	0.314 ± 0.17*
Muscle	11	0.308 ± 0.23	0.337 ± 0.11	0.745 ± 0.42	0.623 ± 0.13
	25	0.307 ± 0.18	0.608 ± 0.19*	0.646 ± 0.16*	0.660 ± 0.07*

Data are means ± SD, $n = 6$. Significant differences compared with control value, * <0.01.

crayfish. Crayfish was used as one with sensitive nontarget aquatic organisms exposed to various pollutants in the environment. Moreover, during the study behavior of crayfish was monitored and the histopathological examination was performed in the hepatopancreas at the end of the test. The information presented in this study will be helpful in fully understanding the mechanism of effect of prometryne on the crayfish.

Conflict of Interests

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

Acknowledgments

This research was supported by the Projects CENAKVA CZ.1.05/2.1.00/01.0024, the project CENAKVA II (the results of the Project LO1205 were obtained with a financial support from the MEYS of the CR under the NPU I program), and the Grant Agency of the University of South Bohemia (087/2013/Z).

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CHAPTER 9

GENERAL DISCUSSION

The widespread and indiscriminate use of pesticides is of great public health concern, as humans are constantly exposed to the chemicals both directly (during the manufacture, application, and sales) and indirectly as residues in crops or animal tissue. Therefore fish are good animal models for the study of aquatic contamination, since altered water chemistry affects not only their behaviour and histology but also their physiology and biochemical processes (Oluah and Chineke, 2014). However, it is important to monitor these effects in other aquatic organisms to obtain a comprehensive view of the effects of xenobiotics on the entire aquatic ecosystem.

Behavioural responses of fish and crayfish

The changes observed in the behaviour of organisms are one of the first signs of toxic effects chemicals. In order to make an accurate assessment of the hazards that a contaminant may pose to a natural system, behavioural indices selected for monitoring must reflect the organism's behaviour in the field (Velisek et al., 2011b). Robinson (2009) defines behaviour as a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment. Nevertheless, behavioural endpoints serve as valuable tools to identify and evaluate effects of exposure to environmental stressors/pollutants, and alterations in behaviour can provide important indices for ecosystem assessment. Generally, fish exposed to high concentrations of chemicals show atypical behaviours such as erratic swimming, sudden rapid movements, and restlessness (Ladipo et al., 2011). Acute toxic effects of triazines on behaviour of fish includes erratic swimming patterns (whirling movement) and had lost their ability to react, they become lethargy, lying on the bottom of the aquaria with intermittent periods of swimming (Mukhi et al., 2005), followed by death (Velisek et al., 2008). In our studies, common carp (Velisek et al., 2011a; Stara et al., 2012a, 2013) and crayfish (Stara et al., 2014) exposed to low concentrations of triazines in chronic tests showed normal feeding habits and exhibited no abnormal behaviour, and no mortalities were observed. Similar results were obtained in tests of early life stages of common carp after chronic exposure to triazines (Velisek et al., 2012a, b; Stara et al., 2012b). In our studies, triazines at environmentally realistic levels were used for long exposure periods. We can assume that the tested organisms adapted to low doses of pesticides, as no typical symptoms of acute triazine poisoning were seen.

Biometric parameters (length and weight- especially on fish early life stages)

Biometric parameters are regarded as general indicators of fish health and the quality of the aquatic environment. We observed important differences in biometric parameters in tests on early life stages of carp at the higher tested concentrations of terbutryn and simazine at the longer exposure times (Velisek et al., 2012a,b). The hepatosomatic index (HSI) is a non-specific biomarker influenced by factors such sex, season, disease, and nutrition (Velisek et al., 2011b). Toxicity tests in embryos, larvae, and juveniles are commonly used to predict environmental damage, as early life cycle stages are usually more sensitive to environmental impacts than are subsequent stages. Adult animals are not as sensitive as juveniles, and effects are not so readily detectible (Kristensen, 1994). Studies of the embryonic development of fishes are important, not only to increase knowledge of the developmental processes, but also to understand species-specific adaptations and their ecological value in the course of speciation (Meijide and Guerrero, 2000).

Biochemical blood plasma parameters

Biochemical profiles of blood can provide important information about the internal environment of the organism. Changes in biochemical parameters can be observed both after acute and long-term exposure to contaminants. In our study, chronic exposure to terbutryn at the highest tested concentration showed important data of biochemical changes in glucose (GLU), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine (CREA), lactate (LACT), and magnesium (Mg) in blood plasma of common carp (Velisek et al., 2011a). Increased levels of glucose may be the result of metabolic stress resulting from exposure to the pesticide (Simon et al., 1983; Li et al., 2010). Li et al. (2011) observed a lower level of LACT in plasma of fish exposed to pesticides, indicating a decrease in the glycolytic process associated with a lower metabolic rate and increasing plasma protein content. This may indicate physiological adaption to combat stress. Activity of plasma enzymes LDH and AST is a relevant stress indicator (Svoboda, 2001; Ishikawa et al., 2007). A significant increase in LDH and AST activity indicates stress-related tissue impairment (Svoboda et al., 2001). Lactate dehydrogenase is a tetrameric enzyme of anaerobic glycolysis recognized as a potential marker for assessing the toxicity of a chemical. Therefore LDH is of crucial to muscle physiology, particularly under conditions of chemical stress when high levels of energy may be required over a short period of time (Monteiro et al., 2007). The increase in LDH level could indicate tissue damage, hypoxic conditions, and a switch to anaerobic metabolism. Mishra and Shukla (2003) suggested that elevated levels of LDH in the haemolymph might be due to the release of isozymes from damaged tissue. Increasing activity of AST indicates amplified transamination processes and compensation for the energy crisis during pesticide stress (Philip et al., 1995). Exposure of carp for 28 days to simazine at 4, 20, and 50 $\mu g.l^{-1}$ was found to increase plasma ammonia (NH_3), creatine kinase (CK), GLU, LDH and CREA levels, and reduce AST activity (Velisek et al, 2009). Velisek et al. (2013) observed similar results as those found in our study on the biochemical blood plasma profile of GLU, AST, alanine aminotransferase (ALT), CREA, LACT, Ca^{2+} (calcium), Mg, and inorganic phosphate (PHOS) with 60 days exposure of carp to prometryne. They found differences in biochemical parameters with exposure to environmental concentrations of prometryne (0.51 $\mu g.l^{-1}$). Ninety-day exposure of carp to simazine at concentrations of 0.06, 2, 3, and 4 $\mu g.l^{-1}$ resulted in reduction of TP (total protein), ALB (albumin), ALT and alkaline phosphatase (ALP) in blood (Velisek et al., 2012c). Changes in biochemical profiles confirm the toxic effects of triazine chronic exposure.

Haematological parameters

Haematological indices, similar to the biochemical profile, are important parameters for the assessment of the fish physiological state. Whereas, their changes depend on the fish species, age, the phase of sexual maturity, and health (Vazquez and Guerrero, 2007). In Velisek et al. (2011a), we investigated the effect of long-term exposure to terbutryn (0.2 and 2 $\mu g.l^{-1}$) on haematological parameters in carp. After 90 days exposure, decrease was observed in mean corpuscular volume (MCV), leukocyte and lymphocyte count, and increase in red blood cell count (RBC), mean corpuscular haemoglobin concentration (MCHC), and neutrophil granulocyte bands. Erythrocytes are the dominant cell type in the blood of the vast majority of fish species. Fish, along with other vertebrates, have a common leucocyte pattern consisting of granulocytes, monocytes, lymphocytes, and thrombocytes (Vazquez and Guerrero, 2007). Decreases in RBC count may be an indicator of anaemia, and changes in leukocyte count are recognized as a sensitive indicator of environmental stress (Cole et al., 2001). Leukocytes are involved in the regulation of immunological function and a protective response to stress in

fish (Velisek et al., 2011b). The reduction in leukocyte count occurs through an alteration in lymphopoesis or altered release of lymphocytes from lymphoid tissues. Velisek et al. (2012c) observed decrease in leukocyte count in carp blood after chronic exposure to simazine. The study indicated a state of stress in the fish subsequent to simazine exposure, which may have produced hypoxia and kidney damage. Nevertheless, the decrease in leukocyte count and the lymphopenia in carp exposed to triazines leads to a reduction in non-specific immunity. Haematological changes may result from the release of immature erythrocytes from the spleen and could be an immediate response to acute stress mediated by catecholamines (Velisek et al., 2011b). However, the haematological changes can represent a compensatory response that increases the O_2 carrying capacity to maintain adequate gas transfer and a change in the water blood barrier for gas exchange in gill lamellae (Sweilum, 2006).

Histopathology

Fish histopathology is increasingly being used to indicate environmental stress, since it provides a definite biological end-point of exposure to pollutants. Histology can be one of the tools in evaluating the effects of herbicides on fish (Stentiford et al., 2003). Our studies report the histopathological alterations observed in selected organs and tissues of early life stages carp after acute exposure to triazines (Velisek et al., 2012a,b). The majority of histological changes were observed in caudal kidney. Fish exposed of simazine and terbutryn showed alteration of the tubular system including destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia, and disintegration of glomeruli. Additionally, terbutryn exposure resulted in diffused steatosis in liver associated with the loss of cellular shape and presence of lipid inclusions in hepatic cells (Velisek et al., 2012b). Kidney and liver perform important functions in implementing immune defence and the maintenance of a stable internal environment.

Wang et al. (2013) studied 40 days exposure to atrazine in common carp. They found altered structure of the spleen and head kidney, as demonstrated by RBC loss in the splenic sinus, splenic cord broadening, RBC haemolysis in the spleen and cortex broadening, RBC and achroacyte loss, and cortex fibrosis in the head kidney. After a 20 day recovery period, the pathological symptoms were improved to some degree. Nevertheless, atrazine exposure was associated with a series of lesions causing severe damage to the fish immune organs that would ultimately have an impact on immune function and physiological activity, thereby inducing disease and possibly death. Velisek et al. (2008, 2009) observed similar pathological changes after exposure to triazines in carp. However, after acute exposure to metribuzin (96hLC50) pathological changes in gill were identified. Histological examination revealed mild proliferation of goblet cells of the respiratory epithelium of secondary gill lamellae and hyaline degeneration of epithelial cells of the renal tubules of caudal kidney. This alteration of kidney resulted in hypoproteinaemia, followed by the formation of transudate in the body cavity (Velisek et al., 2008). The reported studies confirm that histopathology in fish can be induced by environmental contaminants, and that these represent an ecologically relevant biological endpoint of exposure to pollution. Although pathological changes in fish are reliable indicators of environmental stress, studies dealing with the effects of pesticides on fish are rare.

Oxidative stress and antioxidant responses in fish and crayfish exposed to triazines

Contaminants such as the triazines are capable of causing oxidative damage within an organism. In response to oxidative stress, there may be adaptive responses of the protective antioxidant protective systems (GSH – reduced glutathione, GSSG – oxidized glutathione,

CAT – catalase, SOD – superoxide dismutase, GR – glutathione reductase, GPx – glutathione peroxidase, etc.). Modification of cellular macromolecules can serve as bio-indicators of a range of contaminant exposure. Macromolecules that may be affected include lipids, proteins, and nucleic acids (Hofman et al., 2002). Lipid peroxidation and the formation of reactive oxygen species induces a range of effects in fish. Activity of antioxidant enzymes appears to be a reliable indicator of pollutant-mediated oxidative stress (Pavlovic et al., 2010). If the antioxidant system does not sufficiently remove potent oxidants, oxidative stress results (Sevgiler et al., 2004).

In my studies, I found changes in antioxidant systems (SOD, CAT, GR, GPx) for the longer exposure times of the three selected triazines at environmentally realistic exposures. Antioxidant defence mechanisms against oxidative damage were observed in the brain, gill, muscle, liver, and intestine of carp (Velisek et al., 2011a; Stara et al., 2012a, 2013), as well as the hepatopancreas and muscle of crayfish (Stara et al., 2014). Antioxidant response against oxidative damage to tissues after chronic exposure to prometryne was detected in the homogenate of the early life stages of carp (Stara et al., 2012b). However, oxidative damage to brain, muscle, and liver, as indicated by increased production of reactive oxygen species (ROS) was observed only after chronic exposure of carp to simazine (Stara et al., 2012a).

Many studies examining the oxidative response in fish tissue have been conducted on atrazine. Chromcova et al. (2013) tested exposure to atrazine at concentrations of 0.3, 30, 100, and 300 $\mu g.l^{-1}$ on early life stages of carp to 33 days post-hatching. Blahova et al. (2013) investigated whether exposure to atrazine at sublethal concentrations of 0.3, 3, 30, and 90 $\mu g.l^{-1}$ for 28 days induced oxidative stress and changes in the detoxification system in juvenile zebrafish. Al-Sawafi and Yan (2013) exposed zebrafish to atrazine (0.636 and 0.957 $mg.l^{-1}$) for 25 days and reported alterations in acetyl cholinesterase (AChE) level and the antioxidant enzymes CAT and SOD in brain. Nwani et al. (2010) evaluated toxicity of atrazine (4.2, 5.2, and 10.6 $mg.l^{-1}$) to the antioxidant system of the green snakehead (*Channa punctatus*) over a 15 day exposure period. These studies, as did the results of our studies on carp and crayfish, confirmed the influence of triazines on antioxidant defences against oxidative cell damage.

However, it is necessary to conduct further studies of the long-term impact of environmental triazine contamination on non-target aquatic organisms, particularly those typical of freshwater ecosystems of Central Europe. Simultaneously, antioxidant defence enzymes and oxidative damage appear to be reliable bio-indicators of toxicity to non-target species (Hostovsky et al., 2012; Stara et al., 2012a,b, 2013, 2014; Xing et al., 2014).

Conclusion

Fish and crayfish are widely used as biological monitors of environmental levels of anthropogenic pollutants. The present thesis is a contribution to the assessment of the toxicity and effects of long-term effect of triazines on the different developmental stages of common carp (*Cyprinus carpio* L.) and adult red swamp crayfish (*Procambarus clarkii*). The carp was selected as a model fish due to its economic importance, e.g. carp farming contributes about 90% to total fish production in the Czech Republic. Crayfish are easily identified species representing given locality, they are widespread, and they provide a sufficient amount of tissue for individual biochemical and chemical analyses.

The results of these studies provide further data on chronic exposure to triazines for consideration in risk assessment. We selected three active substances of triazine herbicides which are the most frequently detected in surface waters such as prometryne, simazine and terbutryn. The findings contribute to knowledge of the toxic potential of triazine herbicides to carp and crayfish at environmentally relevant concentrations in Czech rivers. There is a scarcity

of information regarding the toxicity of triazines on freshwater organisms. During the tests we monitored several parameters: behaviour, mortality, biometric, haematological, biochemical blood, histopathology, oxidative stress and antioxidants. The data obtained from all tests performed during my thesis are very valuable for assessment and evaluation of long-term effects xenobiotics on aquatic organisms, especially fish and crayfish.

In the future, I would extend the focus of my research for study of possible synergic or amplifying effect of mixtures triazines with other xenobiotics which are often found in the aquatic environment. This approach is recently accented as the aquatic environment is polluted by mixtures of different compounds. Therefore, more research is needed in order to clarify the more detailed effects of xenobiotics on non-target aquatic organisms.

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ENGLISH SUMMARY

The effect of triazine based pesticides on fish

Alžběta Stará

Aquatic environment pollution by pesticides and other anthropogenic substances is one of the major global problems - nowadays. Water is a basic component of all living organisms on the earth and its quality reflects on the ecological value and maintains the balance of aquatic ecosystems. Contamination of water by pesticides may lead directly or indirectly to reduction in productivity, impaired immunity and increased mortality. At the same time we must not forget that the incidence and elevated levels of undesirable chemicals in the aquatic environment, and subsequently in fish and other aquatic organisms, may adversely affect human health. Because, pesticides and their metabolites are still detected in drinking water and food.

Therefore, the main goal of our thesis was to observe the impact of pesticides on non-target aquatic organisms. For the majority of studies, we chose the common carp (*Cyprinus carpio* L.). Carp is one of the most famous and the most common fish of our pond and is also one of most frequently eaten fish by us. In addition, we have chosen as a model species red crayfish (*Procambarus clarkii*). Crayfishs are ecologically important organisms and they are considered as a good indicator of environmental pollution in both standing and flowing waters. In selected organisms, we observed long-term effect of triazine herbicides (prometryne, simazine, terbutryn) in concentrations commonly occurring in Czech rivers. Effects of triazines have been assessed through observation of behavior, histopathological examination and antioxidant parameters and oxidative tissue damage on fish and crayfish. In case of carp were also monitored biometric, haematological and biochemical parameters.

The first part is about monitoring the long-term effects of triazines on the early development stages of carp. We found that chronic exposure to simazine affected growth and led to the histological changes in early developmental stages of carp. However, most of changes were observed only at higher concentrations of simazine (600 and 3000 $\mu g.l^{-1}$), at these concentrations, a reduction of weight and total body length of carp was found. At concentrations simazine of 60, 600 and 3000 $\mu g.l^{-1}$ histopathological changes in the caudal kidney were observed. Chronic exposure to terbutryn on early-life stages of common carp affected their survival, growth rate, early ontogeny, and histology. Some of these changes were observed only at higher exposures (0.02, 0.2, and 2 $mg.l^{-1}$), but growth rate was affected in fish exposed to the lowest tested concentration (0.02 $\mu g.l^{-1}$), which is that reported in Czech rivers in recent years. Chronic exposure of early life stages of carp to prometryne showed no effect on growth and mortality rates. Levels of oxidative damage in fish test groups showed no significant differences from the controls. Glutathione reductase activity at exposure to 0.51 $\mu g.l^{-1}$ was significantly increase ($p < 0.01$) compared to the controls and other exposures.

Another part of the work has included long-term effects of low concentrations of triazines on carp. We observed the effects of terbutryn at concentrations of 0.02 (reported concentration in Czech rivers), 0.2, and 2.0 $\mu g.l^{-1}$, this effect were assessed in one-year-old common carp exposed for 90 days. Exposure to 0.02, 0.2 and 2.0 $\mu g.l^{-1}$ showed significant differences in the oxidative damage to tissue (brain, liver) and in antioxidative biomarkers activity (SOD, CAT, GR, CP, GPx) in tissues. In exposure to 0.2 and 2.0 $\mu g.l^{-1}$ significantly affected biochemical (GLU, AST, LDH, CREA, LACT, Mg) and haematological (RBC, MCV, Leuko, Lymphocytes) profiles were observed. Next the effects of prometryne on oxidative stress and antioxidant status of common carp were investigated. Fish were exposed to sublethal concentrations of prometryne

(0.51, 8 and 80 μg.l^{-1}) for 60 days. Chronic exposure to prometryne was shown to alter the antioxidant status of carp. Significant changes in enzyme activity (SOD, CAT, GR) in tissues of common carp were seen at chronic exposure to prometryne, with no observed oxidative damage to the cells. Changes in SOD and GR activity were observed at commonly occurring environmental concentrations of prometryne (0.51 μg.l^{-1}). We also investigated the chronic effects of simazine on the activity of atioxidant systems and oxidative biomarkers in tissues of carp. In carp exposed to concentrations of simazine 2 and 4 mg.l^{-1} changes in the activity of antioxidant enzymes (SOD, CAT, GPx, GSH) and increased production of reactive oxygen species during 60 days exposure were detected. In the environmental concentrations of simazine 0.06 μg.l^{-1} change in the activity of CAT and GSH in the tissues of carp was detected.

The last part of our study examined the chronic effect of prometryne on red swamp crayfish. Crayfish were exposed to prometryne concentrations of 0.51 μg.l^{-1}, 0.144 mg.l^{-1}, and 1.144 mg.l^{-1} for 25 days. Prolonged exposure of prometryne did not result in oxidative damage to cell lipids and proteins, but it led to the changes in antioxidant activity (SOD, CAT, GR) in crayfish tissues. Changes in antioxidant systems were also observed in the environmental prometryne concentration of 0.51 μg.l^{-1}. We did not see any differences in histopatological examination to hepatopancreas.

These studies provided important results for the evaluation of long-term impact and influence of triazines on the water non-target organisms, even in concentrations commonly measured in surface waters. Effects of triazines were observed on carp and crayfish, by using selected parameters. They appear to be good indicators in the assessment of damage in non-target aquatic organisms and aquatic environments. However, the aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. For detailed elucidation is very important to do next study about the effect of anthropogenic substances, pesticides, on non-target organisms occurring in the aquatic environment.

CZECH SUMMARY

Vliv triazinových pesticidů na ryby

Alžběta Stará

Znečištění vodního prostředí pesticidy a jinými antropogenními látkami je v dnešní době jeden z hlavních celosvětových problémů. Voda je základní složkou všech živých organizmů žijících na Zemi a její kvalita se odráží na ekologické hodnotě a udržení rovnováhy vodních ekosystémů. Kontaminace vody pesticidy může vést přímo, nebo i nepřímo, ke snížení produktivity, oslabení imunity a zvýšení úmrtnosti ryb. Zároveň nesmíme opomenout, že výskyt a zvýšené koncentrace nežádoucích chemických látek ve vodním prostředí následně i v rybách a ostatních vodních organizmech mohou negativně ovlivnit zdraví lidí. Neboť pesticidy a jejich metabolity jsou stále zjišťovány v pitné vodě a v potravinách.

Proto bylo hlavním cílem naší práce sledovat vliv pesticidů na necílové vodní organizmy. Pro většinu studií jsme zvolili kapra obecného (*Cyprinus carpio* L.). Kapr je jednou z nejznámějších a nejběžnějších ryb českého rybníkářství a patří i mezi nejčastěji konzumované ryby v České republice. Dále jsme jako modelový druh vybrali raka červeného (*Procambarus clarkii*). Raci jsou ekologicky významné organizmy a jsou považováni za dobré indikátory při ekologickém znečištění stojatých i tekoucích vod. U zvolených organizmů jsme sledovali dlouhodobý vliv triazinových herbicidů (prometrynu, simazinu, terbutrynu) v běžně se vyskytujících koncentracích v českých řekách. Vliv triazinů na ryby a raky jsme hodnotili pomocí sledování chování, histopatologického vyšetření a antioxidačních parametrů a oxidačního poškození tkání. U kaprů byly navíc sledovány biometrické, hematologické a biochemické parametry.

První část práce se zabývala sledováním dlouhodobého vlivu triazinů na rané vývojové fáze kapra. Zjistili jsme, že chronická expozice simazinu ovlivnila růst a vedla k histologickým změnám v raných vývojových stádiích kapra. Většina změn však byla pozorována pouze při vyšších koncentracích simazinu (600 a 3 000 $\mu g.l^{-1}$), při těchto koncentracích bylo zjištěno snížení hmotnosti a celkové délky těla kaprů. V koncentracích simazinu 60, 600 a 3 000 $\mu g.l^{-1}$ byly zjištěny histopatologické změny v kaudální ledvině. Chronická expozice terbutrynu na raná vývojová stádia kapra měla vliv na jejich přežití, růst, ontogenezi a histologické změny. Některé změny byly pozorované jen u nejvyšších testovaných koncentrací (0,02, 0,2 a 2 $mg.l^{-1}$). Růst ryb byl ale ovlivněn nejnižší testovanou koncentrací 0,02 $\mu g.l^{-1}$ terbutrynu (environmentální koncentrace zjištěná v českých řekách). Chronická expozice prometrynu na raná vývojová stadia kapra neměla žádný vliv na růst, úmrtnost a oxidativní poškození ryb. Byla zjištěna pouze zvýšená aktivita glutation reduktázy ($p < 0,01$) u ryb vystavených koncentraci prometrynu 0.51 $\mu g.l^{-1}$ (reálná koncentrace prometrynu v českých řekách) ve srovnání s kontrolou a ostatními sledovanými expozicemi.

Další část práce zahrnovala dlouhodobý vliv nízkých koncentrací triazinů na kapry. Byly sledovány účinky terbutrynu v koncentracích 0,02, 0,2 a 2,0 $\mu g.l^{-1}$ na rok starých kaprech po dobu 90 dnů. Ryby vystavené těmto koncentracím ukázaly výrazné rozdíly v oxidačním poškození tkání (mozku, jater) a aktivitě antioxidačních biomarkerů (SOD, CAT, GR, CP, GPx) v tkáních. V expozicích 0,2 a 2,0 $\mu g.l^{-1}$ terbutrynu byly významně ovlivněny biochemické (GLU, AST, LDH, CREA, LACT, Mg) a hematologické (RBC, MCV, Leuko, Lymphocyty) ukazatele. Dále jsme studovali účinky prometrynu pomocí oxidačního stresu antioxidační kapacity na kapru. Ryby byly vystaveny subletálním koncentracím prometrynu (0,51, 8 a 80 $\mu g.l^{-1}$) po dobu 60 dnů. Chronická expozice prometrynu prokázala vliv na antioxidační změny (SOD, CAT, GR) v tkáních kaprů, oxidační poškození buněk však nebylo pozorováno. Změny v CAT a SOD byly pozorovány již i v běžně se vyskytující koncentraci prometrynu v životním prostředí (0,51 $\mu g.l^{-1}$).

Zkoumali jsme také chronické účinky simazinu na aktivitu atioxidačních systémů a oxidační biomarkery v tkáních kapra. U kaprů vystavených koncentraci simazinu 2 a 4 $mg.l^{-1}$ byly zjištěny změny v aktivitě antioxidačních enzymů (SOD, CAT, GPx, GSH) a zvýšená tvorba reaktivních kyslíkových radikálů během 60denní expozice. V environmentální koncentraci simazinu 0,06 $\mu g.l^{-1}$ byla zjištěna změna pouze v aktivitě CAT a GSH v tkáních kapra.

Poslední část naší studie se zabývala dlouhodobým vlivem prometrynu na raka červeného. Rak byl vystaven následujícím koncentracím prometrymu 0,51 $\mu g.l^{-1}$, 0,144 $mg.l^{-1}$ a 1,144 $mg.l^{-1}$ po dobu 25 dní. Chronická expozice prometrynu nevedla k oxidačnímu poškození buněk, ale byly zjištěny významné změny v aktivitě antioxidačních enzymů (SOD, CAT, GR) v tkáních raků. Změny v antioxidačních systémech byly pozorovány i v environmentální koncentraci prometrynu 0,51 $\mu g.l^{-1}$. Histopatologickým vyšetřením hepatopankreatu nebylo zjištěno poškození po expozici prometrynu.

Výsledky této studie shrnují a poskytují důležité informace o hodnocení dlouhodobého dopadu a vlivu triazinů na vodní prostředí, dokonce i v koncentracích běžně se vyskytujících v povrchových vodách. Zjištěné účinky triazinů na kapry a raky pomocí zvolených parametrů se zdají být dobrými indikátory při hodnocení poškození necílových vodních organizmů a vodního prostředí. Nicméně vodní prostředí je znečištěno mnoha xenobiotiky a jejich toxický vliv se kombinací může zesilovat. Proto je velmi důležité dále studovat vliv antropogenních látek, pesticidů na necílové organizmy vyskytující se ve vodním prostředí.

ACKNOWLEDGEMENTS

To my heartfelt thanks belongs to all who have helped me throughout my doctoral studies, both in my personal life so in the scientific world, and they contributed to the creation of this work and to develop of my personality skills.

However, my thanks belongs especially to my supervisor dr hab. Dipl.-Ing. Pepa Velíšek, Ph.D., for his encouragement, advice, support, patience, criticism, and his optimistic view of the problems which for me was not always easy to, from the beginning to finish of my study.

I am also a very grateful to my colleagues Jana, Ilonka, Hanka, Tonik, Jitka, Jiří, Christoph, Kateřina, Olina, Veronika, Tomáš, Roman and many other from the Laborotory of Aquatic Toxicology and Ichtyopathology, Laboratory of Environmental Chemistry and Biochemistry and other less important people of the Faculty of Fisheries and Protection of Waters and from the Research Institute of Fish Culture and Hydrobiology in Vodňany. Special thanks belongs to Prof. dr hab. Teresa from Olsztyn, department of Ichthyology, Faculty of Environment Sciences, who was so willing and accepted me for internship and she gave me the opportunity to learn from one of the most respected fish researchers and his advices. I also deeply appreciate the assistance of the Lucidus Consultancy for English correction.

Last but not least, I am very thankful for my family, friends and colleagues who have become friends who shared with me the happy and sad moments full of emotions during this time.

Many times these are unforgettable moments and experiences, thank.

This Ph.D. thesis could have arisen through the financial support of:

- The Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (CZ.1.05/2.1.00/01.0024), and CENAKVA II (project LO1205 under the NPU I program).
- The Grant Agency of the University of South Bohemia in České Budějovice, projects no. 047/2010/Z, 087/2013/Z, 022/2012/Z.
- The Czech Science Foundation, project no. 525/09/P218.
- The National Agency for Agricultural Research, project no. QH82117.

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Seminar days of RIFCH	2011 2012 2013 2013

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Stara, A., Kouba, A., Velisek, J., Masojidek, J., Kozak, P., 2013. Comparing the impact of various forms of selenium (inorganic vs. organic bound in the biomass of microalgae) in the diet of signal crayfish (*Pacifastacus leniusculus*). Regional European Crayfish Meeting – CrayCro. 26–28 September 2013, Rovinj, Craoatia, Book of Abstracts, p. 59. (Poster presentation)	2013
Stara, A., Savonea, A.O., Bezusa, O., Velisek, J., 2013. Effect of zeta-cypermethrin on oxidative stress biomarkers of rainbow trout (*Oncorhynchus mykiss*). Diversification in Inland Finfish Aquaculture II (DIFA II). 24–26 September 2013, Vodňany, Czech Republic, Book of Abstracts, p. 98. (Poster presentation)	2013
Stara, A., Steinbach, Ch., Wlasow, T., Gomulka, P., Ziomek, E., Machova, J., Velisek, J., 2013. Effect of zeta-cypermethrin on common carp. In: Book of abstracts. 18th Interdisciplinary Toxicology Conference TOXCON 2013. 19–21 June 2013, Hradec Králové, Czech Republic, Military Medicine Science Letters 82 (Supll. 1): 32–33. (Poster presentation)	2013
Stara A., Zuskova E., Velisek J., 2012. Effects of chronic exposure to prometryn on common carp (*Cyprinus carpio* L.). World Aquaculture Society Conference AQUA 2012, Abstract Book. Prague Congress Centre, Prague, Czech Republic. (Poster presentation)	2012
Stara, A., Machova, J., Velisek, J., 2012. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response on early life stage of common carp (*Cyprinus carpio* L.). In: Book of abstracts. 17th Interdisciplinary Toxicology Conference TOXCON 2012. 27–31 August 2012, Stará Lesná, Slovakia, Interdisciplinary Toxicology 5 (Supll. 1): 71. (Poster presentation)	2012
Stara, A., Machova, J., Velisek, J., 2011. Effects of chronic exposure to simazine on oxidative stress and antioxidant response in common carp. In: Book of abstracts. 15th Diseases of Fish and Shellfish EAFP 2011. 12–16 September 2011. Split, Croatia, p. 251. (Poster presentation)	2011

Foreign stays during Ph.D.	**Year**
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Andreea-Oana Savonea Effects of acute exposure to zeta-cypermetryn on biomarkers of oxidative stress and antioxidant response on rainbow trout (*Oncorhynchus mykiss*)	2013
Oxana Bezusa Effects of acute exposure to zeta-cypermetryn on biomarkers of oxidative stress and antioxidant response on rainbow trout (*Oncorhynchus mykiss*)	2013
Ivan Car Effects of acute exposure to molluscicides Vanish on biomarkers of oxidative stress and antioxidant response on rainbow trout (*Oncorhynchus mykiss*)	2014
Behzad Pournori Effects of acute exposure to molluscicides Vanish on biomarkers of oxidative stress and antioxidant response on rainbow trout (*Oncorhynchus mykiss*)	2014

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Ph.D. COURSES

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RESPONSIBLE LEADER OF PROJECT

Grant Agency of the University of South Bohemia GAJU 022/2012/Z (2012): Effect of environmental concentrations of prometryn and its metabolites on nontarget organisms of the aquatic ecosystem

KNOWLEDGE OF LANGUAGES

English in a word and writing

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