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

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

ISBN 978-80-7514-082-1

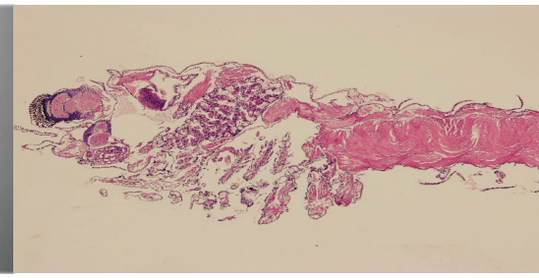
2019

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

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

The effect of triazine based pesticides and their metabolites on no-target aquatic organisms

Vliv triazinových pesticidů a jejich metabolitů na necílové vodní organizmy



Dalibor Koutnik

Czech Republic, Vodňany, 2019

The effect of triazine based pesticides and their metabolites on no-target aquatic organisms

Dalibor Koutnik

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

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

The effect of triazine based pesticides and their metabolites on no-target aquatic organisms

Vliv triazinových pesticidů a jejich metabolitů na necílové vodní organizmy

Dalibor Koutnik

Vodňany, Czech Republic, 2019

I, Dalibor Koutnik, thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

I hereby declare that, in accordance with the § 47b Act No. 111/1998 Coll., as amended, I agree with publicizing of my Ph.D thesis in full version electronically in a publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its web sites, with keeping my copyright to the submitted text of this Ph.D. thesis. I also agree so that the same electronic way, in accordance with above mentioned provision of the Act No. 111/1998 Coll., was used for publicizing reviews of supervisor and reviewers of the thesis as well as record about the progress and result of the thesis defence. I also agree with compering the text of my Ph.D. thesis with a database of theses "Theses.cz" operated by National Register of university theses and system for detecting of plagiarisms.

In Vodňany 10th, May, 2018

Supervisor:
Assoc. Prof. Josef Velíšek
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultant:
Alžběta Stará, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Aquatic Toxicology and Ichthyopathology, USB, FFPW, RIFCH:
Assoc. Prof. Josef Velíšek

Dean of Faculty of Fisheries and Protection of Waters:
Prof. Pavel Kozák

Board of doctorate study defence with reviewers:
Assoc. Prof. Josef Matěna – head of the board
Prof. Petr Ráb – board member
Assoc. Prof. Martin Kocour – board member
Assoc. Prof. Tomáš Policar – board member
Prof. Lukáš Kalous – board member
Assoc. Prof. Radovan Kopp – board member
Assoc. Prof. Zdeněk Adámek – board member

Assoc. Prof. Anna Rymuszka, John Paul II Catholic Univiversity of Lublin, Institut Biotechnology, Department of Physiology & Ecotoxicology, 14 Al Raclawickie St, PL-20950 Lublin, Poland – thesis reviewer
Assoc. Prof. Jana Blahová, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic – thesis reviewer

Date, hour and place of Ph.D. defense:
26th March 2019, 10 a.m., in USB, FFPW, RIFCH, Vodňany

Name: Dipl.-Ing. Dalibor Koutnik

Title of thesis:
The effect of triazine based pesticides and their metabolites on no-target aquatic organisms
Vliv triazinových pesticidů a jejich metabolitů na necílové vodní organizmy

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2019, 123 pages, with the summary in English and Czech.

Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

ISBN 978-80-7514-082-1

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Pesticides

Using various chemical pesticides are spread very rapidly after the Second World War. Already in those years was the requesting to pesticide products have the greatest efficacy on target organisms and low, accompanying side effects (Cremlyn, 1985; Brown et al., 2001; Galbraith and Burns, 2007; Abrantes et al., 2010). The number of substance that fall into major descriptive class of pesticides, and its various chemical and biological subgroups, is enormous. To achieve their ultimate major intended function pesticides are introduced into the environmental to control by harming, usually by killing, those living organisms (pests) that are detrimental, or potentially detrimental, to the existence or health of the human race (Brown et al., 2001). The broad definition of pesticides often excludes those biologically active substances that are used to control or eliminate organisms that directly infect humans and animals, and cause ill health (Marrs and Ballantyne, 2004; Moraes et al., 2009). Pesticides are defined according to the Food and Agricultural Organization (FAO) substances intended for preventing, destroying, suppressing, repel or control of noxious agents, thus undesirable microorganisms, plants and animals during production, storage, transport, distribution and processing of food, agricultural commodities and animal feed. Among the pesticides also include the growth regulators, desiccants and germination inhibitors.

Pesticides enter the cycle when they interact with the everywhere present biochemical and biological, physical and chemical processes in the abiotic and biotic environment. Disrupt these processes and pesticides, but do not themselves subject to the conversion of molecular structure. Pesticides are subject to metabolic (animals, plants, microorganisms), and nonmetabolic (solar radiation, heat, water, air, soil) transformations, which may give rise to specific cases, making products with higher toxicity than was the original compound, or the termination of decomposition (Rozsival and Szokolay, 1983; Marrs and Ballantyne, 2004). Pesticides contribute to physical and chemical changes in water properties, which are reflected in the biological integrity of the aquatic communities (Abrantes et al., 2010).

Many of pesticides are used globally and persist in the environment, and can be transported long ranges to regions where they have never been used. Pesticides are carried as dust by wind over very long distances and contaminate aquatic systems thousands of miles away (e.g. tropical/subtropical pesticides found in Arctic mammals) (Palaniappan et al., 2010). Number of pollutants, pesticides, medications, that are produced in sufficient quantities still stop. These substances are seriously threatening the environment and affect the health of organisms inhabiting these ecosystems, as well as human consumers (Palaniappan et al., 2010; Hernandez-Moreno et al., 2010). Specifically rivers, lakes, ponds and dams are predisposed to receive and accumulate contaminants discharged from industrial sewage, as well as agriculture runoff (Ceyhun et al., 2010; Hernandez-Moreno et al., 2010).

Currently, contamination of environment is one of the key worldwide problems, primarily pollution of water ecosystems. Aquatic ecosystems are endangered by contaminants discharged from areas of intense pesticide use (Scholz et al., 2012). Export of agrochemicals from agricultural fields is threatening of water quality in aquatic systems in large parts of the world (Schwarzenbach, 2006). Pesticides are one of the most commonly discovered organic pollutants in agricultural soils, surface and ground waters (Cerejeira et al., 2003; Rebich et al., 2004; Hayasaka et al., 2013; Morrissey et al., 2015; Bussan et al., 2017; Herrero-Hernandez et al., 2017; Zheng et al., 2017).

1.2. Triazines

Triazine herbicides are among the most commonly used pesticides in the world and were discovered in 1954 (Hostovsky et al., 2014). The structures of the triazine herbicides have a six-member ring containing three nitrogen atoms and three carbon atoms. The chemical structure of triazines is divided into asymmetric (with adjacent nitrogen atoms) and symmetric (with alternating nitrogen and carbon atoms around) (Kamrin, 1997). The symmetrical triazines can be for example atrazine, simazine and prometryne. The asymmetrical triazines can be for example metribuzine and metamitrone (Sanderson et al., 2001).

As a chemical family, triazines are a group of pesticides with a wide range of use. Triazines herbicides have been used increasingly since the 1960s, mainlly on maize crops, in America and Europe (Sanderson et al., 2001). Triazines are used primarily to control broad leaf and grassy weeds. Most are used in selective weed control program, they have nonselective properties which make them suitable for use on industrial sites (Fishel, 2009). The chief mechanism of action is inhibition of photosynthesis (Hogan, 2010).

Even though triazines are subject to photodegradation, soil persistence is notable. Metabolites of the triazines are frequently found in groundwater where the triazines have been applied (Hogan, 2010). Triazines herbicides are persistent, the detectable levels are in drinking water, food and fish (Solomon et al., 1996). Some evidence has linked the mode of toxicity of the triazines compounds in mammals to a disruption of the metabolism of vitamins (Kamrin, 1997) and the ability to disrupt energy metabolism (Hayes and Laws, 1991).

In last 20 years, concerns about the persistence and mobility triazines and their metabolites have been growing, owing to the detection these compounds and their of residual concentrations in different environmental compartments. The detectable levels are in drinking and ground water, food and fish, also their metabolites are frequently found in water ecosystems (Chapadense et al., 2009; Hogan, 2010 Bussan et al., 2017; Herrero-Hernandez et al., 2017; Zheng et al., 2017). Moreover, some of triazine are prohibited in European country. Seven s-triazines have been identified in a study to prioritize substances dangerous to the aquatic environment by the member states of the European Community and are included in the EU Priority Pollutants list and the US Environmental Protection Agency list. According to EU Commission Regulation 196/2010 of 9 March 2010 amending Annex I to Regulation (EC) 689/2008 of the European Parliament, the export and import of these chemicals are banned in the European Union.

Below will be briefly described three most detected triazines (metribuzine, prometryne, terbutylazine) and two triazine metabolites (terbuthylazine-2-hydroxy, terbuthylazine-desethyl) in aquatic ecosystem. The detailed description (environmental fate, toxicity and effect on fish) of these substances is given in Chapter 2, (Koutnik et al., 2015). The effect of selected triazine on fish: a review).

1.2.1. Metribuzine

Metribuzin (4-amino-6-tert-butyl-3-(methythio)-1,2,4-triazin-5-one) is an asymmetrical triazine herbicide. It is distinct from the symmetrical triazines such as atrazine and simazine, in which the central ring structure has alternating carbon and nitrogen atoms, in that metribuzin possesses two nitrogen atoms and two adjacent carbon atoms (Pauli et al., 1990). Metribuzin was first registered as a pesticide in the United States in 1973 (Tomlin, 2003). Metribuzine is used to selectively control certain broadleaf weeds and grassy weed species on a wide range of sites including vegetable and field crops, turf grasses in recreational areas, and noncrop areas (Fairchild and Sappington, 2002).

The degradation of metribuzine is through photochemical, chemical and biochemical deamination. Aqueous photolysis of metribuzin is rapid with a half-life of <1 day, and this clearly contributes to the half-life of <7 days in pond water (Pauli et al., 1990; Wauchope et al., 1992). Measured environmental concentrations of metribuzine in water are usually low, with maximum concentrations below 3 μg/L (Battaglin et al., 2001), but modelling studies have indicated that metribuzine can reach concentrations as high as 390 g/L in surface water runoff (Pauli et al., 1990). The real maximal concentration of metribuzine detected in Czech river was 2.3 μg/L (CHMI, 2018).

Acute toxicity 96hLC50 of metribuzine for fish is ranging from units to hundreds milligrams per liter (Koutnik et al., 2015). The 96hLC50 for rainbow trout (*Oncorhynchus mykiss*) is 42 mg/L (Mayer a Ellersieck, 1986), for bluegill (*Lepomis macrochirus*) is 75.96 mg/L (PED, 2000), and for common carp (*Cyprinus carpio*) is 175.1 mg/L (Velisek et al., 2009).

1.2.2. Prometryne

Prometryne (2,4-bis(isopropylamino)-6-methylthio- s-triazine) a selective herbicide of the s-triazine family was first registered in 1964 and has been used as a pre- or postemergence controller of annual grasses and broadleaf weeds in a variety of crops (U.S. EPA, 1996; LeBaron et al., 2008). Prometryn's mechanism of action inhibits the electron transport in susceptible species (Jiang and Yang, 2009).

The soil half-life of prometryne is 60 days. Halflife of prometryne in water is 500 days (U.S. EPA, 1996). Although prometryne has been banned in Europe since 2004 (Zhou et al., 2012), it still can be found in surface and ground waters. Prometryne has been reported in European surface waters at concentrations from 0.01 to 4.40 μg/L (Papadopoulou-Mourkidou et al., 2004; Vryzas et al., 2011; Caquet et al., 2013). The real maximal concentration of prometryne detected in Czech river was 0.51 μg/L (CHMI, 2018).

Prometryne is toxic to moderately toxic to aqautic organisms. The most sensitive aquatic organisms are freshwater algae (PED, 2000). The 5dEC50 of prometryne for algae *Selenastrum capricornutum* is 0.023 mg/L (PED, 2000). The 96hLC50 for rainbow trout is 2.9 mg/L (U.S. EPA, 1996), for sheepshead minnow (*Cyprinodon variegatus*) is 5.1 mg/L (Kegley et al., 2010), and for common carp is 8 mg/L (Popova, 1976).

1.2.3. Terbuthylazine

Terbuthylazine (N-tert-butyl-6-chloro-N'-ethyl-1,3,5-triazine-2,4-diamine) was registered in the United States in 1975 (Mladinic et al., 2009). Terbuthylazine is used as a substitute for atrazine since the end of 2006 (Nodler et al., 2013). Terbuthylazine is herbicide that belongs to the chlorotriazine family, is used in both pre- and post-emergence treatment of a variety of agricultural crops and in forestry (WHO, 2003; Vryzas et al., 2011).

Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions (U.S. EPA, 1995). Terbuthylazine is a slightly basic, slightly water soluble triazine herbicide or algacide which adsorbs to soil organic matter. In surface water of Europe, terbuthylazine has been recorded at concentrations from 0.01 to 13.0 μg/L (Rodriguez-Mozaz et al., 2004; Fait et al., 2010; Chary et al., 2012; Jurado et al., 2012). The real maximal concentration of terbuthylazine detected in Czech river was 2.9 μg/L (CHMI, 2018). Degradation of terbuthylazine in natural water depends on the presence of sediments and biological activity (WHO, 2006). Terbuthylazine photo-degrades in water, this is likely to be the main degradation pathway. The fate of residues in aerobic and anaerobic aquatic conditions is similar. The major metabolites of terbuthylazine

are terbuthylazine-2-hydroxy, terbuthylazine-desethyl and terbuthylazinedesethyl-hydroxy, which are more mobile than the parent, and exhibit some herbicidal activity when they retain the chlorine atom on the triazine ring plus one alkyl group (Byrnes, 2001; WHO, 2003).

Terbuthylazine is slight toxicity towards fish and shellfish, and variable toxicity towards aquatic crustaceans, from very highly toxic to practically non-toxic (WHO, 2006). Standard toxicity tests with various fish species as nontarget organisms revealed LC50 values between 4.6 and 66 mg/L (Koutnik et al., 2015).

1.2.4. Terbuthylazine-2-hydroxy

Terbuthylazine-2-hydroxy is one of the main degradation products of terbuthylazine (EFSA, 2011). The half-life of terbuthylazine-2-hydroxy is 112 days, 120 days in water at 20–25 °C, and 456 days at 10 °C (Nodler et al., 2013). The degradation products of triazines are usually more polar and thus pose a greater potential risk for ground water contamination, often with considerably higher toxicity, than does the parent compound (Loss et al., 2010). Terbuthylazine-2-hydroxy is highly mobile and have been frequently detected in surface and ground water of Europe (Bozzo et al., 2013; Stipicevic et al., 2015). In surface water terbuthylazine-2-hydroxy has been recorded at concentrations from 0.05 to 9.24 μg/L (Masia et al., 2015; CHMI, 2018). The real maximal concentration of terbuthylazine-2-hydroxy detected in Czech river was 0.75 μg/L (CHMI, 2018). Terbuthylazine-2-hydroxy is moderately toxic to fish. The 96hLC50 for rainbow trout is 15 mg/L and 48hEC50 for Daphnia magna is 16 mg/L (PPDB, 2018).

1.2.5. Terbuthylazine-desethyl

Terbuthylazine-desethyl, another no less important degradation product of terbuthylazine (EFSA 2011). The half-life of terbuthylazine-desethyl is 112 days (Nodler et al., 2013). In surface water of Europe, terbuthylazine-desethyl has been recorded at concentrations from from 0.007 to 2.9 μg/L (Hildebrandt et al., 2008; Benvenuto et al., 2010; Loss et al., 2010; Masia et al., 2015; CHMI, 2018). The real maximal concentration of terbuthylazine-desethyl detected in Czech river was 1.8 μg/L (CHMI, 2018). Terbuthylazine-desethyl is moderately toxicity to fish. The 96hLC50, for rainbow trout is 18 mg/L and 48hEC50 for *Daphnia magna* is 42 mg/L (PPDB, 2018).

1.3. Aims of the thesis

The main aim of this work was to investigate the effects of chronic exposure to triazines (prometryne and terbuthylazine) and theier metabolites (terbuthylazine-2-hydroxy and terbuthylazine-desethyl) at real environmental concetrations on developmental stages of common carp (*Cyprinus carpio* L.) and marbled crayfish (*Procambarus fallax* f. *virginalis*).

The effect was assessed with respect to:
- behaviour and mortality,
- growth,
- ontogenetic development,
- histopathology,
- indices of oxidative stress and antioxidant parameters.

The effects of triazine metribuzine on signal crayfish (*Pacifastacus leniusculus* Dana) as a non-target organism were also investigated with respect to
- behaviour,
- histopathology,
- indices of oxidative stress and antioxidant parameters.

REFERENCES

Abrantes, N., Pereira, R., Gonçalves, F., 2010. Occurrence of pesticides in water, sediments, and fish tissues in a Lake Surrounded by agricultural lands: concerning risks to humans and ecological receptors. Water and Air Pollution 212: 77–88.

Battaglin, W.G., Furlong, E.T., Burkhardt, MR., 2001. Concentrationof selected sulfonylurea, sulfonamide, and imidazolinoneherbicides, other pesticides, and nutrients in 71 streams, 5 reservoiroutflows, and 25 wells in the Midwestern United States, 1998. Denver: US Geological Survey Water Resources Investigations, Report 00–4225.

Benvenuto, F., Marin, J.M., Sancho, J.V., Canobbio, S., Mezzanotte, V., Hernandez, F., 2010. Simultaneous determination of triazines and their main transformation products in surface and urban wastewater by ultra-high-pressure liquid chromatographytandem mass spectrometry. Analytical and Bioanalytical Chemistry 397: 2791–2805.

Bozzo, S., Azimonti, G., Villa, S., Di Guardo, A., Finizio, A., 2013. Spatial and temporal trend of groundwater contamination from terbuthylazine and desethyl-terbuthylazine in the Lombardy Region (Italy). Environmental Science: Processes & Impacts 15: 366–372.

Brown, E.C., Claassen, A., Hathaway, C.R., Holmstead, J., Powell, T., Wehrum, W., Weinstein, K., 2001. Pesticide Regulation Deskbook. Latham & Watkins, 174 pp.

Bussan, D.D., Ochs, C.A., Jackson C.R., Anumol, T., Snyder, S.A., Cizdziel, J.V., 2017. Concentrations of select dissolved trace elements and anthropogenic organic compounds in the Mississippi River and major tributaries during the summer of 2012 and 2013. Environmental Monitoring and Assessment 189: 73.

Byrnes, C., 2001. Evaluation of the active terbuthylazine in the product SWIM-CARE® T SWIMMING POOL ALGAECIDE. National Registration Authority for Agricultural and Veterinary Chemicals, 20 pp.

Caquet, T., Roucaute, M., Mazzella, N., Delmas, F., Madigou, C., Farcy, E., 2013. Risk assessment of herbicides and booster biocides along estuarine continuums in the Bay of Vilaine area (Brittany, France). Environmental Science and Pollution Research 20: 651–666.

Cerejeira, M.J., Viana, P., Batista, S., Pereira, T., Silva, E., Valerio, M.J., Silva, A., Ferreira, M., Silva-Fernandes, A.M., 2003. Pesticides in Portuguese surface and ground waters. Water Research 37: 1055–1063.

Ceyhun, S.B., Senturk, M., Erdogan, O., Kufrevioglu, O.I., 2010. In vitro and in vivo effect of some pesticides on carbonic anhydrase enzyme from rainbow trot (*Oncorhynchus mykiss*) gills. Pesticide Biochemistry and Physiology 97: 177–181.

Chapadense, P.F.G., Castro, F.J., Almeina, J.A., Moron, S.E., 2009. Toxicity of atrazine herbicide in Colossoma macropomum. Revista Brasileira de Saúde e Producato Anima 10: 398–405.

Chary, N.S., Herrera, S., Gómez, M.J., Fernández-Alba, A.R., 2012. Parts per trillion level determination of endocrine-disrupting chlorinated compounds in river water and wastewater effluent by stir-bar-sorptive extraction followed by gas chromatography- triple quadrupole mass spectrometry. Analytical and Bioanalytical Chemistry 404: 1993–2006.

CHMI (Czech Hydrometeorological Institute), 2018. On-line water duality database. Available from: http://hydro.chmi.cz/, (visited online 12.2.2018).

Cremlyn, R., 1985. Pesticidy. Státní nakladatelství technické literatury, Praha, 244 s.

EFSA (European Food Safety Authority), 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance terbuthylazine. EFSA Journal 9: 1969.

Fait, G., Balderacchi, M., Ferrari, F., Ungaro, F., Capri, E., Trevisan, M., 2010. A field study of the impact of different irrigation practices on herbicide leaching. European Journal of Agronomy 32: 280–287.

Fairchild, J.F., Sappington, L.C., 2002. Fate and effects of the triazinone herbicide metribuzin in experimental pond mesocosms. Archives of Environment Contamination and Toxicology 43: 198–202.

Fishel, F.M., 2009. Pesticide Toxicity Profile: Triazine Pasticides. University of Florida, IFAS Extension. Avaliable from: http://edis.ifas.ufl.edu/pdffiles/PI/PI15800.pdf, (visited online 8.2.2018), 3 pp.

Galbraith, L.M., Burns, C.W., 2007. Linking land-use, water body type and water quality in southern New Zealand. Landscape Ecology 22: 231–241.

Hayasaka, D., Suzuki, K., Nomura, T., Nishiyama, M., Nagai, T., Sanchez-Bayo, F., Goka, K., 2013. Comparison of acute toxicity of two neonicotinoid insecticides, imidacloprid and clothianidin, to five cladoceran species. Journal of Pest Science 38: 44–47.

Hayes, W.J., Laws, E.R., 1991. Handbook of Pesticide Toxikology. Classes of Pesticides. New York, NY: Academic Press, 1451 pp.

Herrero-Hernandez, E., Rodriguez-Cruz, M.S., Pose-Juan, E., Sanchez-Gonzalez, S., Andrades, M.S., Sanchez-Martin, M.J., 2017. Seasonal distribution of herbicide and insecticide residues in the water resources of the vineyard region of La Rioja (Spain). Science of the Total Environment 609: 161–171.

Hernandez-Moreno, D., Soler, F., Miguez, M.P., Perez-Lopez, M., 2010. Brain acetylcholinesterase, malondialdehyde and reduced glutathione as biomarkers of continuous exposure of tench, *Tinca tinca*, to carbofuran or deltamethrin. Science of the Total Environment 408: 4976–4983.

Hildebrandt, A., Guillamon, M., Lacorte, S., Tauler, R., Barcelo, D., 2008. Impact of pesticides used in agriculture and vineyards to surface and groundwater quality (North Spain). Water Research 42: 3315–3326.

Hogan, C.M., 2010. Herbicide. The Encyclopedia of Earth, online version. Available from: http://www.eoearth.org/article/Herbicide?topic=49494, (visited online 10.2.2018).

Hostovsky, M., Blahova, J., Plhalova, L., Kopriva, V., Svobodova, Z., 2014. Effects of the exposure of fish to triazine herbicides. Neuroendocrinology Letter 35 (Suppl. 2): 3–25.

Jiang, L., Yang, H., 2009. Prometryne-induced oxidative stress and impact on antioxidant enzymes in wheat. Ecotoxicology and Environmental Safety 72: 1687–1693.

Jurado, A., Vàzquez-Suñé, E., Carrera, J., López de Alda, M.J., Pujades, E., Barceló, D., 2012. Emerging organic contaminants in groundwater in Spain: a review of sources, recent occurrence and fate in a European context. Science of the Total Environment 440: 82–94.

Kamrin, M.A., 1997. Pesticide profiles. Boca Raton Florida: Lewis Publishers, 676 pp.

Kegley, S.E., Hill, B.R., Orme, S., Choi, A.H., 2010. PAN Pesticide Database, Pesticide Action Network, North America (San Francisco, CA, 2010), Pesticide Action Network, North America.

Koutnik, D., Stara, A., Velisek, J., 2015. The effect of selected triazine on fish: a review. Slovenian Veterinary Research 52: 107–131.

LeBaron, H.M., McFarland, J.E., Burnside, O.C., 2008. The Triazine Herbicides, 50 Years Revolutionizing Agriculture. Elsevier, 584 pp.

Loss, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., 2010. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Research 44: 4115–4126.

Marrs, T.C., Ballantyne, B., 2004. Pesticide toxicology and international regulation. John Wiley and Sons, 554 pp.

Masia, A., Campo, J., Navarro-Ortega, A., Barcelo, D., Pivo, Y., 2015. Pesticide monitoring in the basin of Llobregat River (Catalonia, Spain) and comparison with historical data. Science of the Total Environment 503–504: 58–68.

Mayer, F.L., Ellersieck, M.R., 1986. Manual of acute toxicity: Interpretation and data base for 410 chemicals and 66 species of freshwater animals. Resource Publication No. 160, U.S. Department Interior, Fish and Wildlife Services, Washington, USA, 32 pp.

Mladinic, M., Perkovic, P., Zeljezic, D., 2009. Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. Toxicology Letters 189: 130–137.

Moraes, B.S., Loro, V.L., Pretto, A., da Fonseca, M.B., Menezes, C., Marchesan, E., Reimche, G.B., de Avila, L.A., 2009. Toxicological and metabolic parameters of the teleost fish (*Leporinus btusidens*) in response to commercial herbicides containing clomazone and propanil. Pesticide Biochemistry and Physiology 95: 57–62.

Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a Review. Environment International 74: 291–303.

Nodler, K., Licha, T., Voutsa, D.T., 2013. Twenty years later atrazine concentrations in selected coastal waters of the Mediterranean and the Baltic Sea. Marine Pollution Bulletin 70: 112–118.

Palaniappan, M., Gleick, P.H., Allen, L., Cohen, M.J., Christian-Smith, J., Smith, C., 2010. Clearing the Waters, A focus on water quality solutions. United Nations Environment Programe, 88 pp.

Papadopoulou-Mourkidou, E., Karpouzas, D.G., Patsias, J., Kotopoulou, A., Milothridou, A., Kintzikoglou, K., Vlachou, P., 2004. The potential of pesticides to contaminate the groundwater resources of the Axios river basin in Macedonia, Northern Greece. Part I. Monitoring study in the north part of the basin. Science of the Total Environment 321: 127–146.

Pauli, B.D., Kent, R.A., Wong, M.P., 1990. Canadian water quality guidelines for metribuzin. Environmental Canada Sciences Serie 179: 135–145.

PED (Pesticide Ecotoxicity Database), 2000. Office of Pesticide Programs, Environmental Fate and Effects Division, U.S. EPA, Washington, DC.

PPDB (Pesticide Properties Database), 2018. Desethyl-terbuthylazine. http://sitem.herts.ac.uk/aeru/iupac/Reports/1494.htm, (visited online 10.2.2018).

Popova, G.V., 1976. Characteristics of the effect of the herbicide prometryn on fish. N Nauchnye Osnovy Okhrany Prirody 4: 118–125.

Rebich, R.A., Coupe, R. H., Thurman, E.M., 2004. Herbicide concentrations in the Mississippi River Basin, the importance of chloroacetanilide herbicide degradates. Science of the Total Environment 321: 189–199.

Rodriguez-Mozaz, S., Lopez de Alda, M., Barcelo, D., 2004. Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction-liquid chromatographyemass spektrometry. Journal of Chromatography A 1045: 85–92.

Rozsival, L., Szokolay, A., 1983. Cudzorodé látky v poživatinách. Osveta, 648 s.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J.P., van den Berg, M., 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109: 1027–1031.

Scholz, N.L., Fleishman, E., Brown, L., Werner, I., Johnson, M.L., Brooks, M. L., Schlenk, D., 2012. A perspective on modern pesticides, pelagic fish declines, and unknown ecological resilience in highly managed ecosystems. BioScience 62: 428–434.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313: 1072–1077.

Solomon, K.R., Baker, D.B., Richards, R.P., Dixon, D.R., Klaine, S.J., LaPoint, T.W., Kendall, R.J., Weisskopf, C.P., Giddings, J.M., Giesy, J.P., Hall, L.W., Williams, W.M., 1996. Ecological risk asssesment of atrazine in North America surface waters. Environmental Toxicology and Chemistry 15: 31–74.

Stipicevic, S., Galzina, N., Udikovic-Kolic, N., Jurina, T., Mendas, G., Dvorscak, M., Petric, I., Baric, K., Drevenkar, V., 2015. Distribution of terbuthylazine and atrazine residues in crop-cultivated soil: the effect of herbicide application rate on herbicide persistence. Geoderma 259: 300–309.

Tomlin, C., 2003. The Pesticide Manual. A World Compendium. British Crop Protection Council, Hampshire, UK, 1344 pp.

U.S. EPA (U.S. Environmental Protection Agency), 1996. R.E.D. Fact Prometryn. Available from: http://www.epa. gov/oppsrrd1/ REDs/factsheets/0467fact.pdf, (visited online 8.3.2018), 11 pp.

U.S. EPA (U.S. Environmental Protection Agency), 1995. Reregistration Eligibility Decision (RED) Terbuthylazine. Available from: http://www.epa.gov/oppsrrd1/REDs/2645.pdf, (visited online 8.3.2018), 186 pp.

Velisek, J., Svobodova, Z., Piackova, V., Sudova, E., 2009. Eff ects of acute exposure of metribuzin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Bulletin of Environmental Contamination and Toxicology 82: 492–495.

Vryzas, Z., Alexoudis, C., Vassiliou, G., Galanis, K., Papadopoulou-Mourkidou, E., 2011. Determination and aquatic risk assessment of pesticide residues in riparian drainage canals in northeastern Greece. Ecotoxicology and Environmental Safety 74: 174–81.

Wauchope, R.D., Buttler, T.M., Hornsby, A.G., Augustijn-Beckers, P.W.M., Burt, J.P., 1992. The SCS/ARS/CES pesticide properties database for environmental decision- making. Reviews of Environmental Contamination and Toxicology 123: 1–164.

WHO (World Health Organization), 2006. Guidelines for drinking-water quality. Available from: http://www.who.int/water_sanitation_health/dwq/gdwq0506.pdf, (visited online 8.3.2018).

WHO (World Health Organization) 2003. Terbuthylazine (TBA) in Drinking-water: Background document for development of WHO Guidelines for Drinking-water duality. Available from: http://www.who.int/water_sanitation_health/dwq/chemicals/terbuthylazine.pdf, (visited online 8.3.2018).

Zheng, L., Zhang, Y., Yan, Z., Zhang, J., Li, L., Zhu, Y., Zhang, Y., Zheng, X., Wu, J., Liu, Z., 2017. Derivation of predicted no-effect concentration and ecological risk for atrazine better based on reproductive fitness. Ecotoxicology and Environmental Safety 142: 464–470.

Zhou, J.H., Hu, F., Jiao, J.G., Liu, M.G., Li, X.H., 2012. Effects of bacterial-feeding nematodes and prometryne-degrading bacteria on the dissipation of prometryne in contaminated soil. Journal of Soils and Sediments 12: 576–585.

CHAPTER 2

THE EFFECT OF SELECTED TRIAZINE ON FISH: A REVIEW

Koutnik, D., Stara, A., Velisek, J., 2015. The effect of selected triazine on fish: a review. Slovenian Veterinary Research 52: 107–131.

My share on this work was about 40%

It was allowed by publisher on 27th March, 2018 to include the paper in this Ph.D. thesis.

Slov Vet Res 2015; 52 (3): 107-31
UDC 639.2.09:502.51:504.5:615.916:547-304.9

Review Article

THE EFFECT OF SELECTED TRIAZINES ON FISH: A REVIEW

Dalibor Koutnik*, Alzbeta Stara, Josef Velisek

South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice Zatisi 728/II, 389 25 Vodnany, Czech Republic

*Corresponding author, E-mail: dkoutnik@frov.jcu.cz

Summary: Anthropogenic pollution constitutes a worldwide problem of growing concern. Increased environmental pollution can be attributed to a variety of factors associated with industrial and agricultural technologies. Triazine herbicides are among the most commonly used pesticides in the world, and are predominant class of herbicide. In recent years, concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection these herbicides compounds and their of residual concentrations in different environmental compartments. The detectable levels are in drinking and ground water, food and fish, also their metabolites are frequently found in water ecosystems. Moreover, some of triazine pesticides are prohibited in European country. Eight s-triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and they are included in the European Union Priority Pollutants List and the U.S. Environmental Protection Agency's List. Current knowledge about residual triazine in the aquatic environment, including status, toxic effects, and traizne in fish, are reviewed. Based on the above, we identify major gaps in the current knowledge and some directions for future research. A review contains the impact of the seven most frequently detected triazines in water (ametryne, atrazine, metribuzine, prometryne, simazine, terbuthylazine, and terburyne) on fish physiology and acute toxicity. Toxic effect of triazine has influence mainly on growth, early development, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney of fish.

Key words: triazine; fish; toxicity; biochemical profile; hematology; histology

---

Abbreviations & Units: AChE – acetylcholinesterase; ACP – acyl carrier protein; ALB – albumin; ALP – alkaline phosphatase; ALT – alanine aminotransferase; APND – aminopyrine; AST – aspartate aminotransferase; Ca – calcium; CA – carbonic anhydrase; CAT – catalase; CbE – carboxylesterase; CF – condition factor; CK – creatine kinase; CREA – creatine; CYP – cytochrome; DS – distal segments; EC – ceruloplasmin; ERND – erythromycin N-demethylase; EROD – ethoxyresorufin-O-deethylase; FRAP – ferric reducing ability of plasma; GLOB – total globulins; GLU – glucose; GSH – reduced glutathione; GPx – glutathione peroxidase; GR – glutathione reductase; Hb – hemoglobin; MRCs – mitochondria-rich cells; HSI – hepatosomatic index; Hsp – heat shock protein; iNOS – inducible nitric oxide synthase; LACT – lactate; LC50 – lethal concentration; LDH – lactate dehydrogenase; LPO – lipid peroxide; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; MCV – mean corpuscular volume; MDA – malondialdehyde; Mg – magnesium; Na – natrium; NCR – NADPH cytochrome P450 reductase; NH3 – ammonia; P – phosphorus; PCV – hematocrit; PD – proximal segments; PHOS – inorganic phosphate; POD – guaiacol peroxidase; PROD – pentoxyresorufin-O-deethylase; RBC – erythrocyte count; RCs – rodlet cells; ROS – reactive oxygen species; SOD – superoxide dismutase; SSI – spleen somatic index; SW – spleen weight; TAG – triacylglycerols; TBARS – thiobarbituric acid reactive substances; TP – total protein; UDPGT – UDP-glucuronosyltransferase; WBC – leukocyte count; 11-KT – 11-ketotestosterone.

Received: 24 June 2014
Accepted for publication: 2 February 2015

Introduction

Sources of pollution constitute a problem of increasing concern all over the world (1). Increased environmental pollution can be attributed to a variety of factors resulting from different industrial and agricultural technologies (2). Agricultural development has led a parallel growth in the use of chemical agents for plague controls, which are known as pesticides. These compounds are released into the environment and due to their physico-chemical properties, such as water solubility, vapor pressure or partition coefficients between organic matter (soil or sediment) and water, they can disperse in various environmental media provoking serious health problems (3).

Effects of the residues of various substances persisting in the aquatic environment, the most important of those being pesticides, also are monitored. From among pesticides, the most frequently found are residue of triazine herbicides. Triazine herbicides are among the most commonly used pesticides in the world. The triazine was discovered in 1954 (4). The chemical structure of triazines is divided into asymmetric (metribuzine) and symmetric (atrazine, simazine, prometryne, etc.). The structures of all of the triazine herbicides have a six-member ring containing three nitrogen atoms and three carbon atoms (5). Triazines compounds are used against a wide variety of weed species. They are used primarily to selective control broad leaf and grassy weeds (6). As herbicides, the triazines may be used alone or in combination with other herbicide active ingredients to increase the weed control spectrum (7).

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 groundwater and in different environmental compartments (8, 9). Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community (10) and they are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. Triazine are highly toxic to moderately toxic to fish (Tab. 1.). On base of these informations, we decided to write a review about the impact of the seven most frequently detected triazines in water (ametryne, atrazine, metribuzine, prometryne, simazine, terbuthylazine, and terburyne) on fish.

Ametryne

Ametryne (4-N-ethyl-6-methylsulfanyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine) was first registered as a pesticide use to control broadleaf weeds and annual grasses in sugarcane fields in the USA in 1964. Ametryne has also been used as a general herbicide in uncultivated areas, rights of way, and industrial areas and aquatic weeds. Over time, the uses of ametryne have been cancelled so that only four use sites remain: field corn, popcorn, pineapple, and sugarcane. Currently, only one ametryne end use product is registered. In 2005 US EPA has received requests for voluntary cancellation of all other products (37). The extensive use of ametryne in agriculture and some properties of this herbicide such as aerobic soil halflife of 53.2 days, adsorption coefficient of 3.45, and leaching potential of 6.94 (38) suggest that it could be present in the environment as a potential contaminant of soil, surface water and groundwater, and river sediment (39).

Environmental fate

Ametryne is a moderately persistent herbicide which inhibits photosynthesis and other enzymatic processes. The environmental fate of ametryne varies based on the site-specific properties of the soil to which it is applied. Based on packed soil column leaching studies, ametryne and its degradates exhibit moderate to high mobility in most sandy to loamy soils, except for clay where its mobility is low. The major route of degradation of ametryne is aerobic soil metabolism, with an observed half-life range of 9.6 days to 84 days. Ametryne is stable to hydrolysis, and degrades slowly by aquatic photolysis, half-life is 368 days (37). Major metabolite product of ametryne is deethyl ametryne (38).

Ametryne is persistent, it may leach as a result of high rainfall, floods, and furrow irrigation. Given its persistence and mobility, transport of ametryne to ground water and surface water is expected. Monitoring of ametryne concentrations in ground water and surfase water is limited. In Europe rivers ametryne levels can reach values,

Table 1: Acute toxicity of triazines on fish

Exposure 96hLC50 [mg/L] (Reference)							
Species	**Ametryne**	**Atrazine**	**Metribuzine**	**Prometryne**	**Simazine**	**Terbuthylazine**	**Terbutryne**
Guppy (*Poecilia reticulata*)	0.3 (11)	4.3 (13)	-	7.0*** (29)	-	1.6 (13)	-
Japanese eel *(Anguilla japonica*)	1.5** (12)	-	-	-	-	-	-
Rainbow trout (*Oncorhynchus mykiss*)	3.4 (13)	8.8 (13)	42.0 (24)	2.9 (14)	100.0* (14)	3.4 (14)	3.0 (13)
Sheepshead minnow (*Cyprinodon variegatus*)	5.8 (14)	13.4 (14)	85.0 (14)	5.1 (28)	4.3 (14)	-	-
Goldfish (*Carassius auratus*)	14.0 (14)	58.6 (22)	-	4.0 (14)	32.0 (14)	-	-
Fathead minnow (*Pimephales promelas*)	16.0 (14)	4.1 (15)	-	-	-	-	-
Bluegill (*Lepomis macrochirus*)	19.0 (13)	50.0 (13)	76.0 (14)	7.9 (28)	100.0 (34)	7.5 (14)	4.0 (13)
Black bullhead (*Ameiurus melas*)	25.0 (11)	35.0 (11)	-	3.0 (11)	65.0 (11)	7.0 (11)	3.0 (11)
Crucian carp (*Carassius carassius*)	27.0 (11)	100.0** (11)	-	-	100.0 (13)	66.0 (13)	4.0 (11)
Channel catfish (*Ictalurus punctatus*)	-	10.0 (16)	3.4 (23) 100.0 (24)	-	85.0 (14)	-	-
Coho salmon (*Oncorhynchus kisutch*)	-	12.0 (17)	-	-	-	-	-
Common carp (*Cyprinus carpio*)	-	18.8 (18)	175.1 (26)	8.0 (27)	40.0** (33)	-	4.0 (35)
Chinook salmon (*Oncorhynchus tshawytscha*)	-	19.0 (17)	-	-	910.0 (17)	-	-
Fera (*Coregonus fera*)	-	26.3 (19)	-	-	-	-	-
Brown trout (*Salmo trutta*)	-	27.0 (20)	-	-	70.0 (20)	-	-
Zebrafish (*Danio rerio*)	-	40.0** (21)	-	3.0 (27)	12.6 (31)	-	-
Red rasbora (*Rasbora heteromorpha*)	-	-	140.0 (25)	-	-	-	-
Red-tailed rasbora (*Rasbora borapetensis*)	-	-	145.0 (25)	-	-	-	-
Minnow (*Phoxinus phoxinus*)	-	-	-	4.5 (27)	-	-	-
Silver carp (*Hypophthalmichthys molitrix*)	-	-	-	7.0 (27)	-	-	-
Western mosquitofish (*Gambusia affinis*)	-	-	-	10.0* (30)	-	-	-
Tilapia mosambicus (*Oreochromis mossambicus*)	-	-	-	-	3.1 (31)	-	-
Barbus ticto (*Barbus ticto*)	-	-	-	-	24.5 (31)	-	-
Rohu (*Labeo rohita*)	-	-	-	-	26.9** (32)	-	-
Yellow bullhead (*Ameiurus natalis*)	-	-	-	-	110.0 (14)	-	-
genus Bullheads (*Ameiurus sp.*)	-	-	-	-	-	7.0 (13)	-
Perch (*Perca fluviatilis*)	-	-	-	-	-	-	4.0 (11)
Grass carp (*Ctenopharyngodon idella*)	-	-	-	-	-	-	8.9** (36)

* 24hLC50; ** 48hLC50; *** 72hLC50

up to 1.14 μg/L (39-41). In surface water near to Sao Paulo (Brasil) was found contamination from 0.17 to 0.23 μg/L (42, 43).

Acute toxicity

Ametryne is highly toxic to moderately toxic to fish. The lethal concentration (96hLC50) for fish is in range 0.3 to 27.0 mg/L (Tab. 1.). Ametryne is highly toxic to crustaceans and moderately to highly toxic to mollusks (44).

Effect of ametryne on fish

Although the lethal toxicity of fish to ametryne, have been well-documented, there is a dearth of data on the effects of ametryne on fish physiology. Only three studies on effects on fish physiology of ametryne have been conducted. Ametryne caused increase of plasma glucose level, hepatic glucose-6-phosphatase and decreased of muscle and liver glycogen contents in grass carp (*Ctenopharyngodon idella*) during sublethal and lethal (96hLC50) exposure (45). Acute exposure of ametryne inhibited of cholinesterase in juvenile and adult zebrafish (*Danio rerio*). Ametryne caused increase of acivity glutathione S-transferase only in larvae, but not in adult fish. And they conclude that these biomarkers are a useful tool to evaluate the risk of fish exposure of ametryne, even at sublethal levels (46). Mix atrazine and ametryne in concentrations (0.5, 1.0, 1.5, and 2.0 μg/L) exposure caused micronuclei formation and erythrocytic nuclear abnormalities in zebrafish (47).

Atrazine

Atrazine (6-chloro-N2-ethyl-N4-(1-methylethyl)-1,3,5-triazine-2,4-diamine) was used for control of some annual broadleaf and grass weeds in corn, sorghum, sugar cane, orchards, vineyards and non-agricultural areas (48). Atrazine causes blockage of electron transport by Hill´s reaction in plant photosynthesis (49). It is an indirect endocrine disruptor (50, 51) because it can cause convert testosterone to estrogen (52). Atrazine and plant protection products containing this substance were banned in 2005 by Commission Decision 2004/247/CE.

Environmental fate

Atrazine is toxic, persistent and bioaccumulative (53). According to its physical and chemical characteristics of the group of compounds that are moderately resistant and moderately mobile in soils. The half-life of atrazine, depending upon the environment and the amount and frequency of administration, varies between a few days to several months. The photolysis in water is very slow. An estimated half-life is 805 days. In controlled aerobic water-sediment systems atrazine was eliminated from the water with a half-life of 28-134 days, while the degradation half-life was found to be 45-253 days for the whole system (54). In European rivers atrazine levels can reach values, up to 6.47 μg/L (55), but in US rivers was about 20 μg/L (56).

Acute toxicity

Lethal acute toxicity (96hLC50) of atrazine for fish is ranging from units to hundreds milligrams per liter (Tab. 1.). Order of sensitivity to atrazine is: macrophytes > phytoplankton > zooplankton > fish > benthos (57). Fish subjected to acute exposure of atrazine herbicide displayed uncoordinated behavior. At the initial exposure, fish were alert, stopped swimming and remained static in position in response to the sudden changes in the surrounding environment. After some time they tried to avoid the toxic water with fast swimming and jumping. Faster opercula activity was observed as surfacing and gulping for air. They secreted copious amounts of mucus from whole body continuously and soon a thick layer of mucus was found deposited in the buccal cavity and gills. Body pigmentation was decreased. Ultimately fish lost their balance, consciousness, engage in rolling movement and became exhausted and lethargic. Lastly, they remained in vertical position for a few minutes with anterior side or terminal mouth up near the surface of the water, trying to gulp air and tail in a downward direction. Soon they settled at the bottom of the tank, and after some time their bellies turned upward and the fish died (58).

Table 2: The effect of atrazine on common carp

Development stage	Concentration	Exposure	Effects	Reference
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ EROD, PROD, CYP, CYP1A mRNA level in liver	(61)
Juvenile	5 mg/L	96 hours	↑ GLU; ↓ RBC, WBC	(62)
	15 mg/L		↑ GLU, TP, ALB, ALT, ALP, LDH, myclocytes ↓ WBC, lymfocytes	
	20 mg/L		↑ GLU, TP, ALT, ALP, LDH, myclocytes, ↓ P, Ca, WBC, lymfocytes	
	30 mg/L		↑ GLU, ALT, AST, LDH, myclocytes, monocytes; injection of visceral vessels, ↓ PCV, RBC, Hb, WBC, lymfocytes; dystrophic lesions of hepatocytes, teleangiectasis in gill	
Juvenile	4.28 µg/L	40 days	↑ ACP in spleen, ACP in head kidney ↓ Na+/K+-ATPase in head kidney	(63)
	42.8 µg/L		↑ ACP in spleen, ACP in head kidney ,MDA in spleen, ↓ SOD in spleen, SOD in spleen, head kidney, Na+/K+-ATPase in head kidney	
	428 µg/L		↑ ACP in spleen, ACP in head kidney, MDA in spleen, head kidney ↓ ALP in spleen, ALP in head kidney, Na+/K+-ATPase in spleen, Na+/K+-ATPase in head kidney, SOD in spleen, SOD in head kidney	
Juvenile	4.28 µg/L	40 days	↑ HSP90	(64)
	4.28, 42.8, 428 µg/L	40, 80 days	↑ HSP60	
	42.8, 428 µg/L		↑ HSP70	
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ APND, ERND, mRNA levels of CYP1 family (CYP1A, CYP1B, CYP1C) in gill	(65)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ iNOS, production of NO in brain	(66)
Juvenile	428 µg/L	40 days	↓ AChE, mRNA levels of AChE	(67)
Juvenile	42.8, 428 µg/L	40 days	↑ MDA in kidney, MDA in brain; ↓ CAT in kidney, SOD in kidney, SOD in brain, GSH-Px in kidney; GSH-Px in brain; different degrees of granule cell loss in the hippocampus, reduction of Nissl bodies, degeneration of Purkinje cells, neuropil loss; swelling of epithelial cells of renal tubules, necrosis in the tubular epithelium, contraction of the glomerulus and expansion of Bowman's space,	(68)
Juvenile	4.28 µg/L	40 days	↑ CAT in gill; CAT in liver ↓ GSH-Px in liver	(69)
	42.8, 428 µg/L		↑ MDA in liver, MDA in gill ↓ CAT in liver, CAT in gill; SOD in liver, SOD in gill, GSH-Px in liver, GSH-Px in gill; different degrees of hydropic degeneration of liver , vacuolisation, pyknotic nuclei, and fatty infiltration; varied degrees of epithelial hypertrophy in gill, telangiectasis, oedema with epithelial separation from basement membranes, general necrosis, and epithelial desquamation	
Juvenile	428 µg/L	40 days	↑ mRNA levels of IL-1 beta, mRNA levels of IL-1R1	(70)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↓ RNA levels of AChE in brain and muscle	(71)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↓ AChE, CbE in brain and muscle	(72)
Juvenile	< 7 µg/L	14 days	induction cytochrome P4501A1	(73)
	< 100 µg/L		↑ DNA strand breaks	
Embryo - larvae	0.3 µg/L	30 days	↑ GPx, GST, SOD, CAT, GR	(74)
	30 µg/L		↓ GR	
	100, 300 µg/L		↑ TBARS, ↓ GR	

Table 3: The effect of atrazine on zebrafish

Development stage	Concentration	Exposure	Effects	Reference
Juvenile	0.3 µg/L	28 days	↑ GPx, GR; ↓ CAT	(75)
	3 µg/L		↑ GPx; ↓ CAT	
	30 µg/L		↑ GPx, GR, SOD, TBARS; ↓ CAT	
	90 µg/L		↑ GPx, SOD, TBARS; ↓ CAT	
	25 µg/L		scattered lesions in gill	
Juvenile	90 µg/L	28 days	↓ growth rates; dystrophic lesions of hepatocytes; ↑ MRCs in filament epithelium of gill	(76)
Juvenile	2.5 µg/L	21 days	↑ SOD, CAT	(77)
	2.5, 5, 10 µg/L	14, 21 days	↑ POD	
Adult – female	10 µg/L	14 days	↑ SOD in ovary, CAT in ovary; ↓ GSH in liver	(78)
	100 µg/L		↑ SOD in liver, MDA in liver; ↓ GSH in liver	
	1000 µg/L		↑ SOD in liver, CAT in liver, MDA in liver; ↓ GSH in liver	
Adult – female	0.01, 0.1, 1 mg/L	10, 15 days	↑ cytochrome P450 content, APND, ERND	(79)
	0.01, 0.1, 1 mg/L	20, 25 days	↑ APND, ERND, NCR	
Adult – male	0.01, 0.1, 1 mg/L	10, 15 days	↑ cytochrome P450 content, NCR, APND, ERND	
	0.1 mg/L	20, 25 days	↑ cytochrome P450 content, APND	
Embryo - larvae	4 mg/L	48 hours	disturbed the normal development to long pec stage	(80)
	10-20 mg/L		retardations in organogenesis, a slowdown of movements, and functional disturbances of heart and circulatory system	
Embryo - larvae	5 mg/L	48 hours	↑ soluble (s) and microsomal (m) GST	(81)

Effect of atrazine on fish

Effects of atrazine on fish physiology, have been well-documented. Its effect is the best described from all triazines. Atrazine affected hematological, biochemical profile, antioxidant enzymes, oxidative stress indices, growth and caused histopatological changes in tissues. The effects of atrazine are mentioned on carp (Tab. 2.), zebrafish (Tab. 3.), Salmonidae (Tab. 4.), other fish (Tab. 5.). In a study conducted by Ventura et al. (59), it was observed that the herbicide atrazine has a genotoxic and mutagenic effect. In this study, the authors observed that the herbicide can interfere in the genetic material of the organisms exposed, even at doses considered residual, which led the authors to suggest that residual doses of atrazine, resulting from leaching of soils of crops near water bodies, can interfere in a negative form in the stability of aquatic ecosystems. The bioaccumulation factors for atrazine in the liver, muscle, heart, gonads and brain of banded tilapia (*Tilapia sparrmanii*) is ranged from 0.9 to 20.0 (60).

Metribuzine

Metribuzine (4-amino-6-tert-butyl-3-(methythio)-1,2,4-triazin-5-one) is an asymmetrical triazine herbicide. It is distinct from the symmetrical

Table 4: The effect of atrazine on Salmonidae

Species	Concentration	Exposure	Effects	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	555 µg/L	4 days	↑ cortisol, monocytes; ↓ SSI, lymphocytes	(82)
Atlantic salmon (*Salmo salar* L) Smolts	100 µg/L	21 days	↓ feeding, Cl^-, Mg^{2+}, Na^+, Ca^{2+}; ↑ cortisol	(83)
Atlantic salmon (*Salmo salar* L) Smolts	2 µg/L	7 days	↓ Na^+K^+ATPase in gill	(84)
	5, 10 µg/L		↑ cortisol; ↓ Na^+K^+ATPase in gill	
Atlantic salmon (*Salmo salar* L) Smolts	atrazine (1 µg/L) + 4-nonylphenol (5 µg/L)	7 days	↑ Na^+K^+ATPase in gill, plasma Cl^-, Na^+	(85)
	atrazine (2 µg/L) + 4-nonylphenol (10 µg/L)		↑ plasma Cl^-, Na^+; ↓ Na^+K^+ATPase in gill	
Atlantic salmon (*Salmo salar* L.) Adult - male	above 0.04 µg/L	shorten	↓ 17,20 beta-dihydroxy-4-pregnen-3-one in plasma and milt	(86)
Rainbow trout (*Oncorhynchus mykiss*) Renal tubules	10, 20, 40, 80, 160 µg/L	4 weeks	In PS I - proliferation of smooth endoplasmic reticulum, atypical mitochondria and lysosomes, as well as gradual alterations of the apical plasmalemma; In PS II - cells proliferation of peroxisomes, ring- and cup-shaped mitochondria, alterations in the basal labyrinth; in DS cells, proliferation of atypical mitochondria with longitudinally oriented cristae, disorganization of Golgi fields and vacuolization of the cell base.	(87)

triazines such as atrazine and simazine, in which the central ring structure has alternating carbon and nitrogen atoms, in that metribuzin possesses two nitrogen atoms and two adjacent carbon atoms. It was first registered as a pesticide in the U.S. in 1973. Metribuzine is used to selectively control certain broadleaf weeds and grassy weed species on a wide range of sites including vegetable and field crops, turf grasses in recreational areas, and non-crop areas (103). Metribuzine is applied by various methods including aerial, chemigation, and ground application (103, 104).

Environmental fate

Metribuzine, like other triazine and triazinone herbicides, is prone to runoff into surface waters due to its physical and chemical characteristics: water solubility 1.220 mg/L; Koc 41; vapor pressure 1.3 mPa; and soil half-life 30 days (104, 105). The degradation of metribuzine is through photochemical, chemical and biochemical deamination. Aqueous photolysis of metribuzin is rapid with a half-life of <1 day, and this clearly contributes to the half-life of <7 days in natural pond water. Contamination of waters could result from spray and vapour drift, runoff or leaching from treated land, or from accidental spills. Measured environmental concentrations of metribuzine in water are usually low, with maximum concentrations below 1.8 µg/L (106), but modelling studies have indicated that metribuzine can reach concentrations as high as 390 g/L in surface water runoff (104).

Acute toxicity

During the acute exposure of metribuzine fish show increased respiration and loss of movement and coordination. Fish lying on the bottom of the tank and moving in circles, followed by a short excitation stage (convulsions). Necropsy after acute exposure can revealed increased watery mucus on body surfaces, black pigmentation of the skin, and abdominal distention with generalized edema. The body cavity contains transudate, and hyperemia of visceral organs and ascites (26).

Acute toxicity 96hLC50 of metribuzine for fish

Table 5: The effect of atrazine on other fish

Species	Concentration	Exposure	Effects	Reference
Rhamdia quelen Juvenile	2, 10, 100 µg/L	96 hours	↓ CAT, GST, GPx, GR, leukocyte infiltration, hepatocyte vacuolization like steatosis and necrosis areas, leading to raised lesion index levels in all tested concentrations. ↑ free melanomacrophage	(88)
Prochilodus lineatus Juvenile	2, 10 pg/L	24, 48 hours	↓ EROD, ROS, CAT, SOD, GPx, GR, MDA in liver	(89)
Silver catfish (Rhamdia quelen) Juvenile	1.02 mg/L	24 hours	↓ bactericidal activity of the serum, bacteria agglutination, total serum peroxidase activity	(90)
Prochilodus lineatus Juvenile	10 µg/L	14 days	↑ GST, SOD, CAT, LPO	(91)
	25 µg/L		scattered lesions in gill	
Prochilodus lineatus Juvenile	25 µg/L	48 hours	↓ osmolarity	(92)
		14 days	↓ CA; ↑ Na+, Cl-, MRCs in filament epithelium of gill	
Rhamdia quelen Juvenile	0.73 mg/L	96 hours	↓ intracelomatic cells, phagocytic index	(93)
Fathead minnow (*Pimephales promelas*) Adult	0.5, 5.0, 50 µg/L	30 days	↓ production of egg; pathological lesions in testes: granulomatous inflammations, mineralized material in testicular tubules and efferent ducts at rates, variably-sized perinucleolar stage oocytes	(94)
Green Snakehead (*Channa punctata*) Juvenile	4.238 mg/L	5, 7, 10, 15 days	↑ SOD	(58)
	5.3, 10.6 mg/L		↑ SOD, TBARS, CAT	
Rare minnow (*Grobiocypris rarus*) Adult – male	333 µg/L	28 days	↑ HSI, hypertrophy of hepatocytes	(95)
Rare minnow (*Grobiocypris rarus*) Adult	3, 10 µg/L	28 days	lesions in gill including hyperplasia, necrosis in epithelium region, aneurysm and lamellar fusion; lesions in kidney included extensive expansion in the lumen, degenerative and necrotic changes of the tubular epithelia, shrinkage of the glomerulus, increase of the Bowman's space	(96)
Caquetaia kraussii Juvenile	2.5 µg/L	72 hours	hepatocytes lost the cytoarchitecture (the hepatocytes have different diameters and irregular contour); isolated associations between mitochondria and rough endoplasmic reticulum in the cytoplasm	(97)
Rhamdia quelen Juvenile	3.5, 5.25 mg/L Herbimix® (simazine + atzrazine)	96 hours	↑ cortisol	(98)
Goldfish (*Carassius auratus L.*) Juvenile	1 000 µg/L	56 days	↑ 11-KT	(99)
Red drum (*Sciaenops ocellatus*) Larvae	40, 80 µg/L	4 days	↓ growth; behaviour: swam significantly faster, with a higher rate of travel, active swimming speed, hyperactive, swam considerably more convoluted paths compared to control	(100)
Goldfish (*Carassius auratus*) Juvenile	0.5 µg/L	24 hours	↓ sheltering, grouping behavior, burst swimming; ↑ surfacing activity	(101)
Mormyrid fish (*Gnathonemus petersii*) Juvenile	0.5, 5 mg/L	6 hours	breaks in the gill epithelium, which developed into deep pits	(102)

is ranging from units to hundreds milligrams per liter (Tab. 1.).

Effect of metribuzine on fish

The effects of metribuzine on fish physiology have been well-documented. Metribuzine affected hematological, biochemical profile, growth and caused hitopatological changes in tissues (Tab. 6.). During acute poisoning of metribuzin in rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio*), the following clinical symptoms are observed: accelerated respiration, loss of movement coordination, fish lying on their flanks and moving in this position. The subsequent short excitation stage (convulsions, jumps above the water surface, movement in circles) changes into a resting stage and another short-time excitation follows again. In the end, fish fall into damp, moving mainly on their flanks. The respiration is slowed down, and the damp phase and subsequent agony are very long. Fish are produceds of watery mucus on body surfaces, the skin is matt dark in colour and the ventricle expansion. The body cavity contained transudate, and an increased injection of visceral vessels is also obtained (26, 107).

Prometryne

Prometryne (2,4-bis(isopropylamino)-6-methylthio-s-triazine) was the first effective herbicide for several crops, making it a true pioneer herbicide in the methylthiotriazine class of chemistry (112) and was first registered in 1964 by Ciba Crop Protection (113). Prometryne is selective herbicide of the s-triazine chemical 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. Prometryn's mechanism of action inhibits the electron transport in susceptible species (114). Prometryne application is not permitted in Europe, but is widely used in China (115), Australia, Canada, New Zealand, South Africa, and the United States (28).

Environmental fate

Prometryne is usually soil-applied and relatively water soluble, it tends to accumulate in crops (114). Prometryne binds readily to soils with high clay and organic matter content. Available data indicate that this herbicide is mobile in sandy soils and moderately mobile in sandy loam soils. Its mobility appears to be related to organic content of the soil. Prometryne the lower the organic content, the more mobile prometryne is in soil. Prometryne is adsorbed to a greater extent than most other commercial triazine herbicides (116). Prometryn is a persistent chemical, it is persists in the soil from one to three months. Its soil half-life is 60 days. Following multiple annual applications of the herbicide, prometryne activity can persist for 12-18 months after the last application. It will persist longer under dry or cold conditions which are not conducive to chemical or biological activity. It resists abiotic hydrolysis, direct photolysis, and biodegradation under anaerobic conditions. Its half-life under aerobic conditions is in excess of 270 days (117).

Significant traces of prometryne are documented in the environment, mainly in water, soil, and plants used for human and domestic animal consumption. Maximal environmental concentration prometryne is 0.51 μg/L in the Czech rivers (14). In surface waters of Greece, prometryne has been recorded at concentrations from 0.19 to 4.40 μg/L (118). Prometryne to contaminate the groundwater resources of the Axios river basin in Macedonia, Northern Greece, during 1992–1994 were detected at concentrations occasionally exceeding 1 μg/L (118). In surface water of Western France, remains of prometryne were detected at concentrations from 0.1 to 0.44 μg/L (119).

Acute toxicity

Exposure prometryne to nontarget organisms can result from direct applications, spray drift, and runoff from treated areas. Studies indicate that prometryne poses an acute risk to nonendagered and endangered terrestrial and aquatic plants (113). Prometryne is toxic to fish (Tab. 1.). The most sensitive aquatic organisms are freshwater algae (14).

Effect of prometryne on fish

Although the lethal toxicity of fish to prometryne, have been well-documented, there is a dearth of data on the effects of prometryne on fish physiology.

Table 6: Effect of metribuzine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Bluegill (*Lepomis macrochirus*) Juvenile	9, 19, 38, 75 µg/L	6 weeks	No effects on fish survival and growth	(103)
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	89.3 mg/l Sencor 70 WG (active substance 70% of metribuzin)	96 hours	↓ TP, TAG, AST, NH3, Ca, LACT, ALP, RBC, PCV, lymphocyte coun. ↑ MCH, relative and absolute count of neutrophile granulocytes 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 the caudal kidney.	(107)
Common carp (*Cyprinus carpio*) Juvenile	1.75 mg/L	28 days	↑ RBC, PCV	(108)
Common carp (*Cyprinus carpio*) Juvenile	250.2 mg/L Sencor 70 WG (active substance 70% of metribuzin)	96 hours	↑ GLU, NH3, Ca, monocytes, neutrophile granulocytes, developmental forms myeloid sequence,basophiles. ↓ TP, ALB, GLOB, TAG, LDH, LACT, PHOS, PCV, Hb, MCV, WBC, lymphocyte Revealed hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney.	(26)
Common carp (*Cyprinus carpio*) Embryo - larvae	0.9, 4, 14, 32 mg/L	30 days	↑ GST	(109)
	0.9, 4, 14 mg/L		↑ GR	
	0.9 mg/L		↑ TBARS	
Common carp (*Cyprinus carpio*) Embryo - larvae	0.9, 4, 14, 32 mg/L	30 days	↓ specific growth rate, body weight, length	(110)
	32 mg/L		Diffuse vacuolization of the cytoplasm of hepatocytes, often with compression of nuclei at the periphery of the cells. Monocellular necroses of hepatocytes. Eosinophilia of tubular epithelial cells with coagulation of cytoplasm and desquamation of necrotic cells into the lumen of proximal tubules in the caudal kidney.	
Zebrafish (*Danio rerio*) Juvenile	33, 55 mg/L	28 days	↓ specific growth rate, body weight, length	(111)
	55 mg/L		Moderate dystrophic lesions of hepatocytes, initial cell injury represented by diffuse hydropic to vacuolar degeneration of hepatocytes.	

Only three studies on effects of prometryne on carp physiology have been conducted (Tab. 7.). Chronic exposure has no influence on growth, oxidative stress biomarkers and it has influence on hematological, biochemical plasma indices, antioxidant enzymes and caudal kidney (120-122).

Simazine

Simazine (6-chlor-N2,N4-diethyl-1,3,5-triazin-2,4-diamin) is one of the first compound triazines (a six-membered ring containing three carbon and three nitrogen atoms), was introduced by a Swiss company J. R. Geigy in 1956 and was registered in 1957 (5). From 1990 to 1993 are among the most widely used herbicides in the U.S. Simazine belongs to a group of selective triazine herbicides, is used for a pre- and post-emergence control most weeds field crops as well as in non-crop areas. When applied to the soil is absorbed by leaves and roots, causing inhibition of photosynthesis in whole plants (123). It is biodegradable, is metabolized in plants and soil, both chemical, and microbiological processes (112). It is fairly resistant to physical and chemical dissipation processes in the soil. It is persistent and mobile in the environment (124). Even before 1992 simazine was used to kill submerged (growing in water) weeds and algae in large aquariums, ponds, swimming

Table 7: Effect of prometryne on fish

Species	Concentration	Exposition	Effects on fish	Reference
Common carp (Cyprinus carpio) Embryo - larvae	0.51, 80, 1 200 µg/L	35 days	↓ GR activity	(120)
Common carp (Cyprinus carpio) Juvenile	80 µg/L	14 days	↓ GR in brain, SOD in intestine	(121)
	8, 80 µg/L		↓ SOD in gill, ↑ SOD in brain	
	0.51, 8, 80 µg/L		↑ GR in muscle	
	8, 80 µg/L	30 day	↓ SOD in brain	
	0.51, 8, 80 µg/L		↓ SOD in gill	
	80 µg/L	60 days	↑ CAT in intestine, ↓ CAT liver, SOD in gill	
Common carp (Cyprinus carpio) Juvenile	80 µg/L	30 days	↑ GLU	(122)
	8, 80 µg/L	60 days	↑ GLU, MCH, MCHC, Hb ↓ SW, LACT	
	0.51, 8, 80 µg/L	30, 60 days	↑ CK, ALT, ↓ AST, Ca, Mg, PHOS	
		60 days	Hyaline degeneration of the epithelial cells of caudal kidney tubules	

pools or cooling towers (125). 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 (6, 126).

Environmental fate

Simazine in soil and groundwater is moderately persistent with an average field half-life of 60 days. Soil half-lives have been reported of 28-149 days (127). Residual activity may remain for a year after application (2 to 4 kg/ha) in high pH soils. Simazine is moderately to poorly bounds to soils (105). Simazine is metabolized in plants and soil, both chemical, and microbiological processes (125). It does, however, adsorb to clays and mucks. It is low water solubility, however, makes it less mobile, limiting it is leaching potential. Simazine has little, if any, lateral movement in soil, but can be washed along with soil particles in runoff. Simazine is subject to decomposition by ultraviolet radiation, but this effect is small under normal field conditions. Loss from volatilization is also insignificant. In soils, microbial activity probably accounts for decomposition of a significant amount of simazine in high pH soils. In lower pH soils, hydrolysis will occur (48).

Simazine can be persistent in aquatic systems, particularly in shallow, well-mixed lakes and ponds (128). Residues may persist up to 3 years in soil under aquatic field conditions. Dissipation of simazine in pond and lake water has been found to be variable, with half-life ranging from 50 to 700 days (105). Slow biodegradation of simazine may occur in water, similar to that observed in soil. Simazine may undergo hydrolysis at lower pH. It does not readily undergo hydrolysis in water at pH = 7 (48). Simazine and its degradation products are detected less frequently than atrazine in the aquatic environment.

Simazine is the second most commonly detected pesticide in surface and ground waters in the U.S., Europe, and Australia. Simazine, and its major degradation products (deisopropyl atrazine and diamino chlorotriazine), have been extensively monitored in 20 counties in California with concentrations ranging from 0.02 to 49.2 µg/L (129, 130). Simazine levels can reach values, up to 5.0 µg/L in Europe rivers (131-134).

Table 8: Effect of simazine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Seabream (*Sparus aurata*) Larvae	4.5 mg/L	72 hours	Cellular alterations related to loss of cellular shape in hepatocytes, lipid inclusions, focal necrosis and abundant nuclear pyknosis in the hepatocytes.	(136)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	90 days	↑ mucus production during the experiment, Hyperplasia of epithelial cells of seconadary lamellae, slight necrosis	(137)
Goldfish (*Carassius auratus*) Adult	50 µg/L ∑ atrazine +simazine + diuron + isoproturon	4, 8, 12 weeks	↑ plasma lysozyme aktivity; production of O2.– in spleen, kidney; SOD in spleen and liver; ↓ antibody titre, CAT in liver, spleen, kidney	(138)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	90 days	↓ AChE in brain and muscle	(139)
Rhamdia quelen Juvenile	16.6%, 33% 50% 96h LC50 hatrazine + simazine (Herbimix™)	96 hours	Decreased capacity in exhibiting an adequate response to cope with stress and in maintaining the homeostasis, with cortisol level lower than that in the control fish	(140)
Common carp (*Cyprinus carpio*) Juvenile	4, 20, 50 µg/L	28 days	↑ PCV, lymphocytes, developmental phases –myeloid sequence, GLU, LDH, CK, CREA; ↓ MCHC, neutrophil granulocytes bands, NH3, AST Decline in hematopoietic tissue in caudal kidney; steatosis, hyperaemia, and necrosis in liver	(141)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	15, 30, 45, 90 days	No efect on muscle LACT, LDH	(142)
			↑ mucus hyperproduction in gills and skin; No efect on MDA and GSH	(143)
		90 days	↑ PCV, necrotic areas in hematopoietic and excretory tissues of the kidneys; Isolated necrotic areas in liver	(144)
Rhamdia quelen Juvenile	16.6% 96h LC50 hatrazine + simazine (Herbimix™)	96 hours	↑ plama cortisol	(145)
Zebrafish (*Danio rerio*) Juvenile	60 µg/L	28 days	Hypertrophy, hyperplasia of epietelial gill cells with lamellar fusion. Initial cell injury represented by swelling and hydroscopic vacuolar degeneration of hepatocytes). Coagulation of the apical part of the cytoplasma of epithelial cells of the renal tubules	(146)
Common carp (*Cyprinus carpio*) Embryo - larvae	60 µg/L	35 days	Alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules	(147)
	0,6, 3 mg/L		↓ growth; alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules	
Common carp (*Cyprinus carpio*) Juvenile	0.06 µg/L	90 days	↑ ALP; ↓ WBC; hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	(148)
	1, 2 µg/L		↑ HSI, ALP, AST; ↓ WBC; hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	
	4 µg/L		↑ HSI, TP, ALB, AST, ALP; ↓ WBC hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	

Common carp (Cyprinus carpio) Juvenile	0.06 µg/L	28 days	↑ GSH in liver;	(149)
		60 days	↑ CAT in muscle, GSH in liver	
	2 mg/L	14 days	↑ SOD in muscle; CAT in muscle, liver; GSH in liver; ↓ GPx in liver	
		28 days	↑ SOD in muscle CAT in muscle, liver; GSH in liver; ↓ GPx in liver	
		60 days	↑ ROS in liver; GSH in liver, brain; ↓ SOD in muscle; CAT in muscle, liver;	
	4 mg/L	14 days	↑ CAT in liver; SOD in muscle; GSH in liver, brain; ↓ GPx in liver	
		28 days	↑ ROS in liver; SOD in muscle; GSH in liver, brain; ↓ GPx in liver	
		60 days	↑ ROS in muscle, brain, liver; GST in brain; ↓ GST and GPx in liver, SOD in muscle; CAT in brain, liver, muscle	

Acute toxicity

Simazine was identified as relevant a study of the prioritization of substances dangerous to the aquatic environment in the member states of the European Community (10). Lethal acute toxicity for fish is ranging from units to hundreds milligrams per liter (Tab. 1.).

Effect of simazine on fish

The effects of simazine mainly on carp physiology have been well-documented in laboratory studies. Chronic exposure of simazine has influence mainly on growth, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, and caused histopathological changes in gill, liver and kidney (Tab. 8.). Simazine has been recently reported as suspected endocrine disruptors, it is also known to cause multiple types of cancers (135).

Terbuthylazine

Terbuthylazine (N-tert-butyl-6-chloro-N'-ethyl-1,3,5-triazine-2,4-diamine) was registered in the United States in 1975 (150). Terbuthylazine is herbicide that belongs to the chlorotriazine family, is used in both pre- and post-emergence treatment of a variety of agricultural crops and in forestry (118). Terbuthylazine have very similar chemical structure to atrazine. The difference is only iso-butyl and tert-butyl substituent on the amino group. The minimum difference in structure affects the decomposition reactions of these substances in the environment that led to a ban on atrazine in the European Union. The EU had more stringent drinking water standards caused farmers to shift from atrazine to terbuthylazine. Terbuthylazine is used as a substitute for atrazine since the end of 2006 (151). Terbuthylazine breaks down much more rapidly than atrazine in both soil and water, and is therefore believed less likely to contaminate drinking water (152).

Environmental fate

Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions (150). Terbuthylazine is a slightly basic, slightly water soluble triazine herbicide or algacide which adsorbs to soil organic matter. Degradation of terbuthylazine in natural water depends on the presence of sediments and biological activity (124). Under laboratory conditions, aquatic photolytic half-lives ranged from around 3 hours (attenuated) to a more realistic 1.5-5 days under more usual test conditions that seem to be reflected in the recommended use pattern. Usually, the main degradation product was hydroxy-terbuthylazine, although with an attenuator N-dealkylation is favoured. Laboratory studies in soils (sandy loam) gave half-lives of 73-138 days at 20-25 °C, but this extended to 456 days at 10 °C, with hydroxy-terbuthylazine and desethyl-terbuthylazine as the

Table 9: Effect of terbuthylazine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	35.1, 42.9, 45.8 µg/L	7 days	↓ EROD, UDPGT	(159)
European sea bass (*Dicentrarchus labrax* L.) Juvenile	3.55, 5.01, 7.08 mg/L	24 hours	↑ RCs in gills, intestine, kidney histopatological examination displayed cellular and/or ultrastructural alterations in all the organs examined. In the gills necrosis, lamellar and cellular oedema, epithelial lifting, telangectasia, and fusion of secondary lamellae were encountered. The liver presented myelin-like figures, cytoplasmic rarefaction and acute cell swelling of hepatocytes. The renal tubular epithelial cells, exhibited 'blebs'.	(158)
		48 hours	↑ RCs in gills, intestine histopatological examination displayed cellular and/or ultrastructural alterations in all the organs examined. In the gills necrosis, lamellar and cellular oedema, epithelial lifting, telangectasia, and fusion of secondary lamellae were encountered. The liver presented myelin-like figures, cytoplasmic rarefaction and acute cell swelling of hepatocytes. The renal tubular epithelial cells, exhibited 'blebs'.	
Common carp (*Cyprinus carpio*) Juvenile	550 µg/L	91 days	↑ TAG, ALB, Na, TP, EC, FRAP ↓. MCHC, MCH, MCV, AST, P	(160)
	60 µg/L		↑ TAG, ALB ↓. MCH, MCV, AST, P	
	380 ng/L		↑ HSI, CF, TAG, TP	
Common carp (*Cyprinus carpio*) Juvenile	13.0 mg/L Gardoprim Plus Gold 500 SC (corresponding to 2.25 mg/L terbuthylazine and 3.75 mg/L S-metolachlor	96 hours	↑ GLU, AST, NH3, LDH ↓ lymphocyte counts, WBC, PCV, PHOS, TAG, chlorides lesions in gills and liver	(161)
Common carp (*Cyprinus carpio*) Embryo - larvae	520 µg.L^{-1}	30 days	↑ GR	(109)
Zebrafish (*Danio rerio*) Juvenile	400 µg/L	28 days	↑ GST	(162)
	700 µg/L		↑ GR, GST, pathological changes in the liver	
	1000 µg/L		↑ GR, GST, TBARS, pathological changes in the liver	
Common carp (*Cyprinus carpio*) Embryo - larvae	520, 820 µg/L	30 days	↓ specific growth and body weight, delay in development, mild leisons in liver including diffuse formation of smal round to oval vacuoles in the cytoplasma of hepatocates	(163)
Common carp (*Cyprinus carpio*) Juvenile	3.3 mg/L	24 hours	↑ GLU, AST, ALT, natrium, chlorides, phosporus, Ca, circulation disorders in gills represented by abundant presence of capillary aneurysms in gill filaments and a local hyperplasia of respirátory epithelium	(164)
Common carp (*Cyprinus carpio*) Embryo - larvae	0.0029, 0.07, 1.4, 3.5 mg/L terbuthylazine-2-hydroxy	26, 35 days	↓ SOD, specific growth and body weight	(165)
	1.4, 3.5mg/L terbuthylazine-2-hydroxy	35 days	damage to caudal kidney tubules, delay in development	

Table 10: Effect of terbutryne on fish

Species	Concentration	Exposition	Effects on fish	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	28.3 29.2, 32.6 µg/L	7 days	↓ EROD, UDPGT	(159)
Seabream (*Sparus aurata*) Larvae	2.5 mg/L terbutryn+triasulfuron	72 hours	cellular alterations related to loss of cellular shape of hepatocytes and intense nuclear pyknosis in the hepatocytes	(175)
Zebrafish (*Danio rerio*) Juvenile	0.6 mg/L	28 days	↓ specific growth; wieght, damage to tubular system of kidneys	(176)
Common carp (*Cyprinus carpio*) Juvenile	2, 20, and 40 µg/L	28 days	↑ RBC, NH3, AST, LDH, CK, LACT ↓ MCV, MCH, CK Diffused steatosis of the liver - the loss of cellular shape and the presence of lipid inclusions in hepatic cells; damage to caudal kidney tubules	(177)
Common carp (*Cyprinus carpio*) Juvenile	0.2, 2 µg/L	90 days	↑ RBC, MCHC, neutrophil granulocyte bands, GLU, AST, LDH, LACT, TBARS in brain, liver; CP in brain, gill; SOD in liver, brain ↓ WBC, MCV, CK, Mg, GR in liver, intestine	(178)
	0.02 µg/L		↑ TBARS in brain, liver, SOD in liver ↓ GR in liver	
Common carp (*Cyprinus carpio*) Embryo - larvae	2 mg/L	30, 36 days	↓ CF	(179)
	0.2, 2 mg/L		delay in development	
	0.02, 0.2, 2 mg/L		Alteration of tubular system in caudal kidney included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomeruli	
	0.00002, 0.02, 0.2, 2 mg/L		↓ mass and total length; damage to caudal kidney tubules	

main degradation products (153). Terbuthylazine photo-degrades in water this is likely to be the main degradation pathway. The fate of residues in aerobic and anaerobic aquatic conditions is similar. The major metabolites of terbuthylazine are the de-chlorinated and N-dealkylated products, which are more mobile than the parent, and exhibit some herbicidal activity when they retain the chlorine atom on the triazine ring plus one alkyl group (152, 153).

Terbuthylazine levels can reach values up to 2.9 µg/L in Europe rivers (40, 154, 155). The groundwater situation in different countries was surveyed by the French Ministry of Agriculture and Fisheries. In Germany and Sweden 22 out of 3204 samples and 6 out of 230 samples were positive for terbuthylazine (above 0.1 µg/L), respectively (156).

Acute toxicity

The ecotoxicity profile of terbuthylazine is typical for a herbicide, with toxic effects mostly apparent towards plants/algae. However, terbuthylazine shows slight toxicity towards fish and shellfish, and variable toxicity towards aquatic crustaceans, from very highly toxic to practically non-toxic (124). Standard toxicity tests with various fish species as nontarget organisms revealed LC50 values between 4.6 and 66 µg/L (Tab. 1.). As a consequence, terbuthylazine might be considered as a moderately or slighty toxic. The acute exposure to terbuthylazine, however, leads to significant alterations of the average swimming velocity on the fish. After a nonuniform initial phase of swimming irritation, an increase in motility can be observed. With every exposure tested, this hyperactivity exceeded any preexposure motility (157).

Effect of terbuthylazine on fish

Exposure to terbuthylazine affected on growth, oxidative stress biomarkers, hematological, biochemical plasma indices, antioxidant enzymes, detoxification enzymes and caused the histopatological changes in gill, liver, intestine and kidney (Tab. 9.). Fish during the terbuthylazine intoxication showed uncoordinated swimming and hyporeflexia increasing (158).

Terbutryne

Terbutryne (N2-tert-butyl-N4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) was used as a selective pre- and early post- emergence controll agent of most grasses and many annual broadleaved weeds for a variety of crops, such as cereals, legumes, and tree fruits. It is also used as a herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs, and fish ponds (166, 167). Large quantities of terbutryne have been used since the mid-1980s (168). Terbutryne and plant protection products containing this substance were banned in 2005 by Commission Decision 2004/247/CE.

Environmental fate

Terbutryne degrades slowly, with a half-life of 240 and 180 days in pond and river sediments, respectively (169). Its tendency to move from treated soils into water compartments through water runoff and leaching has been demonstrated, and residual amounts of terbutryne and its metabolites have been found in drinking water and industrial food products long after application (170). The application of terbutryne has been banned in many countries because it has the potential to bioaccumulate in organisms, but it has been still detected in water environment (171). The highest concentration reported in surface water in the Weschnitz River, Germany, at a maximal concentration of 5.6 μg/L from September 2003 to September 2006 (172). Terbutryn was also detected in Mediterranean coastal waters at a concentration of 5-184 ng/L (173).

Acute toxicity

Acute toxicity 96hLC50 of terbutryne for fish is ranging from units of milligrams per liter. Terbutryne is toxic to fish (Tab. 1.).

Effect of terbutryne on fish

The effects of terbutryne mainly on carp, zebrafish and rainbow trout, physiology have been documented in laboratory studies. Chronic exposure of terbutryne has influence mainly on growth, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney (Tab. 10.). The results demonstrate that the terbutryne accumulated to a somewhat greater extent in the viscera (liver, intestine, and pyloric caeca) than in the muscle tissue of the carp and trout during exposure (169, 174). Bioconcentration factors (BCFs) of terbutryne for fish were estimated 312 (169).

Conclusion

Triazines are predominant class of herbicide. They are most frequently detected pesticide in aquatic environment. Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and they are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. All of above cited seven triazines are banned or severely restricted in EU (180). Acute toxicity was assessment on 28 fish species. Toxic effect of triazine has influence mainly on growth, early development, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney. Investigation of triazine and their metabolites properties in connection with environment, chronic effects and potential bioaccumulation must continue thoroughly. Research on non-target species should be really detailed and should continue because as can be seen in the previous text, triazines are able to cause pathological changes in fish. We assume

that triazines and their metabolites have similar effects on other non-target organisms as to have on fish. As shown some studies on crayfish (181-183). It is necessary to focus on the research of triazines metabolites using new molecular techniques and gene expression.

Acknowledgements

The study was financially supported by the projects „CENAKVA "(No.CZ.1.05/2.1.00/01.0024), "CENAKVA II "(No. LO1205 under the NPU I program), and by the GAJU No. 018/2014/Z.

References

1. Abrantes N, Pereira R, Gonçalves F. Occurrence of pesticides in water, sediments, and fish tissues in a lake surrounded by agricultural lands: concerning risks to humans and ecological receptors. Water Air Pollut 2010; 212: 77–88.

2. Figueiredo-Fernandes A, Fontainhas-Fernandes A, Peixoto F, et al. Effects of gender and temperature on oxidative stress enzymes in Nile tilapia Oreochromis niloticus exposed to paraquat. Pestic Biochem Physiol 2006; 85: 97–103.

3. Bermudez-Saldana JM, Escuder-Gilabert L, Medina-Hernandez MJ, et al. Chromatographic evaluation of the toxicity in fish of pesticides. J Chromatogr B 2005; 814: 115–25.

4. Modra H, Svobodova Z. Incidence of animal poisoning cases in the Czech Republic: current situation. Interdiscip Toxicol 2009; 2: 48–51.

5. Kamrin MA. Pesticide profiles. Boca Raton: Lewis Publishers; 1997: 676 p.

6. Sanderson JT, Letcher RJ, Heneweer M, et al. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ Health Persp 2001; 109: 1027–31.

7. Fishel FM. Pesticide toxicity profile: triazine pesticides. Gainesville: University of Florida, IFAS Extension, 2009: 3 p.

8. Chapadense PFG, Castro FJ, Almeina JA, et al. Toxicity of atrazine herbicide in Colossoma macropomum. Rev Bras Saúde Prod Anim 2009; 10: 398–405.

9. Hogan CM. Herbicide. The encyclopedia of earth. Washington: National Council for Science and the Environment, 2010. (online) http://www.eoearth.org/article/Herbicide?topic=49494 (7. 2. 2014)

10. European Commission. Study on the prioritisation of substances dangerous to the aquatic environment. Luxembourg: Office for Official Publications of the European Communities, 1999: 264 p.

11. Bathe R, Sachsse K, Ullmann L, et al. The evaluation of fish toxicity in the laboratory. Proc Eur Soc Toxicol 1975; 16: 113–24.

12. Yokoyama T, Saka H, Fujita S, et al. Sensitivity of Japanese eel, *Anguilla japonica*, to 68 kinds of agricultural chemicals. Bull Agric Chem Insp Stn (Tokyo) 1988; 28: 26–33.

13. Bathe R, Ullmann L, Sachsse K. Determination of pesticide toxicity to fish. Schriftenr Ver Wasser Boden Lufthyg Berlin – Dahlem 1973; 37: 241–56.

14. Pesticide Ecotoxicity Database (online) (Formerly: Environmental Effects Database (EEDB)). Washington.: Environmental Fate and Effects Division, U.S. EPA, 2000.
http://www.ipmcenters.org/ecotox/DataAccess.cfm (7. 2. 2014)

15. Prost M, Studnicka M, Niezgoda J. Porownanie toksycznosci blekitu metylenowego i zieleni malachitowej dla narybku Pstraga teczowego. Med Weter 1975; 31: 226–9.

16. Sastry KV, Sharma K. Effects of mercuric chloride on the activities of brain enzymes in a fresh water Teleost, *Ophiocephalus* (Channa) *punctatus*. Arch Environ Contam Toxicol 1980; 9: 425–30.

17. Hanazato T, Yasuno M. Influence of overwintering Daphnia on spring zooplankton communities: an experimental study. Ecol Res 1989; 4: 323–38.

18. Neskovic NK, Elezovic I, Karan V, et al. Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.). Ecotoxicol Environ Safe 1993; 25: 173–82.

19. Gunkel G, Kausch H. Acute toxicity of atrazine (S-Triazine) on *Coregonus fera* under starvation conditions. Arch Hydrobiol 1976; 48: 207–34.

20. Cossarini-Dunier M. Effects of the pesticides atrazine and lindane and of manganese ions on cellular immunity of carp, *Cyprinus carpio*. J Fish Biol 1987; 31: 67–73.

21. Koenst WM, Smith LL Jr, Broderius SJ. Effect of chronic exposure of brook trout to sublethal concentrations of hydrogen cyanide. Environ Sci Technol 1977; 11: 883–7.

22. Schmid OJ, Mann H. Action of a detergent (dodecylbenzenesulphonate) on the gills of the trout. Arch Fischereiwiss 1961; 1: 41–51.

23. Clemens HP, Sneed KE. Lethal doses of several commercial chemicals for fingerling channel catfish. Washington: U.S. Department of Interior, Fish and Wildlife Service, 1959: 10 p. (Sci Rep Fisheries, no. 316)

24. Mayer FL Jr, Ellersieck MR. Manual of acute toxicity: iterpretation and data base for 410 chemicals and 66 species of freshwater animals. Washington: U.S. Department of Interior, Fish and Wildlife Service, 1986: 63 p. (Res Publ no. 160)

25. Tooby TE, Hursey PA, Alabaster JS. Acute toxicity of 102 pesticides and miscellaneous substances to fish. Chem Ind (Lond.) 1975; 21: 523–6.

26. Velisek J, Svobodova Z, Piackova V, et al. Effects of acute exposure to metribuzin on some hematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Bull Environ Contam Toxicol 2009; 82: 492–5.

27. Popova GV. Characteristics of the effect of the herbicide prometryn on fish. Nauchn Osn Okhr Prir 1976; 4: 118–25.

28. Kegley SE, Hill BR, Orme S, et al. PAN Pesticide Database, Pesticide Action Network, North America. San Francisco, CA: Pesticide Action Network, North America, 2010. http://www.pesticideinfo.org/ (7. 2. 2014)

29. Tscheu-Schluter M. On the acute toxicity of herbicides to selected aquatic organisms. Part 2: triazine herbicides and amitrole. Acta Hydrochim Hydrobiol 1976; 4: 153–70.

30. Fabacher DL, Chambers H. Resistance to herbicides in insecticide-resistant mosquitofish, *Gambusia affinis*. Environ Lett 1974; 7: 15–20.

31. Rao KS, Dad NK. Studies of herbicide toxicity in some freshwater fishes and ectoprocta. J Fish Biol 1979; 14: 517–22.

32. Ku CC, Kapoor IP, Rosen JD. Metabolism of cytrolane (mephosfolan) systemic insecticide [(diethoxyphosphinyl)dithiomidocarbonic acid, cyclic propylene ester] in a simulated rice paddy. J Agric Food Chem 1978; 26: 1352–7.

33. Hashimoto Y, Nishiuchi Y. Establishment of bioassay methods for the evaluation of acute toxicity of pesticides to aquatic organisms. J Pestic Sci 1981; 6: 257–64.

34. Johnson WW, Finley MT. Handbook of acute toxixcity of chemicals to fish and aquatic invertebrates. Washington: U. S. Deprtment Interor, Fish and Wildlife Service, 1980: 106 p. (Res Publ No. 137)

35. NHI (National Health Institute). Guidelines of the Italian CCTN (National Advisory Toxicological Committee) for the classification of some effects of chemical substances. In: Mucci N, Camoni I., eds. Rome: National Health Institute, 1996: 12–6. (National Health Institute Rep Ser No. 2)

36. Tooby TE, Lucey J, Stott B. The tolerance of grass carp, *Ctenopharyngodon idella* Val., to aquatic herbicides. J Fish Biol 1980; 16: 591–7.

37. U.S. EPA. Reregistration eligibility decision (RED) for ametryn. Washington: U. S. Environmental Protection Agency, 2005: 95 p. http://www.epa.gov/pesticides/reregistration/REDs/ametryn_red.pdf (13. 2. 2014)

38. U. S. EPA. Pesticide reregistration status, 2014. (online) Washington: U. S. Environmemntal Protection Agencyhttp://www.epa.gov/pesticides/reregistration/status.htm (13. 2. 2014)

39. Jacomini AE, de Camargo PB, Avelar WEP, et al. Assessment of ametryn contamination in river water, river sediment, and mollusk Bivalves in São Paulo State, Brazil. Arch Environ Contam Toxicol 2010; 60: 452–61.

40. CHMI. On-line water quality database. Prague: Czech Hydrometeorological Institute, Department of Water Quality, 2005. http://hydro.chmi.cz/oj (2. 2. 2011)

41. Bocquene G, Franco A. Pesticide contamination of the coastline of Martinique. Mar Pollut Bull 2005; 51: 612–9.

42. Cerejeira MJ, Viana P, Batista S, et al. Pesticides in Portuguese surface and ground waters. Water Res 2003; 37: 1055–63.

43. Laabs V, Amelung W, Pinto AA, et al. Pesticides in surface water, sediment, and rainfall of the northeastern Pantanal basin, Brazil. J Environ Qual 2002; 31: 1636–48.

44. Ametryn: material safety data sheet. Wenzhou Zhejiang, China: Zhejiang Rayfull Chemicals service http://www.rayfull.com/UploadFiles/PDF/201368843203.pdf (12. 4. 2014)

45. Abohegas S, Assem H, Kandil A. Toxic effects of environmental pollutants on the carbohydrate metabolism in grass carp (*Ctanopharyngodon Idella*). Zool Jahrbuch Abteil Allgem Zoolog Physiol Tier 1992; 2: 255–62.

46. Moura MAM, Domingues I, Oliveira R, et al. Efeito da ametrina a em larves e adultos de paulistinha (*Danio rerio*). Biol São Paulo 2011; 73: 330–5.

47. Botelho RG, Rossi ML, Maranho LA, et al. Evaluation of surface water quality using an eco-

toxicological approach: a case study of the Piracicaba river (Sao Paulo, Brazil). Environ Sci Pollut Res 2013; 20: 4382–95.

48. Ahrens WH, Hatzios KK, Edwards MT. Herbicide Handbook Committee. Lawrence, Kansas: Weed Science Society of America, 1994: 352.

49. Moreland DE. Mechanisms of action of herbicides. Ann Rev Plant Physiol 1980; 31: 597–638.

50. Petit F, Le Goff P, Cravedi J, et al. Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J Molecul Endocrinol 1997; 19: 321–35.

51. Dodson SI, Merritt CM, Shannahan J, et al. Low exposure concentrations of atrazine increase male production in *Daphnia pulicaria*. Environ Toxicol Chem 1999; 18: 1568–73.

52. Sanderson JT, Seinen W, Giesy JP, et al. 2-chloro-S-triazine herbicides induce aromatase (CYP-19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity. Toxicol Sci 2000; 54:121–7.

53. Fernando MD, Alcaron V, Ferandez-Casalderrey A, et al. Persistence of some pesticides in the aquatic environment. Bull Environ Contam Toxicol 1992; 48: 747–55.

54. Radosevich M, Traina SJ, Tuovinen OH. Biodegradation of atrazine in surface soils and subsurface sediments collected from an agricultural research farm. Biodegradation 1996; 7: 137–49.

55. Bishop CA, Mahony NA, Struger J, et al. Anuran development, density and diversity in relation to agricultural activity in the Holland River watershed, Ontario, Canada (1990–1992). Environ Monitor Assess 1999; 57: 21–43.

56. Perry C. Source, extent, and degradation of herbicides in a shallow water aquifer near Hesston, Kansas. Water-resources investigations report 91-4019. Lawrence: US Geological Survey, Water Resource Division; Kansas City University, 1990: 30 p.

57. Hall LW Jr, Anderson RD, Kilian J, et al. Concurrent exposure assessments of atrazine and metolachlor in the mainstem, major tributaries and small streams of the Chesapeake bay watershed: indicators of ecological risk. Environ Monit Assess 1999; 59: 155–90.

58. Nwani CD, Lakra WS, Nagpure NS, et al. Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa Punctatus* (Bloch). Int J Environ Res Public Health 2010; 7: 3229–312.

59. Ventura BC, Angelis DF, Marin-Morales MA. Mutagenic and genotoxic effects of the atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic Biochem Physiol 2008; 90: 42–51.

60. du Preez HH, van Vuren JH. Bioconcentration of atrazine in the banded tilapia, *Tilapia sparrmanii*. Comp Biochem Physiol C 1992; 101: 651–5.

61. Xing HJ, Zhang ZW, Yao HD. Effects of atrazine and chlorpyrifos on cytochrome P450 in common carp liver. Chemosphere 2014; 104: 244–50.

62. Blahova J, Modra H, Sevcikova M, et al. Evaluation of biochemical, haematological, and histopathological responses and recovery ability of common carp (*Cyprinus carpio* L.) after acute exposure to atrazine herbicide. BioMed Res Int 2014; 2014: e980948 (8 p.) http://www.hindawi.com/journals/bmri/2014/980948/ (12. 4. 2014)

63. Wang X, Xing HJ, Jiang Y. Accumulation, histopathological effects and response of biochemical markers in the spleens and head kidneys of common carp exposed to atrazine and chlorpyrifos. Food Chem Toxicol 2013; 62: 148–58.

64. Liu T, Zhang ZW, Chen DC, et al. Effect of atrazine and chlorpyrifos exposure on heat shock protein response in the brain of common carp (*Cyprinus carpio* L.). Pestic Biochem Physiol 2013; 107: 277–83.

65. Fu Y, Li M, Liu C, et al. Effect of atrazine and chlorpyrifos exposure on cytochrome P450 contents and enzyme activities in common carp gills. Ecotoxicol Environ Saf 2013; 94: 28–36.

66. Wang LL, Liu T, Wang C, et al. Effects of atrazine and chlorpyrifos on the production of nitric oxide and expression of inducible nitric oxide synthase in the brain of common carp (*Cyprinus carpio* L.). Ecotoxicol Environ Saf 2013; 93: 7–12.

67. Xing HJ, Wu HD, Sun G, et al. Alterations in activity and mRNA expression of acetylcholinesterase in the liver, kidney and gill of common carp exposed to atrazine and chlorpyrifos. Environ Toxicol Pharmacol 2013; 35: 47–54.

68. Xing HJ, Li S, Wang ZL, et al. Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. Chemosphere 2012; 88: 377–83.

69. Xing HJ, Li S, Wang ZL, et al. Oxidative

stress response and histopathological changes due to atrazine and chlorpyrifos exposure in common carp. Pestic Biochem Physiol 2012; 103: 74–80.

70. Wang X, Xing HJ, Li XL, et al. Effects of atrazine and chlorpyrifos on the mRNA levels of IL-1 and IFN-gamma 2b in immune organs of common carp. Fish Shellfish Immun 2011; 31: 126–33.

71. Xing HJ, Han Y, Li S, et al. Alterations in mRNA expression of acetylcholinesterase in brain and muscle of common carp exposed to atrazine and chlorpyrifos. Ecotoxicol Environ Saf 2010; 73: 1666–70.

72. Xing HJ, Wang JT, Li JL, et al. Effects of atrazine and chlorpyrifos on acetylcholinesterase and carboxylesterase in brain and muscle of common carp. Environ Toxicol Pharmacol 2010; 30: 26–30.

73. Chang LW, Toth GP, Gordon DA, et al. Responses of molecular indicators of exposure in mesocosms: common carp (*Cyprinus carpio*) exposed to the herbicides alachlor and atrazine. Environ Toxicol Chem 2005; 24: 190–7.

74. Chromcova L, Blahova J, Plhalova L, et al. The effects of atrazine exposure on early life stages of common carp (*Cyprinus carpio*). Neuroendocrinol Lett 2013; 34: 95–101.

75. Blahova J, Plhalova L, Hostovsky M, et al. Oxidative stress responses in zebrafish Danio rerio after subchronic exposure to atrazine. Food Chem Toxicol 2013; 61: 82–5.

76. Plhalova L, Blahova J, Mikulikova I, et al. Effects of subchronic exposure to atrazine on zebrafish (*Danio rerio*). Polish J Vet Sci 2012; 15: 417–23.

77. Zhu LS, Shao B, Song, Y, et al. DNA damage and effects on antioxidative enzymes in zebra fish (*Danio rerio*) induced by atrazine. Toxicol Mech Methods 2011; 21: 31–6.

78. Jin YX, Zhang XX, Shu LJ, et al. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). Chemosphere 2010; 78: 846–52.

79. Dong XL, Zhu LS, Wang JH, et al. Effects of atrazine on cytochrome P450 enzymes of zebrafish (*Danio rerio*). Chemosphere 2009; 77: 404–12.

80. Wiegand C, Krause E, Steinberg CT, et al. Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). Ecotoxicol Environ Saf 2001; 49: 199–205.

81. Wiegand C, Pflugmacher S, Giese, M, et al. Uptake, toxicity, and effects on detoxication enzymes of atrazine and trifluoroacetate in embryos of zebrafish. Ecotoxicol Environ Saf 2000; 45: 122–31.

82. Shelley LK, Ross PS, Miller KM, et al. Toxicity of atrazine and nonylphenol in juvenile rainbow trout (*Oncorhynchus mykiss*): effects on general health, disease susceptibility and gene expression. Aquatic Toxicol 2012; 124: 217–26.

83. Nieves-Puigdoller K, Bjornsson BT, McCormick SD. Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon. Aquat Toxicol 2007; 84: 27–37.

84. Waring CP, Moore A. The effect of atrazine on Atlantic salmon (*Salmo salar*) smolts in fresh water and after sea water transfer. Aquat Toxicol 2004; 66: 93–104.

85. Moore A, Scott AP, Lower, N, et al. The effects of 4-nonylphenol and atrazine on Atlantic salmon (*Salmo salar* L) smolts. Aquaculture 2003; 222: 1–4.

86. Moore A, Waring CP. Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. Pestic Biochem Physiol 1998; 62: 41–50.

87. Oulmi Y, Negele RD, Braunbeck T. Segment specificity of the cytological response in rainbow trout (*Oncorhynchus mykiss*) renal tubules following prolonged exposure to sublethal concentrations of atrazine. Ecotoxicol Environ saf 1995; 32: 39–50.

88. Mela M, Guiloski IC, Doria HB, et al. Effects of the herbicide atrazine in neotropical catfish (*Rhamdia quelen*). Ecotoxicol Environ Saf 2013; 93: 13–21.

89. Santos TG, Martinez CBR. Atrazine promotes biochemical changes and DNA damage in a Neotropical fish species. Chemosphere 2012; 89: 1118–1125.

90. Kreutz LC, Barcellos LJG, dos Santos ED. Innate immune response of silver catfish (Rhamdia quelen) exposed to atrazine. Fish Shellfish Immunol 2012; 33: 1055–1059.

91. Paulino MG, Souza NES, Fernandes MN. Subchronic exposure to atrazine induces biochemical and histopathological changes in the gills of a neotropical freshwater fish, *Prochilodus lineatus*. Ecotoxicol Environ Saf 2012; 80: 6–13.

92. Paulino MG, Sakuragui MM, Fernandes MN. Effects of atrazine on the gill cells and ionic

balance in a neotropical fish, *Prochilodus lineatus*. Chemosphere 2012; 86: 1–7.

93. Kreutz LC, Barcellos LJG, Marteninghe A, et al. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol 2010; 29: 694–7.

94. Tillitt DE, Papoulias DM, Whyte JJ, et al. Atrazine reduces reproduction in fathead minnow (*Pimephales promelas*). Aquatic Toxicol 2010; 99: 149–59.

95. Yang LH, Zha JM, Zhang XY, et al. Alterations in mRNA expression of steroid receptors and heat shock proteins in the liver of rare minnow (*Grobiocypris rarus*) exposed to atrazine and p,p '-DDE. Aquatic Toxicol 2010; 98: 381–7.

96. Yang LH, Zha JM, Li W, et al. Atrazine affects kidney and adrenal hormones (AHs) related genes expressions of rare minnow (*Gobiocypris rarus*). Aquatic Toxicol 2010; 97: 204–11.

97. de Bravo MIS, Medina J, Marcano S, et al. Ultrastructural alterations of hepatocytes in *Caquetaia kraussi* (Pisces: Cichlidea) due to atrazine. Acta Microscop 2009; 18: 81–4.

98. Cericato L, Machado JG, Fagundes M, et al. Cortisol response to acute stress in jundia *Rhamdia quelen* acutely exposed to sub-lethal concentrations of agrichemicals. Comp Biochem Physiol C 2008; 148: 281–6.

99. Nadzialek S, Spano L, Mandiki SNM, et al. High doses of atrazine do not disrupt activity and expression of aromatase in female gonads of juvenile goldfish (*Carassius auratus* L.). Ecotoxicology 2008; 17: 464–70.

100. Alvarez MD, Fuiman LA. Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae. Aquat Toxicol 2005; 74: 229–41.

101. Saglio P, Trijasse S. Behavioral responses to atrazine and diuron in goldfish. Arch Environ Contam Toxicol 1998; 35: 484–91.

102. Alazemi BM, Lewis JW, Andrews, EB. Gill damage in the freshwater fish Gnathonemus petersii (family: Mormyridae) exposed to selected pollutants: an ultrastructural study. Environ Technol 1996; 17: 225–38.

103. Fairchild JF, Sappington LC. Fate and effects of the triazinone herbicide metribuzin in experimental pond mesocosms. Arch Environ Contam Toxicol 2002; 43: 198–202.

104. Pauli BD, Kent RA, Wong MP. Canadian water quality guidelines for metribuzin. Ottawa : Inland Waters Directorate, Water Quality Branch, 1990: 44 p. (Environ Can Sci Ser no. 179)

105. Wauchope R D, Buttler TM, Hornsby AG, et al. The SCS/ARS/CES pesticide properties database for environmental decision-making. Rev Environ Contam Toxicol 1992; 123: 1–155.

106. Battaglin WA, Furlong ET, Burkhardt MR, et al. Concentrations of selected sulfonylurea, sulfonamide, and imidazolinone herbicides, and other pesticides in storm runoff from 71 streams, outflow from 5 reservoirs, and ground water from 25 Wells in the Midwestern United States, 1998. Denver: U.S. Department of the Interior, U. S. Geological Survey, 2001: 123 p. (Water-Resources Investigations Report 00-4225)

107. Velisek J, Svobodova Z, Pickova V, et al. Effects of metribuzin on rainbow trout (*Oncorhynchus mykiss*). Vet Med 2008; 53: 324–32.

108. Modra H, Haluzova I, Blahova J, et al. Effects of subchronic metribuzin exposure on common carp (*Cyprinus carpio*). Neuroendocrinol Lett 2008; 29: 669–74.

109. Hostovsky M, Blahova J, Plhalova L, et al. Oxidative stress parameters in early developmental stages of common carp (*Cyprinus carpio* L.) after subchronic exposure to terbuthylazine and metribuzin. Neuroendocrinol Lett 2012; 33: 124–9.

110. Stepanova S, Dolezelova P, Plhalova L, et al. The effects of metribuzin on early life stages of common carp (*Cyprinus carpio*). Pestic Biochem Physiol 2012; 103: 152–8.

111. Plhalova L, Stepanova S, Praskova E, et al. The effects of subchronic exposure to metribuzin of *Danio rerio*. ScientificWorldJournal 2012; 2012: e728189 (5 p.) http://www.hindawi.com/journals/tswj/2012/728189/ (12. 4. 2014)

112. LeBaron HM, McFarland JE, Burnside OC. The triazine herbicides: 50 years revolutionizing agriculture. Amsterdam: Elsevier, 2008: 584 p.

113. U.S. EPA. R.E.D. facts prometryn. Wahington: Environmental Protection Agency R.E.D, 1996: 11 p. http://www.epa.gov/pesticides/reregistration/REDs/factsheets/0467fact.pdf (12. 1. 2011)

114. Jiang L, Yang H. Prometryne-induced oxidative stress and impact on antioxidant enzymes in wheat. Ecotoxicol Environ Saf 2009; 72: 1687–93.

115. Zhou J, Chen J, Cheng Y, et al. Determi-

nation of prometryne in water and soil by HPLC–UV using cloud-point extraction. Talanta 2009; 79: 189–93.

116. Beste CE. Herbicide handbook of the Wed Science Society of America. 5th ed. Champaign: The Society, 1983: 515 p.

117. U.S. EPA. Reregistration Eligibility Decision (RED) Prometryn. Washington: U. S. Environmental Protection Agency, 1996: 117 p. http://www.epa.gov/oppsrrd1/REDs/0467.pdf (12. 1. 2014)

118. Vryzas Z, Alexoudisa C, Vassilioua G, et al. Determination and aquatic risk assessment of pesticide residues in riparian drainage canals in northeastern Greece. Ecotoxicol Environ Saf 2011; 74: 174–81.

119. Caquet T, Roucaute M, Mazzella N, et al. Risk assessment of herbicides and booster biocides along estuarine continuums in the Bay of Vilaine area (Brittany, France). Environ Sci Pollut Res Int 2013; 20: 651–66.

120. Stara A, Machova J, Velisek J. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett 2012; 33: 130–5.

121. Stara A, Kristan J, Zuskova E, et al. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.). Pestic Biochem Physiol 2013; 105: 18–23.

122. Velisek J, Zuskova E, Stara A, et al. Use of biometric, hematological, and plasma biochemical variables and histopathology to assess the chronic effects of the herbicide prometryn on common carp. Vet Clin Pathol 2013; 42: 508–15

123. Manahan SE. Environmental chemistry. Boca Roton: CRC Press, 2005: 783 p.

124. WHO. Guidelines for drinking-water quality: incorporating first addendum. (elektronski vir)Vol. 1: recommendation. 3rd ed. Geneva: World Health Organization, 2006: 595 p. http://www.who.int/water_sanitation_health/dwq/gdwq0506.pdf (12. 1. 2014).

125. U.S. EPA. Reregistration eligibility decision for simazine. Washington: United States Environmental Protection Agency, Prevention, Pesticides and Toxic Sudstances 2006: 266 p.

126. Zorrilla LM, Gibson EK, Stoker TE. The effects of simazine, a chlorotriazine herbicide, on pubertal development in the female Wistar rat. Reprod Toxicol 2010; 29: 393–400.

127. Arndt E. Pesticide use practices and the impact on water quality in Oregon communities. Corvallis: College of Agricultural Sciences, Oregon State University, 2009. SBI summer internship. http://sbi.oregonstate.edu/education/su09interns/Arndt_Eva.pdf (14. 2. 2014).

128. Bester K, Hiihnerfuss H. Triazines in the Baltic and North-sea. Mar Pollut Bull 1993; 26: 423–7.

129. Gunasekara AS. Environmental fate of simazine. Environmental Monitoring Branch Department of Pesticide Regulation. Sacramento: California Environment Protection Agency, 2004: 36 p.

130. U.S. EPA (U.S. Environmental protection agency). Atrazine, simazine and cyanazine: notice of initiation of special review. Federal Register U. S. Government Publishing Office 1994; 59: 30–60.

131. Beitz H, Schmidt F, Herzel F. Occurrence, toxicological and ecotoxicological significance of pesticides in groundwater and surface water. In: Borner H, ed. Pesticides in ground and surface water. Chem Plan Prot 1994; 9: 3–56.

132. Drevenkar V, Fingler S, Mendas G, et al. Levels of atrazine and simazine in waters in the rural and urban areas of north-west Croatia. Int J Environ Analyt Chem 2004; 84: 207–16.

133. Belmonte A, Garrido A, Martinez JL. Monitoring of pesticides in agricultural water and soil samples from Andalusia by liquid chromatography coupled to mass spectrometry. Anal Chim Acta 2005; 538: 117–27.

134. Martınez-Bueno MJ, Hernando MD, Aguera A, et al. Application of passive sampling devices for screening of micro-pollutants in marine aquaculture using LC–MS/MS. Talanta 2009; 77: 1518–27.

135. Strandberg MT, Fordsmand JJS. Field effects of simazine at lower trophic levels: a review. Sci Total Environ 2002; 296: 117–37.

136. Arufe MI, Arellano J, Moreno MJ, et al. Comaprative toxic effects of formulated simazine on *Vibrio fischeri* and gilthead seabream (*Sparus aurata* L.) larvae. Chemosphere 2004; 57: 1725–32.

137. Oropesa-Jimenez AL, Garcia-Cambero JP, Gomez-Gordo L, et al. Gill modifications in the freshwater fish *Cyprinus carpio* after subchronic exposure to simazine. Bull Environ Contam Toxicol 2005; 74: 785–92.

138. Fatima M, Mandiki SNM, Douxfils J, et al. Combined effects of herbicides on biomarkers reflecting immune-endocrine interactions in gold-

fish immune and antioxidant effects. Aqaut Toxicol 2007; 81: 159–67.

139. Oropesa AL, Cambero JPG, Soler F. Effect of long-term exposure to simazine on brain and muscle acetylcholinesterase activity of common carp (*Cyprinus carpio*). Environ Toxicol 2008; 23: 285–93.

140. Cericato L, Machado JG, Fagundes MC, et al. Cortisol response to acute stress in jundia *Rhamdia quelen* acutely exposed to sub-lethal concentrations of agrichemicals. Comp Biochem Physiol C 2008; 148: 281–6.

141. Velisek J, Stastna K, Sudova E, et al. Effects of subchronic simazine exposure on some biometric, biochemical, hematological and histopathological parameters of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett 2009; 30: 236–41.

142. Oropesa AL, Garcia-Camber JP, Soler F. Effect of a subchronic exposure to simazine on energetic metabolism of common carp (*Cyprinus carpio*). J Environ Sci Health 2009; 44: 144–56.

143. Oropesa AL, Garcia-Cambero JP, Soler F. Glutathione and malondialdehyde levels in common carp after exposure to simazine. Environ Toxicol Pharm 2009; 27: 30–8.

144. Oropesa AL, Garcia-Cambero JP, Gomez L, et al. Effect of long-term exposure to simazine on histopathology, hematological, and biochemical parameters in *Cyprinus carpio*. Environ Toxicol 2009; 24: 187–99.

145. Cericato L, Neto JGM, Kreutz LC, et al. Responsiveness of the interrenal tissue of Jundia (*Rhamdia quelen*) to an in vivo ACTH test following acute exposure to sublethal concentrations of agrichemicals. comp Biochem Physiol C 2009; 149: 363–7.

146. Plhalova L, Haluzova I, Macova S, et al. Effects of subchronic exposure to simazine on zebrafish (*Danio rerio*). Neuroendocrinol Lett 2011; 32: 89–94.

147. Velisek J, Stara A, Machova J, et al. Effects of low-concentrations of simazine on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett 2012; 33: 90–5.

148. Velisek J, Stara A, Machova J, et al. Effects of long-term exposure to simazine in real concentrations on common carp (*Cyprinus carpio* L.). Ecotoxicol Environ Safe 2012; 76: 79–86.

149. Stara A, Machova J, Velisek J. Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L). Environ Toxicol Pharmacol 2012; 33: 334–43.

150. U.S. EPA. Reregistration Eligibility Decision (RED) Terbuthylazine. Washington: U.S. Environmental Protection Agency, marec 1995: 186 p. (EPA 738-R-95-005) http://www.epa.gov/oppsrrd1/REDs/2645.pdf (12.1.2014).

151. Mladinic M, Perkovic P, Zeljezic D. Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. Toxicol Lett 2009; 189: 130–7.

152. WHO. Terbuthylazine (TBA) in drinking-water: background document for development of WHO Guidelines for drinking-water quality. Geneve: World Health Organization, 2003: 13 p. http://www.who.int/water_sanitation_health/dwq/chemicals/terbuthylazine.pdf (12. 5. 2014)

153. Byrnes C. Evaluation of the active terbuthylazine in the product Swim-Care® T swimming pool algaecide. Canberra, Australia: National Registration Authority for Agricultural and Veterinary Chemicals 2001: 20 p.

154. Buser HR. Atrazine and other s-triazine herbicides in lakes and in rain in Switzerland. Environ Sci Technol 1990; 24: 1049–58.

155. Brambilla A, Rindone B, Polesello S, et al. The fate of triazine pesticides in River Po water. Sci Total Environ 2003; 132: 339–48.

156. Dabene E. Recherche de produits phytosanitaires dans les eaux souterraines. Premiers résultats pour quelques pays: Allemagne, États-Unis, Grande-Bretagne, Italie, Pays-Bas, Suède. Paris: Ministère de l'Agriculture et des Pêches maritimes, Bureau de l'Agriculture et des Ressources naturelles, 1993 : 30 p.

157. Steinberg CEW, Mayr C, Lorenz R, et al. Dissolved humic material amplifies irritant effects of terbutylazine (triazine herbicide) on fish. Naturwissenschaften 1994; 81: 225–7.

158. Dezfuli BS, Simoni E, Giari L, et al. Effects of experimental terbuthylazine exposure on the cells of *Dicentrarchus labrax* (L.). Chemosphere 2006; 64: 1684–94.

159. Tarja N, Kirsti E, Marja L, et al. Thermal and metabolic factors affecting bioaccumulation of triazine herbicides by rainbow trout (*Oncorhynchus mykiss*) Environ Toxicol 2003; 18: 219–26.

160. MikulikovaI, Modra H, Blahova J, et al. The effects of Click 500 SC (terbuthylazine) on common carp, *Cyprinus carpio* under (sub)chronic conditions. Neuroendocrinol Lett 2011; 32: 15–42.

161. Dobsikova R, Blahova J, Modra H, et al.

The effect of acute exposure to herbicide Gardoprim Plus Gold 500 SC on haematological and biochemical indicators and histopathological changes in common carp (*Cyprinus carpio* L.). Acta Vet Brno 2011; 80: 359–63.

162. Plhalova L, Stepanova S, Blahova J, et al. The effects of subchronic exposure to terbuthylazine on zebrafish. Neuroendocrinol Lett 2012; 33: 113–9.

163. Stepanova S, Plhalova L, Dolezelova P, et al. The Effects of subchronic exposure to terbuthylazine on early developmental stages of common carp. ScientificWorldJournal 2012; 2012: e615920 (7 p.) http://www.hindawi.com/journals/tswj/2012/615920/ (14. 1. 2014)

164. Mikulikova I, Modra H, Blahova J, et al. Recovery ability of common carp (*Cyprinus carpio*) after a short-term exposure to terbuthylazine. Pol J Vet Sci 2013; 16: 17–23.

165. Velisek J, Stara A, Koutnik D, et al. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed Res Int 2014; 2014: e621304 (7 p.) http://www.hindawi.com/journals/bmri/2014/621304/ (14. 1. 2014)

166. Nilson EL, Unz RF. Antialgal substances for iodine-disinfected swimming pools. Appl Environ Microbiol 1977; 34: 815–22.

167. Tomlin C. The pesticide manual: a world compendium. Hampshire: British Crop Protection Council, 2003: 600 p.

168. Larsen L, Sorensen SR, Aamand J. Mecroprop, isoproturon, and atrazine in and above a sandy aquifer: vertical distribution of mineralization potential. Environ Sci Technol 2000; 34: 2426–30.

169. Muir DCG, Grift NP, Townsend BE, et al. Comparison of the uptake and bioconcentration of fluridone and terbutryn by rainbow trout and Chironomus tentans in sediment and water systems. Arch Environ Contam Toxicol 1982; 11: 595–602.

170. Konstantinou IK, Hela DG, Albanis TA. The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. Environ Pollut 2006; 141: 555–70.

171. Rioboo C, Prado R, Herrero C, Cid A. Population growth study of the rotifer Brachionus sp. Fed with triazine-exposed microalgae. Aquat Toxicol 2007; 83: 247–53.

172. Quednow K, Puttmann W. Monitoring terbutryn pollution in small rivers of Hesse, Germany. J Environ Monit 2007; 12: 1337–43.

173. Tolosa I, Readman JW, Blaevoet A, et al. Contamination of Mediterranean (Costed'Azur) coastal waters by organotins and Irgarol 1051 used in antifouling paints. Mar Pollut Bull 1996; 22: 335–41.

174. Bathe R. A dynamic system for long-term toxicity studies in fish under laboratory conditions. Arch Toxicol 1979; 41: 417–23.

175. Arufe MI, Arellano J, Moreno MJ, et al. Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. Ecotoxicol Environ Saf 2004; 59: 209–16.

176. Plhalova L, Macova S, Haluzova I, et al. Terbutryn toxicity to *Danio rerio*: effects of subchronic exposure on fish growth. Neuroendocrinol Lett 2009; 30: 242–7.

177. Velisek J, Sudova E, Machova J, et al. Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). Ecotoxicol Environ Saf 2010; 73: 384–90.

178. Velisek J, Stara A, Macova J, et al. Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pestic Biochem Physiol 2011; 102: 102–8.

179. Velisek J, Stara A, Kolarova J, et al. Biochemical, physiological and morfological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryn in real environmental concentration. Pestic Biochem Physiol 2012; 100: 305–13.

180. PAN UK. Pesticide Action Network UK. Which pesticide are banned in Europe? Food & Fairness Briefing April 2008; (1): 8 p. http://www.pan-europe.info/Resources/Links/Banned_in_the_EU.pdf

181. Koutnik D, Stara A, Zuskova E, et al. The effect of subchronic metribuzin exposure to signal crayfish (*Pacifastacus leniusculus* Dana 1852). Neuroendocrinol Lett 2014; 35: 102-5.

182. Stara A, Kouba A, Velisek J. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in red swamp crayfish (*Procambarus clarkii*). BioMed Res Int 2014.; 2014: e680131 (6 p.) http://www.hindawi.com/journals/bmri/2014/680131/ (12. 1. 2014)

183. Velisek J, Stara A, Koutník D, et al. Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax f. virginalis*). Neuroendocrinol Lett 2014; 35: 106–10.

UČINEK TRIAZINSKIH HERBICIDOV NA RIBE: PREGLED

D. Koutnik, A. Stara, J. Velisek

Povzetek: Onesnaževanje okolja je svetovni problem, ki povzroča vse večjo zaskrbljenost in je posledica različnih človekovih dejavnosti povezanih z industrijo in kmetijstvom. Triazinski herbicidi so med najpogosteje uporabljenimi pesticidi. V zadnjem času vse bolj naraščata zavedanje in zaskrbljenost zaradi njihove široke uporabe, saj so ostanki in presnovki triazinov zelo obstojni in se kopičijo v različnih delih okolja. Triazini so bili zaznani tudi v vodnih ekosistemih, v pitni vodi in podzemnih vodah ter tudi v ribah. Zato je uporaba določenih triazinskih pesticidov v evropskih državah že prepovedana. Osem s-triazinov je bilo uvrščeno v študijo za pripravo prednostnega seznama snovi, nevarnih za vodno okolje v državah članicah Evropske unije in so že vključeni v prednostni seznam onesnaževalcev okolja v Evropski unije in ZDA (*European Union Priority Pollutants List* in *U.S. Environmental Protection Agency's List*). V preglednem članku je predstavljeno trenutno poznavanje stanja ostankov triazina v vodnem okolju in njihovi strupeni učinki na ribe. Na osnovi pregleda dosedanjega poznavanja problematike smo opredelili glavne vrzeli v trenutnem znanju in nekatere usmeritve za prihodnje raziskave. Pregled vsebuje vpliv sedmih najpogosteje odkritih triazinov v vodi (ametrin, atrazin, metribuzine, prometrin, simazin, terbutilazin in terburine) na fiziologijo rib in njihovo akutno strupenost. Toksični učinki triazinov vključujejo vpliv na rast rib, njihov zgodnji razvoj, oksidativni stres in izražanje antioksidantnih encimov, pa tudi na krvne in biokemične parametre v plazmi ter na histopatološke spremembe v jetrih in ledvicah rib.

Ključne besede: triazini; ribe; strupenost; biokemični profil; hematologija; histologija

CHAPTER 3

EFFECT OF PROMETRYNE ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

Velisek, J., Stara, A., Koutnik, D., Machova, J., 2015. Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.). Pesticide Biochemistry and Physiology 118: 58–63.

My share on this work was about 25%

It was allowed by publisher on 5th May, 2018 to include the paper in this Ph.D. thesis.

Pesticide Biochemistry and Physiology 118 (2015) 58–63

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Effects of prometryne on early life stages of common carp (*Cyprinus carpio* L.)

Josef Velisek *, Alzbeta Stara, Dalibor Koutnik, Jana Machova

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

ARTICLE INFO

Article history:
Received 13 August 2014
Accepted 26 November 2014
Available online 1 December 2014

Keywords:
Triazine
Embryo
Larvae
Early development
Histopathology

ABSTRACT

Toxicity of prometryne to early life stages of common carp was assessed. On the basis of accumulated mortality in the experimental groups lowest observed-effect concentration (LOEC) was estimated as 1100 μg/l; and no observed-effect concentration (NOEC) was 850 μg/l. Fulton's condition factor was significantly lower than in controls in fish exposed to 4000 μg/l after 7, 14, and 21 days. By day 14, fish exposed to 4000 μg/l prometryne showed significantly lower mass and total length compared to controls. Fish exposed the 1200 and 4000 μg/l showed delay in development, severe hyperaemia in gill, liver, and caudal and cranial kidney. Subchronic prometryne exposure of early-life stages of common carp at concentrations of 1200 and 4000 μg/l affected their survival, growth rate, early ontogeny, and histology.

1. Introduction

The aquatic environment continues to be under threat by the use of pesticides, resulting in high risk to non-target organisms [1]. Pesticides used in agro-ecosystems and forests enter aquatic environments such as streams, rivers, and lakes if applied in adjacent areas or if an accidental spill occurs [2]. Such pesticides are carried into aquatic environments by surface runoff from sites of application and can negatively affect the health of aquatic organisms [3–8].

Eight s-triazines (atrazine, cyanazine, prometryne, propazine, sebuthylazine, simazine, terbuthylazine, and terbutryne) have been identified as relevant for testing, based on a compilation of freshwater monitoring data in the member states of the European Community [9]. Prometryne [2,4-bis (isopropylamino)-6-methylthio-s-triazine], a selective herbicide of the s-triazine chemical family, has been utilized for pre- and post-emergence control of annual grasses and broadleaf weeds in a variety of crops, including cotton, celery, pigeon peas, and dill [10]. Prometryne was first registered in the United States of America by Ciba Crop Protection in 1964 [11]. Prometryne persists in the soil from one to three months and has a soil half-life of 60 days. With multiple annual applications, prometryne activity can persist for 12–18 months following the most recent application [12]. It is slightly to moderately toxic to fish. Acute toxicity 96 h LC50 for common carp (*Cyprinus carpio* L.) is reported as 8 mg/l [13].

Although prometryne has been banned in Europe since 2004 [14], it can still be found in surface and ground waters. Prometryne has been reported in European surface waters at concentrations from 0.01 to 4.40 μg/l [15–17]. Prometryne is still being widely used in China [14], Australia, Canada, New Zealand, South Africa, and the United States [18].

Although the effects of acute and subchronic exposure of juvenile and adult fish to prometryne, another s-triazine herbicide, have been well documented, there is a dearth of data on the subchronic toxicity of prometryne at environmentally realistic concentrations in embryos and larvae of common carp. Prometryne caused changes in haematological, biochemical profile, caudal kidney [19], and antioxidant enzymes in juvenile carp [20,21]. Fish metamorphose in surface waters, and it is presumed that the embryo-to-larva transformation is sensitive to chemicals in the environment [22]. The aim of the present study was to describe the effects of prometryne on embryos and larvae of common carp, a well-established model species for testing chemical effects on early development [23]. The toxicity of prometryne was assessed on the basis of mortality, early ontogeny, growth rate, Fulton's condition factor (FCF), and occurrence of morphological anomalies during, and at the conclusion of the test.

2. Materials and methods

2.1. Experimental animals

Fertilized eggs of carp were obtained from the breeding station of the Faculty of Fisheries and Protection of Waters Vodnany, Czech Republic. Eggs were fertilized according to standard methods

* Corresponding author. University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic. Fax: +420 383 382 396.
E-mail address: velisek@frov.jcu.cz (J. Velisek).

http://dx.doi.org/10.1016/j.pestbp.2014.11.011
0048-3575/

described by Kocour et al. [24]. The study was conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the Faculty of Fisheries and Protection of Waters Vodnany, based on the EU-harmonized animal welfare act of Czech Republic.

2.2. Water parameters

Eggs and larvae were maintained in aerated tap water with the following parameters: dissolved oxygen, >91%; temperature, 18.7–20.6 °C; pH, 7.1–8.1; acid neutralization capacity ($ANC_{4.5}$), 0.99 mmol/l; chemical oxygen demand (COD_{Mn}), 1.1 mg/l; total ammonia, 0.01 mg/l; NO_3^-, 6.75 mg/l; NO_2^-, <0.02 mg/l; $Ca^{2+} + Mg^{2+}$, 8.2 mg/l. The test baths were continuously gently aerated. Temperature, oxygen saturation and pH were measured daily.

To ensure agreement between nominal and actual compound concentrations, water in the aquaria was analysed during the experimental period by liquid chromatography–tandem mass spectrometry (LC–MS/MS) [25]. Water samples were collected from the aquaria immediately before (24 h after application) and after renewing the test solutions (0 h). The mean concentration of prometryne in the water samples was always within differentiation 2% of the intended concentration.

2.3. Experimental protocol

The trial was carried out using the modified test No. 210 – Fish, Early-Life Stage Toxicity Test OECD [23]. At 24 h post-fertilization, unfertilized eggs were discarded, and 100 fertilized eggs were transferred into each of fifteen glass crystallization basins with the prometryne solution, plus control dishes. Prometryne (chemical purity 99.3%) was obtained from Sigma–Aldrich Corporation (USA). Four concentrations of test solutions and a control were used, each with 100 fertilized eggs, in triplicate. The concentrations were: 0.51 μg/l (environmental concentration in Czech rivera), 80 μg/l, 1200 μg/l, and 4000 μg/l. Prometryne concentrations of 80 μg/l, 1200 μg/l, and 4000 μg/l corresponded to 1% of the 96 h LC50, 15% of 96 h LC50, and 50% of 96 h LC50 for carp [13].

The water for each treatment was renewed daily by gently draining each chamber and adding new solution slowly to avoid disturbing embryos and larvae. Control of hatching, mortality, and behaviour was made twice daily, and dead fish were removed. From 6 day larvae were fed freshly hatched brine shrimp *Artemia salina* nauplii *ad libitum* daily prior to water exchange.

On days 7, 14, 21, 28, and 35 samples of fish (30 per concentration groups and control) were collected to monitor development, occurrence of morphological anomalies, growth rate, FCF, and the length/mass relationship. Determination of development periods and stages followed Penaz et al. [26]. Final evaluations included accumulated mortality, mass and total length (TL) of fish with no deformities. The total length was measured by stereomicroscopy using a micrometer. Mass to 0.1 mg was measured with a Mettler-Toledo balance.

2.4. Trial schedule

The experiment schedule was: day 1, trial initiation (1 day post-fertilization); day 7, hatching complete; day 9, initiation of exogenous feeding; day 35, end of the experiment. To 35 day, the majority of control fish had become first stage juveniles.

2.5. Growth rate evaluation

The mean specific growth rate (SGR) for fish in each experimental group was calculated for the period from day 7 to day 35 and compared with controls using the method described by Kroupova et al. [27].

2.6. Statistical analysis

One-way ANOVA was conducted to compare differences among the test groups using the software program Statistica 12 for Windows (StatSoft). The differences in cumulative mortality among groups were assessed using contingency tables (χ^2) [28].

2.7. Evaluation of 35 day LC50, LOEC, and NOEC

For the evaluation of LC50, lowest observed-effect concentration (LOEC), and no observed effect concentration (NOEC) at the completion of the test, a probit analysis EKOTOX 5.1 software (Ingeo Liberec) was conducted based on mortality at different prometryne concentrations. The day 35 LC5 and day 35 LC10 values were used to express the NOEC and LOEC values, respectively.

2.8. Histopathology

Histopathology was evaluated in all groups at the end of the trial. Six fish from each group and control were placed in 10% buffered formalin, prepared with standard histological techniques, stained with haematoxylin and eosin, examined by light microscopy.

3. Results

3.1. Hatching

Hatching began 5 days following onset of exposure, and the majority of eggs in all treatment groups hatched by day 7. Significantly ($p < 0.01$) lower hatching and embryo viability were found in fish exposed to the two highest prometryne concentrations, 1200 and 4000 μg/l, compared with controls and other concentrations (0.51 and 80 μg/l).

3.2. Cumulative mortality

Significant ($p < 0.01$) differences from controls in cumulative mortality were found in fish exposed to 1200 (15% 96 h LC50) and 4000 μg/l prometryne (50% 96 h LC50) (Fig. 1). Massive mortality in those groups occurred on days 6 and 7. Based on mortality in the experimental groups, prometryne concentrations were estimated at day 35 to be LC50 = 2314 μg/l, LOEC = 1100 μg/l, and NOEC = 850 μg/l with 95% confidence interval.

3.3. Growth parameters

Beginning on day 14 of exposure, fish exposed to prometryne at 4000 μg/l showed significantly ($p < 0.01$) lower mass (Fig. 2) and total length (Fig. 3) than did controls. The FCF values were significantly ($p < 0.01$) lower in the 4000 μg/l group after 7, 14, and 21 days compared to controls (Table 1). Inhibition of specific growth in the group exposed to the 4000 μg/l was 41.68% compared to controls (Table 2).

3.4. Early ontogeny

Fish exposed to 1200 and 4000 μg/l were delayed in development (Table 3) compared with the control group. At the conclusion of the trial the percent of individuals remaining in larval stages (L4b or L6) was elevated with higher concentrations of prometryne, whereas the majority of control fish reached the juvenile stage.

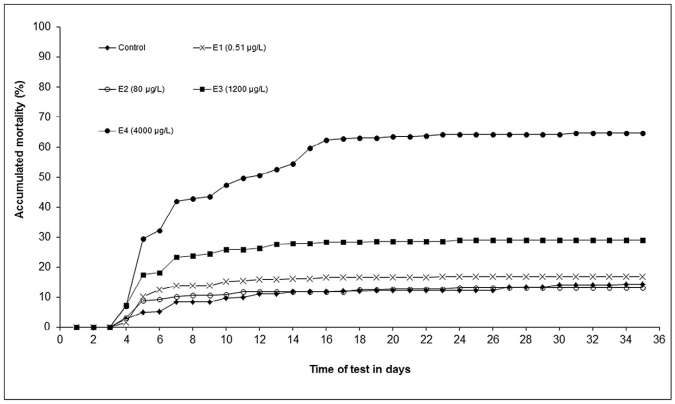


Fig. 1. Cumulative percent mortality of common carp embryos, larvae, and juveniles following prometryne exposure.

3.5. Morphological anomalies

Morphological anomalies (<1%) were found in fish in experimental and control groups. Morphological anomalies included axial and/or lateral curvature of the spine and body shortening. These morphological anomalies could be considered a spontaneous appearance.

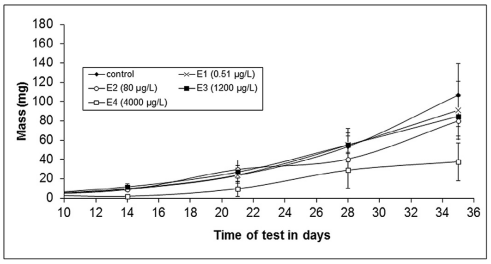


Fig. 2. Mean mass ± SD of common carp larvae and juveniles following prometryne exposure, $N = 30$.

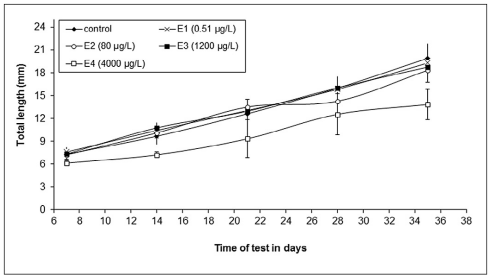


Fig. 3. Total length ± SD of common carp larvae and juveniles following prometryne exposure, $N = 30$.

3.6. Histology

Histological examination revealed focal and diffused hyperaemia of the gills, liver (Fig. 4), and caudal and cranial kidney in groups of carp exposed to the two highest prometryne concentrations (1200 and 4000 µg/l). Liver structure was altered by diffused steatosis associated with loss of cellular shape and the presence of lipid inclusions in hepatic cells. Described pathologies in liver were found in fish within all the experimental groups and control. Therefore, the pathological changes of liver could not be attributed to prometryne exposure. It is possible that these changes could be caused by nutritional factors.

4. Discussion

Toxicity tests on early life stages of fish are commonly used to predict xenobiotic effects. Embryos and larvae are often the life stages most sensitive to toxic effects, although they may differ in susceptibility due to physiological and biochemical differences [29,30]. This study provides data on subchronic exposure to prometryne for consideration in risk assessment. The findings contribute to knowledge of the toxic profile of prometryne on the early life stage of common carp.

In subchronic toxicity tests, decreased growth rate and delayed early development are commonly found [31]. The present study revealed significant negative effects of prometryne on hatching and embryo viability at the higher concentrations tested (1200 and 4000 µg/l). Our result differ from those of Velisek et al. [7,32] who found no differences in carp hatching following exposure to triazine pesticides terbutryne and simazine. Larval mortality of 65% was observed at the prometryne concentration of 4000 µg/l and 29% at 1200 µg/l with mortality peaking on days 7 and 16 of the experiment. The critical periods were soon after the beginning of the test, at the time of hatching, and within 10 days post-hatching, which includes the period of change from endogenous to exogenous nutrition. After 17 days, only accidental mortality was observed, indicating higher tolerance of the older larvae to prometryne.

Table 1
Mean Fulton's condition factor for common carp larvae and juveniles after prometryne exposure.

Prometryne (μg/l) Times (day)	Control Mean ± SD	0.51 Mean ± SD	80 Mean ± SD	1200 Mean ± SD	4000 Mean ± SD
7	0.72 ± 0.20	0.78 ± 0.22	0.94 ± 0.26	0.88 ± 0.21	0.52 ± 0.61*
14	1.11 ± 0.69	0.86 ± 0.15	0.86 ± 0.27	0.91 ± 0.14	0.49 ± 0.13*
21	1.10 ± 0.12	1.04 ± 0.13	1.18 ± 0.25	1.22 ± 0.17	0.84 ± 0.23*
28	1.29 ± 0.20	1.33 ± 0.15	1.41 ± 0.64	1.32 ± 0.14	1.23 ± 0.14
35	1.30 ± 0.10	1.21 ± 0.35	1.27 ± 0.09	1.26 ± 0.09	1.26 ± 0.21

* Experimental groups significantly ($p < 0.01$) different from the control group, $N = 30$.

Table 2
Growth rate and fish mortality among 35 day embryo–larva prometryne toxicity test of common carp.

Prometryne (μg/l)	Control	0.51	80	1200	4000
m_7 (Mean ± SD, mg)	2.76 ± 0.45	3.27 ± 0.47	3.44 ± 0.50	3.39 ± 0.64	4.05 ± 1.16
m_{35} (Mean ± SD, mg)	106.73 ± 32.52	90.98 ± 29.88	80.01 ± 19.83	84.77 ± 20.79	37.63 ± 19.53*
SGR	12.93	11.68	11.18	11.46	7.54
I (%)	–	9.67	13.53	11.37	41.68
Mortality (%)	14	17	13	29*	65*

am7, m35, mean fish mass in selected group after 7 and 35 days exposure; SGR, mean specific growth rate in selected group after 28 days exposure; I, inhibition of specific growth in selected group after 28 days exposure; SD, standard deviation; $N = 30$.
* Experimental groups significantly ($p < 0.01$) different from the control group.

Based on cumulative mortality, the 35 day LC50 was estimated at 2314 μg/l prometryne. The values obtained were lower than that reported in short-term tests on adult specimens, 8 mg/l prometryne [13]. Prometryne acute toxicity (96 h LC50) values for the rainbow trout *Oncorhynchus mykiss* is 2.9 mg/l [12], for bluegill sunfish *Lepomis macrochirus*, 7.9 mg/l and, for sheepshead minnow *Cyprinodon variegates*, 5.1 mg/l [18]. On the basis of cumulative mortality, the values for NOEC and LOEC were estimated at 1100 μg/l and 850 μg/l prometryne, respectively. It appears that prometryne may not be a serious problem for early-life stages of carp in the wild in the Europe, since reported concentrations in rivers of Europe (0.01–4.40 μg/l) usually do not exceed NOEC levels [15–17].

Beginning on day 14 of exposure, fish exposed to 4000 μg/l prometryne showed significantly lower mass and total length than controls. These results agree with Velisek et al. [7,32].who found significantly lower mass and total length of carp after terbutryne and simazine exposure. Plhalova et al. [33] reported significantly reduced zebrafish *Danio rerio* growth associated with a terbutryne concentration of 0.6 mg/l. Erickson and Turner [34], reported adverse effects on growth of fathead minnows *Pimephales promelas* exposed to 1–2 mg/l prometryne for 32 days. Decreased growth rate is a common subchronic toxicity response [31,35].

Developmental stage is a sensitive parameter for evaluation of impacts of toxicants on fish [7,32,36]. Chemically induced adverse effects on early life stages are based on developmental events, such as organogenesis. Although we have information on the toxicity of prometryne in adult fish, little is known of effects of this compound on embryonic development in carp. Fish exposed 1200 and 4000 μg/l prometryne were delayed in development compared with the control group and concentrations of 0.51 and 80 μg/l. In other studies, the significantly delayed development has been observed in carp exposed to simazine at concentrations 0.06, 60, 600, and 3000 μg/l [7], terbutryne at concentrations 0.2 and 2 mg/l [32], and terbuthylazine-2-hydroxy at concentrations 1.4 and 3.5 mg/l [37].

Table 3
Development periods during the 35 day embryo–larva toxicity test on common carp.

Prometryne (μg/l) Times (day)	Control	0.51	80	1200	4000
7	ec9-L1	ec9-L1	ec9-L1	ec9-L1	ec9
14	L2-L3b	L2-L3b	L2-L3b	L2-L3a	L1-L2
21	L4a-L5	L4a-L5	L4a-L5	L4a-L4b	L3-L4a
28	L5-L6	L5-L6	L5-L6	L4a-L5	L4a-L4b
35	L6-J1	L6-J1	L6-J1	L5-L6	L4b-L5

In fish, developmental malformations have been linked to the presence of environmental pollutants such as pesticides [38], but body malformation was not observed in the present study. This is in agreement with Velisek et al. [7,32] who found no malformation in carp after terbutryne respectively simazine exposure.

The gill, liver, and caudal and cranial kidney of carp exposed to 1200 and 4000 μg/l prometryne showed severe hyperaemia. This may be a regulatory response allowing alteration in blood supply to different tissues through vasodilation. We observed massive diffused steatosis in liver of all examined fish; however, similar changes were found to a lesser extent in the control group. Therefore, the pathological changes of liver could not be attributed to prometryne exposure. It is possible that these changes could be caused by nutritional factors.

Triazine has been reported to be associated with damage to fish kidney structure [4–6,39–41]. Prometryne in the two highest tested concentrations resulted in severe hyperaemia in the gill, liver, caudal and cranial kidney. Gross morphological anomalies in gill epithelium of yearling coho salmon *Oncorhynchus kisutch* exposed to the herbicide atrazine (15 μg/l for 114 h) included necrosis, desquamation, hypertrophy and hyperlasia, and telangiectasia [42]. Hyperplasia of gill epithelial cells was also reported by Neskovic et al. [40] in carp exposed to atrazine at 1500 μg/l. Similar changes to gill epithelial cells were reported by Oropesa-Jimenaz et al. [43] in carp following acute exposure to simazine. Arufe et al. [44] described alterations in liver in gilthead seabream *Sparus aurata* after exposure to a combination of terbutryn and triasulfuron. Fischer-Scherl et al. [45] observed changes in renal corpuscles and tubules of rainbow trout after subchronic exposure to atrazine. Biagianti-Risbourg and Bastide [46] reported that atrazine affects liver, particularly, 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. Necrosis of liver and changes in the endoplasmic reticulum and hepatocellular compartment were associated with 40 mg/l atrazine in rainbow trout with 5 weeks exposure, whereas zebrafish were more sensitive, with 1 mg/l atrazine associated with similar disturbances [47].

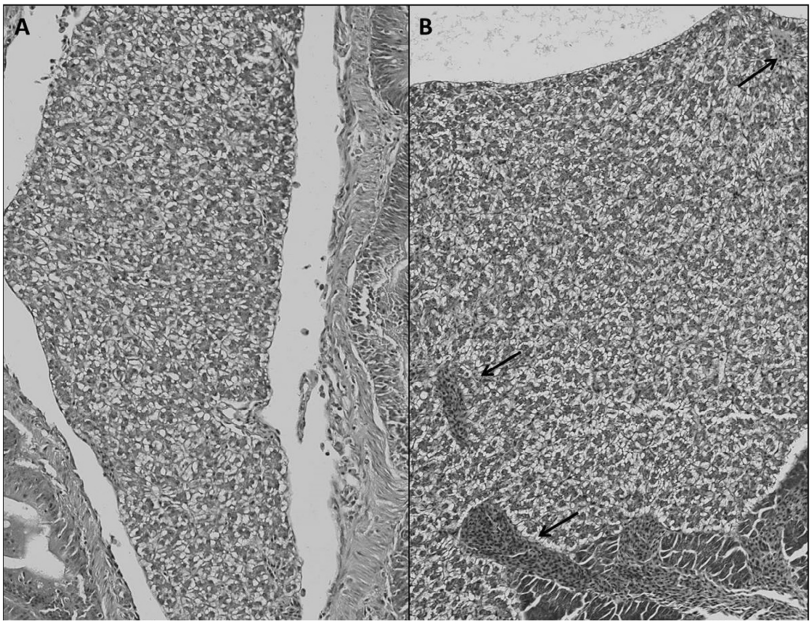


Fig. 4. Histological sections of liver of common carp. Representative sections from control (A) and prometryne-exposed (4 mg/l) groups (B) show cytoplasmic vacuolation of parenchyma. Arrows indicate blood congestion associated with vasodilatation of vessels (HE ×200).

5. Conclusions

The information obtained in this study may be of use in efforts to assess the ecological risk of prometryne in the aquatic environment. Subchronic prometryne exposure of early-life stages of common carp affected their survival, growth rate, early ontogeny, and histology. The changes were observed only at concentrations of 1200 and 4000 μg/l prometryne. Concentrations of prometryne in European surface waters have been reported to generally range from 0.10 to 4.40 μg/l. For detailed elucidation of prometryne effects further research is necessary. This research should be focused not only on the studies of effects of prometryne alone, but also in combination with other pollutants.

Acknowledgments

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program) and by the Grant Agency of the University of South Bohemia (project No. 018/2014/Z).

References

[1] A. Kumar, M.R. Prasad, K. Srivastava, S. Tripathi, A.K. Srivastava, Branchial histopathological study of catfish *Heteropneustes fossilis* following exposure to purified neem extract, azadirachtin, World J. Zool. 5 (2010) 239–243.
[2] R.N. Singh, P.K. Pandey, N.N. Singh, V.K. Dass, Acute toxicity and behavioral responses of common carp *Cyprinus carprio* (Linn.) to an organophosphate (Dimethoate), World J. Zool. 5 (2010) 183–188.
[3] R. Dobsikova, J. Velisek, T. Wlasow, P. Gomulka, Z. Svobodova, L. Novotny, Effects of cypermethrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.), Neuroendocrinol. Lett. 27 (Suppl. 2) (2006) 91–95.
[4] J. Velisek, Z. Svobodova, V. Piackova, L. Novotny, J. Blahova, E. Sudova, et al., Effect of metribuzin on rainbow trout (*Oncorhynchus mykiss*), Vet. Med. 5 (2008) 324–332.
[5] J. Velisek, E. Sudova, J. Machova, Z. Svobodova, Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.), Ecotoxicol. Environ. Saf. 73 (2010) 384–390.
[6] J. Velisek, A. Stara, J. Kolarova, Z. Svobodova, Biochemical, physiological and morphological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryne in real environmental concentration, Pest. Biochem. Physiol. 100 (2011) 305–313.
[7] J. Velisek, A. Stara, J. Machova, P. Dvorak, E. Zuskova, Z. Svobodova, Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L, Neuroendocrinol. Lett. 33 (2012) 90–95.
[8] J. Velisek, A. Stara, J. Machova, Z. Svobodova, Effects of long-term exposure to simazine in real concentration on common carp (*Cyprinus carpio* L.), Ecotoxicol. Environ. Saf. 76 (2012) 79–86.
[9] European Commission, Study on the Prioritisation of Substances Dangerous to the Aquatic Environment, Office for Official Publications of the European Communities, Luxembourg, 1999.
[10] M.A. Kamrin, Pesticide Profiles, Lewis Publishers/CRC Press, Boca Raton, FL, 1997, 686.
[11] U.S. EPA (Environmental Protection Agency), R.E.D. Fact Prometryn, 11 pp, 1996.
[12] U.S. EPA (Environmental Protection Agency), Reregistration Eligibility Decision (RED) Prometryn, 117 pp, 1996.
[13] G.V. Popova, Characteristics of the effect of the herbicide prometryn on fish, Nauchn. Osn. Okhr. Prir. 4 (1976) 118–125.
[14] J.H. Zhou, F. Hu, J.G. Jiao, M.G. Liu, X.H. Li, Effects of bacterial-feeding nematodes and prometryne-degrading bacteria on the dissipation of prometryne in contaminated soil, J. Soil. Sediment. 12 (2012) 576–585.
[15] Z. Vryzas, C. Alexoudisa, G. Vassilioua, K. Galanisa, E. Papadopoulou-Mourkidoub, Determination and aquatic risk assessment of pesticide residues in riparian drainage canals in northeastern Greece, Ecotoxicol. Environ. Saf. 74 (2011) 174–181.
[16] T. Caquet, M. Roucaute, N. Mazzella, F. Delmas, C. Madigou, E. Farcy, et al., Risk assessment of herbicides and booster biocides along estuarine continuums in

the Bay of Vilaine area (Brittany, France), Environ. Sci. Pollut. Res. Int. 20 (2013) 651–666.
[17] CHI (Czech Hydrometeorological Institute), On-line water duality database. <http://hydro.chmi.cz/oj/>, 2014 (accessed 02.01.14).
[18] S.E. Kegley, B.R. Hill, S. Orme, A.H. Choi, PAN Pesticide Database, Pesticide Action Network, North America (San Francisco, CA, 2010), Pesticide Action Network, North America, 2010.
[19] J. Velisek, E. Zuskova, A. Stara, Z. Svobodova, Use of biometric, hematological, and plasma biochemical variables and histopathology to assess the chronic effects of the herbicide prometryn on common carp, Vet. Clin. Pathol. 42 (2013) 508–515.
[20] A. Stara, J. Machova, J. Velisek, Effect of chronic exposure to prometryne on oxidative stress and antioxidant response on early life stages of common carp (*Cyprinus carpio* L, Neuroendocrinol. Lett. 33 (Suppl. 3) (2012) 130–135.
[21] A. Stara, J. Kristan, E. Zuskova, J. Velisek, Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L, Pest. Biochem. Physiol. 105 (2013) 18–23.
[22] D. Giulio, D.E. Hinton (Eds.), The Toxicology of Fishes, CRC Press, New York, 2008, p. 1096.
[23] OECD (Organization for Economic Cooperation and Development), Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems TG- No. 210: Fish, Early-Life Stage Toxicity Test. Paris, France, pp. 24, 2013.
[24] M. Kocour, D. Gela, M. Rodina, O. Linhart, Testing of performance in common carp *Cyprinus carpio* L. under pond husbandry conditions I: top-crossing with Northern mirror carp, Aquacul. Res. 36 (2005) 1207–1215.
[25] D. Barcelo, M.C. Hennion, Trace Determination of Pesticides and Their Degradation Products in Water, Mass Spectrometric Methods, LC–MS, Elsevier, Amsterdam, The Netherlands, 1997, p. 556.
[26] M. Penaz, M. Prokes, J. Kouril, J. Hamackova, Early development of the carp, *Cyprinus carpio*, Acta Sci. Nat. Brno. 17 (1983) 1–39.
[27] H. Kroupova, M. Prokes, S. Macova, M. Penaz, V. Barus, L. Novotny, et al., Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L, Environ. Toxicol. Chem. 29 (2010) 535–540.
[28] J.H. Zar, Biostatistical Analysis, third ed., Prentice-Hall, New Jersey, USA, 1996, p. 663.
[29] P. Kristensen, Sensitivity of embryos and larvae in relation to other stages in the life cycle of fish: a literature review, in: R. Muller, R. Lloyd (Eds.), Sublethal and Chronic Effects of Pollutants on Freshwater Fish, United Nation Organization, Fishing News Books, New York, USA, 1994, pp. 339–352.
[30] J.M. McKim, Early life stage toxicity tests, in: G.M. Rand (Ed.), Fundamentals of Aquatic Toxicology, Effects, Environmental Fate and Risk Assessment, Taylor & Francis, Washington, DC, 1995, pp. 974–1011.
[31] D. Woltering, The growth response in fish chronic and early life stage toxicity tests: a critical review, Aqua. Toxicol. 5 (1984) 1–21.
[32] J. Velisek, A. Stara, J. Machova, P. Dvorak, E. Zuskova, M. Prokes, et al., Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.), Pest. Biochem. Physiol. 102 (2012) 102–108.
[33] L. Plhalova, S. Macova, I. Haluzova, A. Slaninova, P. Dolezelova, P. Marsalek, et al., Terbutryn toxicity to *Danio rerio*: effects of subchronic exposure on fish growth, Neuroendocrinol. Lett. 30 (2009) 242–247.
[34] W. Erickson, L. Turner, Prometryn analysis of risks to endangered and threatened Salmon and Steelhead, Environmental Field Branch, Office of Pesticide Programs, pp. 71, 2002.
[35] J.H.S. Blaxter, Pattern and variety in development, in: W.S. Hoar, R.J. Randall (Eds.), Fish Physiology, Harcourt Brace Jovanovich, London, UK, 1988, pp. 1–58.
[36] Q.H. Pickering, J.M. Lazorchak, Evaluation of the robustness of the Fathead minnow, *Pimephales promelas*, larval survival and growth test, US EPA Method-1000.0, Environ. Toxicol. Chem. 14 (1995) 653–659.
[37] J. Velisek, A. Stara, D. Koutnik, J. Machova, Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.), BioMed Res. Int. 2014 (2014) Article ID 621304.
[38] H. von Westernhagen, Sublethal effects of pollutants on fish eggs and larvae, in: W.S. Hoar, D.J. Randall (Eds.), Fish Physiology, vol. XI, Part A, Academic Press, San Diego, 1988, pp. 253–347.
[39] V.G. Gunkel, Bioaccumulation of a herbicide (atrazine, s-triazine) in the whitefish (*Coregonus fera* J.): uptake and distribution of the residue in fish, Arch. Hydrobiol. Suppl. 59 (1981) 252–287.
[40] N.K. Neskovic, I. Elezovic, V. Karan, V. Poleksic, M. Budimir, Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.), Ecotoxicol. Environ. Saf. 25 (1993) 173–182.
[41] J. Velisek, Z. Svobodova, V. Piackova, E. Sudova, Effects of acute exposure of metribuzin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.), Bull. Environ. Contam. Toxicol. 82 (2009) 492–495.
[42] T.R. Meyer, J.D. Hendricks, Histopathology, in: G.M. Rand, S.R. Petrocelli (Eds.), Fundamentals of Aquatic Toxicology, Methods and Applications, Hemisphere Publishing Corp., Washington, USA, 1985, p. 283.
[43] A.L. Oropesa-Jimenaz, J.P. García-Cambero, L. Gomez-Gordo, V. Roncero-Cordero, F. Soler-Rodríguez, Gill modifications in the freshwater fish *Cyprinus carpio* after subchronic exposure to simazine, Bull. Environ. Contam. Toxicol. 74 (2005) 785–792.
[44] M.I. Arufe, J. Arellano, M.J. Moreno, C. Sarasquetec, Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test, Ecotoxicol, Environ. 59 (2004) 209–216.
[45] T. Fischer-Scherl, A. Veseer, R.W. Hoffman, C. Kuhnhauser, R.D. Negele, T. Ewringmann, Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*), Arch. Environ. Contam. Toxicol. 20 (1991) 454–461.
[46] S. Biagianti-Risbourg, J. Bastide, Hepatic perturbations induced by a herbicide (atrazine) in juvenile grey mullet *Liza ramada* (Mugilidae. Teleostei): an ultrastructural study, Aquat. Toxicol. 31 (1995) 217–229.
[47] T. Braunbeck, P. Burkhardt-Holm, G. Gorge, R. Nagel, R.D. Negele, V. Storch, Rainbow trout and zebra fish, two models for continuous toxicity tests: relative sensitivity, species and organ specificity in cytopathologic reaction of liver and intestines to atrazine, Schriftenr. Ver. Wasser Boden Lufthyg. 89 (1992) 109–145.

CHAPTER 4

EFFECTS OF TERBUTHYLAZINE ON EARLY LIFE STAGES OF COMMON CARP

Velisek, J., Stara, A., Koutnik, D., Zuskova, E., 2015. Effects of terbuthylazine on early life stages of common carp. Neuroendocrinology Letters 36: 120–125.

My share on this work was about 20%

It was allowed by publisher on 17th April, 2018 to include the paper in this Ph.D. thesis.

ORIGINAL ARTICLE

Neuroendocrinology Letters Volume 36 Suppl. 1 2015
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

Effects of terbuthylazine on early life stages of common carp

Josef Velisek, Alzbeta Stara, Dalibor Koutnik, Eliska Zuskova

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic

Correspondence to: Assoc. Prof. Dipl.-Ing. Josef Velisek, PhD.
University of South Bohemia in Ceske Budejovice,
Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses,
Research Institute of Fish Culture and Hydrobiology
Zatisi 728/II 389 25 Vodnany, Czech Republic.
TEL: +420-383382402; FAX: +420-383382396; E-MAIL: velisek@frov.jcu.cz

Submitted: 2015-07-18 Accepted: 2015-09-09 Published online: 2015-10-15

Key words: **triazine; embryolarval toxicity test; early development; histology; fish**

Neuroendocrinol Lett 2015; **36**(Suppl. 1):120–125 **PMID:** 26757117 NEL360915A21

Abstract

OBJECTIVES: The aim of this study was to assess the toxicity of terbuthylazine 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 methodology. The developmental stages of carp were exposed to terbuthylazine at four concentrations, 2.9 (reported environmental concentration in Czech rivers); 70; 1,400; and 3,500 $\mu g.L^{-1}$ for 35 days and compared to carps in a non-treated control group.
RESULTS: Terbuthylazine in concentration 1,400 and 3,000 $\mu g.L^{-1}$ caused significant ($p<0.01$) decrease of mass, total length and delayed in development of carp. Fish exposed to terbuthylazine showed alteration of tubular system of caudal kidney. On the basis of histopathological changes the values of LOEC = 2.9 $\mu g.L^{-1}$ terbuthylazine were estimated.
CONCLUSIONS: Chronic terbuthylazine exposure of early-life stages of common carp affected their growth rate, early ontogeny and histology. Some of the changes were observed only at higher exposures, but change founded in caudal kidney was affected in fish exposed to the real environmental concentration tested (i.e., 2.9 $\mu g.L^{-1}$).

Abbreviations

96hLC50	- lethal concentration
$ANC_{4.5}$	- acid neutralization capacity
COD_{Mn}	- chemical oxygen demand
FWC	- Fulton's weight condition factor
LOEC	- lowest observed effect concentration
OECD	- Organization for Economic Cooperation and Development
SD	- standard deviation
SGR	- specific growth rate
TL	- total length

To cite this article: **Neuroendocrinol Lett** 2015; **36**(Suppl. 1):120–125

INTRODUCTION

Sources of pollution constitute a problem of increasing concern all over the world (Abrantes *et al.* 2010). Increased environmental pollution can be attributed to a variety of factors resulting from different industrial and agricultural technologies (Figueiredo-Fernandes *et al.* 2006). Effects of the residues of various substances persisting in the aquatic environment, the most important of those being pesticides, also are monitored. From among pesticides, the most frequently found are residue of triazine herbicides. Triazine herbicides are among the most commonly used pesticides in the world. Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community (European Commission 1999) and they are included in the EU Priority Pollutants List (EP 2013).

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine), a triazine herbicide, is a selective systemic herbicide which acts as a photosynthesis inhibitor. It is used as a broad spectrum herbicide in maize, sorghum, vines, citrus, coffee, potatoes, legumes, and forestry. Terbuthylazine was registered in the United States in 1975. Terbuthylazine has very similar chemical structure to atrazine. The difference is only iso-butyl and tert-butyl substituent on the amino group (Roberts *et al.* 1998). The minimum difference in structure affects the decomposition reactions of these substances in the environment that led to a ban on atrazine in the European Union. Terbuthylazine is used as a substitute for atrazine since the end of 2006.

Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions The half-life of terbuthylazine was reported to 8 days at pH 1, 86 days at pH 5, >200 days at pH 9 in water at 20 °C (Roberts *et al.* 1998). The fate of residues in aerobic and anaerobic aquatic conditions is similar. The major metabolites of terbuthylazine are the de-chlorinated and N-dealkylated products, which are more mobile than the parent, and exhibit some herbicidal activity when they retain the chlorine atom on the triazine ring plus one alkyl group (WHO 2003). Terbuthylazine levels can reach values up to 2.9 $\mu g.L^{-1}$ in Europe rivers (Buser 1990; Brambilla *et al.* 2003; CHMI 2014).

Studies of the toxicity of various triazine herbicides to aquatic organisms indicate that it can cause growth retardation and morphological, biochemical, haematological, histopathological, and antioxidant enzymes alteration (Stara *et al.* 2013, 2014; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a, b, 2015), but less is known about the specific effects of terbuthylazine in real concentration on fish. The toxicity of terbuthylazine 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 carp 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 described by Kocour *et al.* (2005).

Water parameters

Aerated tap water was used in the present study, with the following parameters: dissolved oxygen >95%, temperature 19.1–20.5°C, pH 7.3–8.0, $ANC_{4.5}$ 1.11 $mmol.L^{-1}$, COD_{Mn} 1.2 $mg.L^{-1}$, total ammonia 0.02 $mg.L^{-1}$, NO_3^- 6.00 $mg.L^{-1}$, NO_2^- 0.01 $mg.L^{-1}$, sum of Ca^{2+} + Mg^{2+} 9.81 $mg.L^{-1}$. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature were measured daily. Terbuthylazine concentrations were checked daily by high performance liquid chromatography. Water samples were assayed using the method of Papadopoulos *et al.* (2012). The values measured did not differ from the value stated for test purposes by more than 6%.

Experimental protocol

The trial was carried out using the modified test design of Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals No. 210 (OECD 2013). At 24 h post-fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into each crystallization basins containing tested solution of terbuthylazine (Sigma Aldrich, Czech Republic, chemical purity 99.4%) 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: 2.9 $\mu g.L^{-1}$ (real environmental concentration – group 1 – E1); 70 $\mu g.L^{-1}$ (group 2 – E2); 1,400 $\mu g.L^{-1}$ (group 3 – E3); and 3,500 $\mu g.L^{-1}$ (group 4 – E4). Selected terbuthylazine concentrations of 70; 1,400; and 3,500 $\mu g.L^{-1}$ corresponded to the 1% of 96 hour half lethal concentration (96hLC50), 20% 96hLC50 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), and the arrangement of basins was random. The water for each treatment was renewed daily by gently draining each chamber and adding new solution slowly to avoid disturbing embryos and larvae. Control of hatching, and mortality, were made twice daily, and dead fish were removed. From 6 day larvae were fed freshly hatched brine shrimp *Artemia salina* nauplii *ad libitum* one daily.

On days 7, 14, 21, 28, and 35 samples of fish (30 per concentration groups and control) were collected

to monitor development, occurrence of morphological anomalies, growth rate, Fulton's weight condition factor (FWC), and the length/mass relationship. Determination of development periods and stages followed Penaz *et al.* (1983). Final evaluations included accumulated mortality, mass and total length (TL) of fish. The total length was measured by stereomicroscopy using a micrometer. Mass to 0.1 mg was measured with a Mettler-Toledo balance.

The experiment schedule was: day 1, trial initiation (1 day post-fertilization); day 7, hatching complete; day 9, initiation of exogenous feeding; day 35, end of the experiment. To 35 day, the majority of control fish had become first stage juveniles.

The mean specific growth rate (SGR) for fish in each experimental group was calculated for the period from day 7 to day 35 and compared with controls using the method described by OECD (2000).

Evaluation of 35 day LC50 and LOEC

For the evaluation of 35 day LC50 values, a probit analysis EKOTOX 5.1 software (Ingeo Liberec) was used based on mortality at different terbuthylazine concentrations. For the evaluation of LOEC value, the probit analysis was based on histopathological changes at different terbuthylazine concentrations.

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.

Statistical analysis

One-way analysis of variance was conducted to compare differences among the test groups using the software program Statistica 12 for Windows (StatSoft). The differences in cumulative mortality among groups were assessed using contingency tables (χ^2).

RESULTS

Hatching began 5 day after the onset of exposure, on days 5 to 7. The majority of eggs in all treatment groups hatched by day 7. No significantly negative effects of terbuthylazine on hatching and embryos viability were observed compared to controls.

Accumulated mortality

Cumulative mortality of common carp samples exposed to terbuthylazine and the control sample was between 10–58% (Figure 1). Significant (p<0.01) differences in total accumulated mortality were found in fish exposed to the two highest terbuthylazine concentration (1,400 and 3,500 µg.L^{-1}), compared with controls. Based on mortality in the experimental groups, terbuthylazine concentrations were estimated at day 35 to be LC50 = 2,992 µg.L^{-1}.

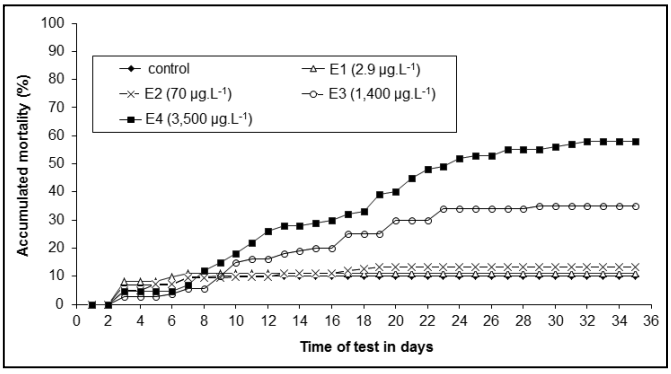


Fig. 1. Accumulated mortality (percentage) of common carp embryos, larvae, and juveniles after terbuthylazine exposure.

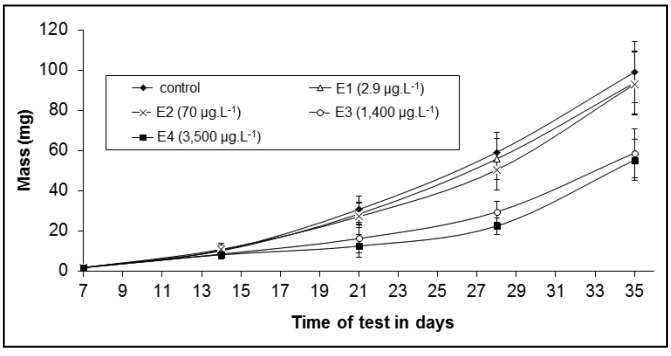


Fig. 2. Mean mass of larvae and juveniles common carp after terbuthylazine exposure. Data are means ± standard deviation (SD).

Tab. 1. Growth rate and fish mortality results of the 35 day embryo-larval toxicity test on common carp after terbuthylazine exposure.

Fish Group	Control	E1	E2	E3	E4
Terbuthylazine (μg.L^{-1})	–	2.9	70	1,400	3,500
m_7 (mean ± SD, mg)	1.72±0.18	1.67±0.24	1.60±0.21	1.58±0.19	1.59±0.25
m_{35} (mean ± SD, mg)	98.85±20.18	93.59±24.36	92.84±20.51	58.64±18.14*	55.16±21.12*
SGR	14.47	14.38	14.50	12.92	12.67
I (%)	–	0.62	–0.21	10.71	12.44
Total mortality (%)	10	11	13	35	58

m_7, m_{35} = mean fish mass in selected group after 7 and 35 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<0.01) different from the control group.

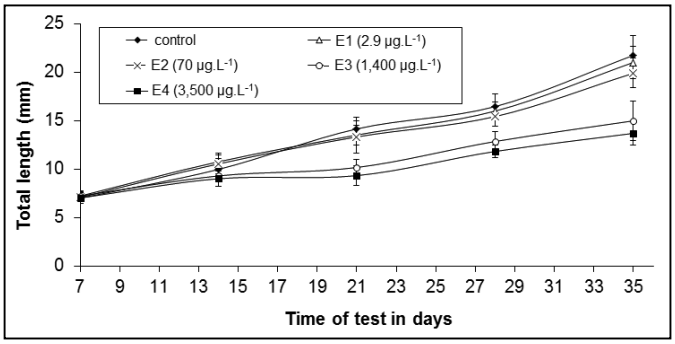


Fig. 3. Total length of larvae and juveniles common carp after terbuthylazine exposure. Data are means ± standard deviation (SD).

Tab. 2. Developmental periods during the 35 day embryo–larva toxicity test on common carp.

Fish Group	Control	E1	E2	E3	E4
Terbuthylazine (μg.L^{-1})	-	2.9	70	1,400	3,500
Times (day)					
7	ec9–L1	ec9–L1	ec9–L1	ec9–L1	ec9–L1
14	L2–L3b	L2–L3b	L2–L3b	L1–L3a	L1–L2
21	L4a–L5	L4a–L5	L4a–L5	L3a–L4a	L3a–L3b
28	L5–L6	L5–L6	L5–L6	L4a–L4b	L3b–L4b
35	J1	J1	J1	L4b–L6	L4b–L5

Length and weight growth parameters

Mass and total length of fish related to terbuthylazine concentrations in water are depicted in Figures 2 and 3. From 21 days of exposure, fish exposed to the two highest tested groups E3 – 1,400 μg.L^{-1}, and E4 – 3,500 μg.L^{-1} terbuthylazine showed significantly (p<0.01) lower mass and total length compared with controls. Terbuthylazine in the tested concentrations had no negative influence on FWC. Specific growth rates and inhibition of growth were calculated for 35 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to the two highest tested concentrations (1,400 and 3,500 μg.L^{-1}) was 10.71% and 12.44%, respectively, compared to control.

Early ontogeny

Fish from two highest tested concentrations (1,400 and 3,500 μg.L^{-1}) of terbuthylazine were significantly (p<0.01) delayed in early development compared with the control group (Table 2). No significant differences in the type and occurrence of morphological abnormalities were observed in tested embryos and larvae of carp during the test.

Histopatology

The histological changes were observed only in caudal kidney in all experimental groups compared to control fish. Fish exposed to terbuthylazine showed alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules. On the basis of histopathological changes of caudal kidney in the experimental groups, values of LOEC = 2.9 μg.L^{-1} terbuthylazine were estimated.

DISCUSSION

Triazines are serious pollutants of the aquatic environment that can have harmful effects on aquatic organisms alteration (Stara *et al.* 2013; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a). Aquatic organisms are subjected to prolonged exposure of small doses of contaminants in water that do not cause death but which could induce retardation of growth, early developmental and histopatological changes (Stepanova *et al.* 2012;

Stara *et al.* 2013; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a,b, 2015). The results of this study provide further data on long term exposure to terbuthylazine for consideration in risk assessment. The findings contribute to improved knowledge of the toxic profile of terbuthylazine at actual concentrations in the Czech rivers on early life stage of carp.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). Based on cumulative mortality, the 35 day LC50 was estimated at 2,992 $\mu g.L^{-1}$ terbuthylazine. The values obtained were lower than that reported in short-term tests on adult specimens, 7 $mg.L^{-1}$ terbuthylazine (Tomlin 2002). Terbuthylazine acute toxicity (96hLC50) values for the goldfish (*Carassius auratus*), 9.4 $mg.L^{-1}$ (Hartley & Kidd 1987); for rainbow trout (*Oncorhynchus mykiss*), is 3.8–4.6 $mg.L^{-1}$; for bluegill sunfish (*Lepomis macrochirus*), 7.5 $mg.L^{-1}$; and for common carp (*Cyprinus carpio*), 7 $mg.L^{-1}$ (Tomlin 2002).

The present study revealed no significant negative effects of terbuthylazine on hatching and embryos viability, morphology at the concentrations tested (2.9–3,500 $\mu g.L^{-1}$). In the present study, beginning on day 21 of exposure, fish exposed to the two highest tested groups terbuthylazine showed significantly lower mass and total length compared with controls. Growth reductions after terbuthylazine exposure might delay maturation and reproduction as well as increase the susceptibility of young fish to predation and disease. This is in agreement with studies Davies *et al.* (1994) who found growth reduction of common galaxias (*Galaxias maculates*) after exposure to low concentrations of atrazine. Plhalova *et al.* (2009) reported growth reduction of zebrafish (*Danio rerio*) after 28 days of exposure to terbutryne. Growth reduction were reported in carp after simazine (Velisek *et al.* 2012a), terbutryne (Velisek *et al.* 2012b) and terbuthylazine-2-hydroxy (Velisek *et al.* 2014b).

The advantage of toxicity tests on early-life stages of fish is the opportunity to observe developmental and morphological changes during exposure of xenobiotics. These tests are a sensitive method, since it covers two developmental stages (embryo and larvae) which differ in susceptibility as a result of physiological and biochemical differences (Penaz *et al.* 1983; McKim 1985; Stepanova *et al.* 2012). In our study terbuthylazine in concentrations 1,400 and 3,500 $\mu g.L^{-1}$ cause delayed in ontogenetic development. These findings are in accord with other studies which describe delay of ontogenetic development in common carp after exposure to terbuthylazine (Stepanova *et al.* 2012), prometryne (Velisek *et al.* 2015), terbutryne (Velisek *et al.* 2012b) and terbuthylazine-2-hydroxy (Velisek *et al.* 2014b).

Triazine pesticides have a direct effect on kidney structure and function in freshwater fish (Velisek *et al.* 2012a, 2014b, 2015). The caudal kidney of carp exposed to terbuthylazine all tested concentrations showed alteration of tubular system of caudal kidney. On the basis of our findings it is possible to describe terbuthylazine 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; Velisek *et al.* 2012a, 2014b, 2015). Histopathological changes were used for estimation of LOEC. The value for LOEC was estimated at 2.90 $\mu g.L^{-1}$ terbuthylazine. It appears that terbuthylazine may be a serious problem for early-life stages of carp in the polluted rivers of terbuthylazine. Because we found, that terbuthylazine in real concentration in Czech rivers has influence on caudal kidney of early-life stages of carp. According to our results of embryolarval test on carp, histopathological changes in caudal kidney seem to be the most sensitive parameter.

Chronic terbuthylazine exposure of early-life stages of common carp affected their growth rate, early ontogeny and histology. Some of the changes were observed only at higher exposures (1,400 and 3,500 $\mu g.L^{-1}$), but change founded in caudal kidney was affected in fish exposed to the real environmental concentration tested (i.e., 2.9 $\mu g.L^{-1}$). According to results of this present study, the histopathological changes in caudal kidney could provide useful information for evaluating the physiological effects on early life stages carp, but the application of these findings will need more detailed laboratory study before they can be established as special indicators for monitoring aquatic environment contaminated to triazine pesticides.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program) and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 018/2014/Z). We would like to thank Alan Pike & Kathleen Hills for manuscript improvement and English correction.

REFERENCES

1 Abrantes N, Pereira R, Gonçalves F (2010). Occurrence of pesticides in water, sediments, and fish tissues in a lake surrounded by agricultural lands: concerning risks to humans and ecological receptors. Water Air Pollut. **212**: 77–88.
2 Arufe MI, Arellano J, Moreno MJ, Sarasquetec C (2004). Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. Ecotoxicol Environ Safe. **59**: 209–216.
3 Buser HR (1990). Atrazine and other s-triazine herbicides in lakes and in rain in Switzerland. Environ Sci Technol. **24**: 1049–1058.
4 Brambilla A, Rindone B, Polesello S, Galassi A, Balestriniet R (2003). The fate of triazine pesticides in River Po water. Sci Total Environ. **132**: 339–348.

5 CHMI – Czech Hydrometeorological Institute (2014). On-line water duality database. Available from: http://hydro.chmi.cz/oj/, (visited online 18/12/2014).
6 Davies PE, Cook LSJ, Goenarso D (1994). Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. Environ Toxicol Chem. **13**: 1341–1354.
7 European Commission (1999). Study on the Prioritisation of Substances Dangerous to the Aquatic Environment. 98/788/ 3040/ DEB/E1, Office for Official Publications of the European Communities, Luxembourg.
8 EP – European Parliament (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy**.**
9 Figueiredo-Fernandes A, Fontainhas-Fernandes A, Peixoto F, Rochad E, Reis-Henriquesd MA (2006). Effects of gender and temperature on oxidative stress enzymes in Nile tilapia *Oreochromis niloticus* exposed to paraquat. Pestic Biochem Physiol. **85**: 97–103.
10 Fischer-Scherl T, Veseer A, Hoffman RW, Kuhnhauser C, Negele RD, Ewringmann T (1991). Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*). Arch Environ Contam Toxicol. **20**: 454–461.
11 Hartley D, Kidd H (1987). The Agrochemicals Handbook. The Royal Society of Chemistry, Lechworth, Herts.
12 Kocour M, Gela D, Rodina M, Linhart O (2005). Testing of performance in common carp *Cyprinus carpio* L. under pond husbandry conditions I: top-crossing with Northern mirror carp. Aquacul Res. **36**: 1207–1215.
13 Koutnik D, Stara A, Zuskova E, Kouba A, Velisek J (2014). The effect of long-term metribuzine exposure to signal crayfish (*Pacifastacus leniusculus* Dana). Neuroendocrinol Lett. **35**(Suppl. 2): 51–56.
14 McKim JM (1985). Early life stage toxicity tests. *In*: Rand GM, Petrocelli SR (Eds.), Fundamentals of Aquatic Toxicology. Hemisphere Publishers Corporation, New York, USA, pp. 58–95.
15 OECD – Organization for Economic Cooperation and Development (2000). Guideline for Testing of Chemicals 215. Fish juvenile growth test, Paris, France, 16 pp.
16 OECD – Organization for Economic Cooperation and Development (2013). Guidelines for the testing of chemicals. Section 2: Effects on Biotic Systems TG- No. 210: Fish, Early-Life Stage Toxicity Test. Paris, France, pp. 24.
17 Oropesa AL, Garcia-Cambero JP, Gomez L, Roncero V, Soler F (2009). Effect of long-term exposure to simazine on histopathology, hematological, and biochemical parameters in *Cyprinus carpio*. Environ Toxicol. **24**: 187–99.
18 Papadopoulos NG, Gikas E, Zalidis G, Tsarbopoulos A (2012). Determination of herbicide terbuthylazine and its major hydroxy and dealkylated metabolites in constructed wetland sediments using solid phase extraction and high performance liquid chromatography array detection. Inter J Environ Anal Chem. **92**: 1429–1442.
19 Penaz M, Prokes M, Kouril J, Hamackova J (1983). Early development of the carp, *Cyprinus carpio*. Acta Sci Natural Brno **17**: 1–39.
20 Plhalova L, Macova S, Haluzova I, Slaninova A, Dolezelova P, Marsalek P, et al (2009). Terbutryn toxicity to *Danio rerio*: Effects of subchronic exposure on fish growth. Neuroendocrinol Lett. **30**(Suppl. 1): 242–365.
21 Roberts TR, Hutson DH, Lee PW, Nicholls PH, Plimmer JR (1998). Metabolic pathways of agrochemicals. In Part 1: Herbicides and Plant Growth Regulators, The Royal Society of Chemistry, Cambridge, UK.
22 Tomlin CDS (2002). Terbuthylazine (5915-41-3). In: The e-Pesticide Manual, Version 2.2. Surrey UK, British Crop Protection Council.
23 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 Res Int. **2014**: Article ID 680131.
24 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.). Pest Biochem Physiol. **105**: 18–23.
25 Stepanova S, Plhalova L, Dolezelova P, Prokes M, Marsalek P, Skoric M, et al (2012). The effects of subchronic exposure to terbuthylazine on early developmental stages of common carp. Sci World J. **2012**: Article ID 615920.
26 Velisek J, Kouba A, Stara A (2013). Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*). Neuroendocrinol Lett. **34**(Suppl. 2): 31–36.
27 Velisek J, Stara A, Koutnik D, Zuskova E, Kouba A (2014a). Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Neuroendocrinol Lett. **35**(Suppl. 2): 93–98.
28 Velisek J, Stara A, Koutnik D, Machova J (2014b). Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed Res Int. **2014**: Article ID 621304.
29 Velisek J, Stara A, Koutnik D, Machova J (2015). Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.). Pest Biochem Physiol. **118**: 58–63.
30 Velisek J, Stara A, Machova J, Dvorak P, Zuskova E, Svobodova Z (2012a). Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **33**(Suppl. 3): 90–95.
31 Velisek J, Stara A, Machova J, Dvorak P, Zuskova E, Prokes M, et al (2012b). Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pest Biochem Physiol. **102**: 102–108.
32 Woltering D (1984). The growth response in fish chronic and early life stage toxicity tests: A critical review. Aqua Toxicol. **5**: 1–21.
33 WHO – World Health Organization (2003). Terbuthylazine (TBA) in Drinking-water: Background document for development of WHO Guidelines for Drinking-water duality. WHO, Geneva, 13 pp.

CHAPTER 5

EFFECT OF PROMETRYNE ON EARLY LIFE STAGES OF MARBLED CRAYFISH (*PROCAMBARUS FALLAX* F. *VIRGINALIS*)

Velisek, J., Stara, A., Koutnik, D., Zuskova, E., Kouba, A., 2014. Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Neuroendocrinology Letters 35: 93–98.

My share on this work was about 15%

It was allowed by publisher on 17th April, 2018 to include the paper in this Ph.D. thesis.

Neuroendocrinology Letters *Volume 35 Suppl. 2 2014*

ORIGINAL ARTICLE

Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*)

Josef Velisek, Alzbeta Stara, Dalibor Koutnik, Eliska Zuskova, Antonin Kouba

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic

Correspondence to: Assoc. Prof. Dipl.-Ing. Josef Velisek, PhD.
University of South Bohemia in Ceske Budejovice,
Faculty of Fisheries and Protection of Waters,
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology,
Zatisi 728/II, CZ-389 25 Vodnany, Czech Republic.
tel: +420 383 382 402; fax: +420 383 382 396; e-mail: velisek@frov.jcu.cz

Submitted: 2014-09-23 Accepted: 2014-11-08 Published online: 2014-11-30

Key words: **triazine; embryo-larval toxicity test; histopathology**

Neuroendocrinol Lett 2014; **35**(Suppl. 2):93–98 **PMID:** 25638372 NEL351014A10

Abstract

OBJECTIVES: The aim of this study is to assess the toxicity of prometryne in early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) on the basis of mortality, early ontogeny, growth rate, and histopathology during and at the end of the test.

DESIGN: The early life stages of marbled crayfish were exposed to prometryne at four concentrations, 0.51, (reported concentration in Czech rivers), 144, 1 440, and 4 320 μg.l^{-1} for 53 days and compared to crayfish in a non-treated control group.

RESULTS: Prometryne in concentration 144, 1 444 and 4 320 μg.l^{-1} caused decrease of weight and specific growth rates of crayfish. Crayfish exposed the highest concentration 4 320 μg.l^{-1} showed delay in ontogeny development. All crayfish groups exposed to prometryne showed histopathological changes in gill. On the basis of histopathological changes the values of LOEC=0.51 μg.l^{-1} and NOEC=for 0.10 μg.l^{-1} of prometryne for marbled crayfish juveniles was estimated.

CONCLUSIONS: Chronic exposure of prometryne on early life stages of crayfish has affected their mortality, growth rate, and histology. Some of the changes were observed only at higher exposures (144, 1 444 and 4 320 μg.l^{-1}), but histopathological changes in gills were observed also in crayfish exposed to the real environmental concentration in Czech rivers (i.e. 0.51 μg.l^{-1}), which is about 9 times lower than maximal concentration (4.40 μg.l^{-1}) reported in surface waters of Greece. Concentrations of prometryne in World rivers have been reported to generally vary in the range of 0.1–4.40 μg.l^{-1}.

Abbreviations:

ANOVA	- analysis of variance
$ANC_{4.5}$	- acid neutralization capacity
COD	- chemical oxygen demand
LC50	- lethal concentration
LOEC	- lowest observed effect concentration
NOEC	- no observed effect concentration
OECD	- Organization for Economic Cooperation and Development
SGR	- specific growth rate

To cite this article: **Neuroendocrinol Lett** 2014; **35**(Suppl. 2):93–98

INTRODUCTION

Environmental pollution caused by pesticides, especially in aquatic ecosystems, has become a serious problem. Many water ecosystems are contaminated with industrial, domestic, and agricultural chemicals such as pesticides, which are ubiquitous and can spread globally as well as regionally (Flynn & Spellman 2009). Pesticides are liable to affect non-target organisms, including fish, and crayfish leading to dramatic ecological changes in the aquatic environment (Velisek *et al.* 2012a, 2013; Stara *et al.* 2012, 2013, 2014).

Prometryne (2,4-bis (isopropylamino)-6-methylthio-s-triazine) was registered in the United States in 1964 as 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. 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 1996). 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). Prometryne has been banned in Europe since 2004, it still can be found in waters. In surface waters of Europe, 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).

Crayfish are important benthic invertebrates in the ecosystem, and they are considered an appropriate model organism (Monot 1995; Buric *et al.* 2013). Although the effects of sub-chronic exposure on oxidative stress and antioxidative enzymes of adult crayfish to prometryne have been well-documented in study Stara *et al.* (2014), there is a dearth of data on the sub-chronic toxicity of prometryne at environmentally realistic concentrations in early life stages of crayfish. Native species of crayfish are endangered and protected in the Czech Republic (Kozak *et al.* 2011). For this reason, we have chosen the invasive marbled crayfish (*Procambarus fallax* f. *virginalis*), which became established in Europe (Kouba *et al.* 2014), as a model species for this study. The marbled crayfish, which was discovered in the mid-1990s, meets researchers' demands for a vigorous, genetically identical and eurytopic laboratory model very well. Its most prominent advantages are production of high numbers of genetically identical offspring, stepwise alteration of the phenotype by moulting, complex morphology and behaviour (Vogt 2008). The aim of the present study was to describe lethal and sublethal effects of prometryne on early life stage of non-target aquatic organism, the marbled crayfish using a 53 day toxicity test.

MATERIALS AND METHODS

Experimental animals

Eggs from single marbled crayfish (*Procambarus fallax* f. *virginalis*) female (carapace length 31.22 mm, postorbital carapace length 23.62 mm, and weight 9.19 g) were gently stripped with tweezers from pleopods. Female originated from own laboratory culture.

Experimental protocol

Three hundred eggs (mean weight 2.27 mg) in IX–X stage of embryonic development were put into petri dishes with tap water. From petri dishes were randomly transferred separately into plastic macroplates containing one of four experimental solutions of prometryne (Sigma Aldrich, Czech Republic, and chemical purity 99.3%) and water which served as a control. Each trial comprised 60 eggs held as single individuals to eliminate the transfer of fungal infection between incubated eggs. The concentrations were marked as follows: 0.51 $\mu g.l^{-1}$ (reported environmental concentration in Czech rivers – group 1 – E1), 140 $\mu g.l^{-1}$ (group 2 – E2), 1 440 $\mu g.l^{-1}$ (group 3 – E3), and 4 320 $\mu g.l^{-1}$ (group 4 – E4). The prometryne concentrations of 140 $\mu g.l^{-1}$, 1 440 $\mu g.l^{-1}$, and 4 320 $\mu g.l^{-1}$ corresponded to the 1% of 96 hour half lethal concentration (96h LC50), 10% 96h LC50 and 30% 96h LC50 for juvenile signal crayfish (*Pacifastacus leniusculus*) (Velisek *et al.* 2013).

Water parameters

Water quality parameters were as follows: temperature 22.8±1.5 °C, dissolved oxygen >60%, pH 7.5–8.0, $ANC_{4.5}$ 0.91 $mmol.l^{-1}$, COD_{Mn} 0.31 $mg.l^{-1}$, total ammonia 0.01 $mg.l^{-1}$, NO_2^- 0.02 $mg.l^{-1}$, NO_3^- 4.38 $mg.l^{-1}$, and sum of Ca^{2+}+Mg^{2+} 32.22 $mg.l^{-1}$. Temperature was measured hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). To ensure agreement between nominal and actual compound concentrations, water was analysed during the experimental period by liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Barcelo & Hennion 1997). The values measured did not differ from the value stated for test purposes by more than 10 %.

Experimental protocol

The macroplates were placed in a laboratory (open-air conditions) with the light exposure (11:13 h light: dark). The exposure water for each treatment was renewed three times weekly until the third developmental stage (24 days) was reached. Water was gently by drained from each chamber, then a new solution was slowly added to prevent disturbance. Observations of survival were made daily and dead eggs were removed. From the third development stage, juvenile were kept individually in small boxes made from clear plastic to prevent cannibalism. Each box, 40 mm in height, was divided into18 separated chambers, with the bottom area of each individual chamber 45 × 30 mm (Kozak *et al.* 2009)

was placed in an aquaria containing 20 l of respective solutions. Animals were fed by freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii *ad libitum* one time daily. The nauplii were rinsed with tap water to avoid contaminating the exposure water with chloride. During and at the end of the experiment, early development stages were observed to monitor development, occurrence of morphological anomalies and body weight. Determination of developmental periods and stages followed Vogt *et al.* (2004), who subdivided into embryonic, juvenile, adolescent and adult phases. The embryonic period starts with oviposition and ends 17–28 days later with hatching. The juvenile phase includes approximately seven to eight stages, which are characterized by a spotted pigmentation pattern. The seven to 10 adolescent stages are increasingly marbled and have clearly visible female characters.

Weight, to 0.1 mg, was measured by using a Mettler-Toledo analytical balance after removing excess water on filter paper. The mean specific growth rate (SGR) for crayfish in each of the experimental groups was calculated for the period beginning at day 24 (the first sampling time) and ending at day 53 (end of the trial) using the method described by Kroupova *et al.* (2010).

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

$$I_x[\%]=[SGR_x(control)-SGR_x(group)]/[SGR_x(control)]\times 100$$

where is I_x = inhibition of specific growth in selected experimental group of crayfish 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 of crayfish.

Evaluation of 53 day LC50, LOEC, and NOEC

For the evaluation of 53 day LC50 values, a probit analysis was used based on mortality at different prometryne concentrations. For the evaluation of LOEC and NOEC values, the probit analysis was based on histopathological changes in tissues at different prometryne concentrations and the EKOTOX 5.1 software (INGEO Liberec, Czech Republic) was used.

Histopathology examination

Histopathology was evaluated in groups E1–E3 and control at the end of the experiment (day 53), the group E4 was no sampled for histology because 30 days all juvenile died. Ten whole crayfish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, and stained with haematoxylin and eosin, examined by light microscopy, and photographed using a digital camera.

Statistical analysis

One-way ANOVA was conducted to compare differences among the test groups using the software program Statistica version 12.0 for Windows (StatSoft).

RESULTS

Accumulated mortality

Significant ($p<0.01$) differences in total accumulated mortality were found in crayfish exposed to the three highest prometryne concentrations, compared with controls (Figure 1). Prometryne in concentration 4 320 µg.l^{-1} caused 100% mortality of crayfish. Massive mortality in this group occurred on day 15 and 30 (developmental stage 2 and 3). Based on accumulated mortality in the experimental groups, values of lethal concentrations of prometryne was estimated at 53 day LC50 = 40 µg.l^{-1}.

Growth parameters

Mean body weight of crayfish related to prometryne concentration in water is depicted in Figure 2. No significantly negative effects on body weight and specific growth rate were observed when environmental concentration of prometryne (E1; 0.51 µg.l^{-1}) was compared with the control. From 24 days of exposure, crayfish exposed to the highest tested groups E4 – 4 320 µg.l^{-1} prometryne showed significantly ($p<0.01$) lower mass compared with control. Beginning on day 38 of expo-

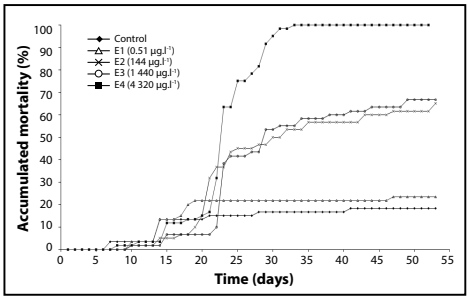


Fig. 1. Accumulated mortality (percentage) of early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) after prometryne exposure.

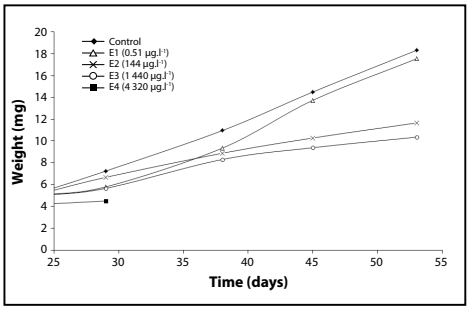


Fig. 2. Mean body weight of early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) after prometryne exposure.

sure, also crayfish exposed to the tested groups E3 – 1 440 μg.l^{-1}, and E2 – 144 μg.l^{-1} prometryne showed significantly (p<0.01) lower mass compared with control. Specific growth rates and inhibition of growth were calculated for 53 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to prometryne in concentrations 144 (E2) and 1 440 μg.l^{-1} (E3) was 34.8 and 42.6 %compared to control, respectively.

Early ontogeny

Only crayfish exposed the highest concentration 4 320 μg.l^{-1} showed delay in ontogeny development compared with the control group. Furthermore, no significant differences in the type and occurrence of morphological abnormalities were observed in tested crayfish during the test.

Histopatology

There were no apparent differences in hepatopancreas tissue between control and group E1. The morphology of the hepatopancreas in these two groups was normal, with tubules tightly arranged. Different cell types were both easily recognized and reasonably uniform in shape and size. The marked alterations in hepatopancreas tubules were observed in groups E2 and E3. The pathologies were as follows: tubular dilatation with the predominance of mononuclear cells in interstitial tissue and focal dystrophic tissue destruction with more pronounced changes in group E3 (Figure 3).

Large fragmental alterations with pseudocystical formations in gills of all experimental groups were apparent. The most pronounced changes were represented by dilatations of filaments into pseudocystical structures filled with fine-grained substance. All described changes were more frequent in the group exposed to higher concentration of promethryne (E3).

On the basis of histopathological changes of gills in the experimental groups, values of LOEC = 0.51 μg.l^{-1} and NOEC = 0.10 μg.l^{-1} of prometryne for marbled crayfish juveniles were estimated.

DISCUSSION

Crayfish can serve as an excellent model species to increase the knowledge-base for invertebrate ecotoxicology (Burggren & McMahon 1983). They are large invertebrates and easily reared in a laboratory setting. This makes them useful not only for toxicity testing, but as an invertebrate model for a variety of physiological experiments. The eggs are very large for a crustacean and can easily be counted and assessed for fertilization status, mortality, etc., thus making them useful for early life stage studies. Studies of the embryonic development of crayfishes 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 & Guerrero 2000). The sensitivity of embryos of the marbled crayfish to pollutant was tested with testosterone (Vogt 2007). Although we have information on the toxicity of the prometryne in adult of crayfish, there is no information on its toxicity in early life stages. This is the first study investigating toxicity of prometryne on early life stage of crayfish.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). Prometryne in concentration 4 320 μg.l^{-1} caused total mortality of crayfish during 15 and 30 day (stage 2 and 3). The most of the external sense organs become functional in juvenile stage 2, that the digestive system becomes functional in stage 3 (Voght 2008). This is in accordance with data concerning the so called "point of no return" the moment when the juvenile irreversibly lose ability to feed, and die even if provided with food. In marbled crayfish juvenile the point of no return occurs about day 10–22 days (Voght 2008) and starvation-induced mortality occurs after that time.

The present study revealed no significant negative effects of prometryne on growth and mortality in early life stages at the environmental concentration

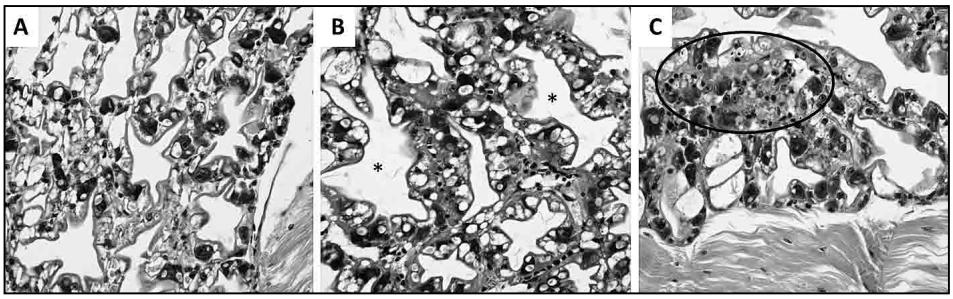


Fig. 3. Transversal sections of juvenile s marbled crayfish (*Procambarus fallax* f. *virginalis*) hepatopancreas. A – control group; B – E2 group exposed to 1% of 96 hour half lethal concentration (96h LC50) of prometryne for 53 days; C – E3 group exposed to 10% of 96h LC50 prometryne for 53 days (400×). Asterisks (*) mark noticeable tubular dilatation; ellipse indicates.

(0.51 μg.l^{-1}). Crayfish exposed the highest concentration 4 320 μg.l^{-1} showed delay in ontogeny development compared with the control group. Delay in ontogenetic development is described after prometryne exposure in concentrations ranging from 200 to 2 000 μg.l^{-1} in early life stages of fish (Velisek *et al.* 2012b). In the present study, crayfish exposed to the 144, 1 440 and 4 320 μg.l^{-1} prometryne showed significantly lower mean body weight compared with the control. Growth reductions might delay maturation and reproduction as well as increase the susceptibility of young crayfish to predation. Their ability to obtain food and to compete for suitable habitats might also be reduced (Woltering 1984). Velisek *et al.* (2012a,b) reported a significant decrease growth after triazines pesticide (terbutryne and simazine) exposure in common carp (*Cyprinus carpio*). Significant decrease of growth in juvenile Australian red claw crayfish (Cherax quadricarinatus) after exposure mixture glyphosate (22.5 mg.l^{-1}) and polyoxyethylenamine (15 mg.l^{-1}) reported Frontera *et al.* (2011).

The effect of chronic exposure to triazine at low concentrations on histopathology of early life stages has not yet been studied. In our study, crayfish exposed to prometryne in all tested concentrations (0.51; 144; 1 440 and 4 320 μg.l^{-1}) resulted in histopathological changes of gills and hepatopancreas. Crustacean gills are a vital organ as they play an important role in diffusion and transport of respiratory gases and regulation of osmotic and ionic balance. Gills are a primary target organ for most pollutant, uptake from water is the most important route, and may be one of the first organs to exhibit symptoms of toxicity (Desouky *et al.* 2013). Changes caused of prometryne in gills may affect osmoregulatory gills function and gases exchange. Observed pathological changes are in accord with those described in Desouky *et al.* (2013) in red swamp crayfish (*Procambarus clarkii*) gills after exposure of organophosphorus insecticide ethion (0.36 mg.l^{-1}). The crustacean hepatopancreas is likely the main organ for the detoxification of pollutants (Vogt 2002) and produces and secretes all digestive enzymes involved in carbohydrate metabolism, production of emulsifiers, excretion, and calcium, and heavy metal storage (Holdich & Reeve 1988). These changes in our experiment are probably due to accumulation of the prometryne in the cells of hepatopancreas or due to increasing the activity of lysosomal enzymes which are capable of destroying cell organelles. Similar histopathological changes in hepatopancreas were also reported in red swamp crayfish after exposure of ethion (Desouky *et al.* 2013); malathion (Garo *et al.* 1998); diazinon (Heiba 1999) and fenthion (Aly 2000). On the other hand, Stara *et al.* (2014) found no histopatological changes in hepatopancreas of adult red swamp crayfish after prometryne exposure in concentrations (0.51–1 440 μg.l^{-1}).

Histopathological changes were used for estimation of NOEC and LOEC. The values for NOEC and LOEC were estimated at 0.1 and 0.51 μg.l^{-1} prometryne, respectively. It appears that prometryne may be a serious problem for early life stages of crayfish in the wild. Some of the changes were observed only at higher exposures (144, 1 444 and 4 320 μg.l^{-1}), but histopathological changes in gills and hepatopancreas were observed in crayfish exposed the real environmental concentration in Czech rivers (i.e., 0.51 μg.l^{-1}), which is about 9 times lower than maximal concentration (4.40 μg.l^{-1}) reported in surface waters of Greece (Vryzas *et al.* 2011; Caquet *et al.* 2013).

Histopathological changes in gills and hepatopancreas are potential biomarkers for monitoring residual triazine pesticides in early life stages of crayfish. For detailed elucidation of prometryne effects, further research is necessary. Aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. This research should be focused not only on the studies of effects of prometryne alone, but in view of possible synergic or potentiation effects.

ACKNOWLEDGMENTS

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects „CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program); by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 018/2014/Z).

REFERENCES

1 Aly RH (2000). Effect of natural and chemical insecticides on some organs of the female crayfish, *Procambarus clarkii* (Crustacea: Decapoda) from the River Nile, Egypt. Egypt J Aquat Biol Fish. **4**: 83–103.
2 Barcelo D, Hennion MC (1997). Trace determination of pesticides and their degradation products in water, Mass Spectrometric Methods, LC–MS. Elsevier, Amsterdam, pp. 225–234.
3 Burggren WW, McMahon BR (1983): An analysis of scaphognathite pumping performance in the crayfish *Orconectes virilis* - Compensatory changes to acute and chronic hypoxic exposure. Physiol Zool. **56**: 309–318.
4 Buric M, Kouba A, Machova J, Mahovska I, Kozak P (2013). Toxicity of the organophosphate pesticide diazinon to crayfish of differing age. Int J Environ Sci Technol. **10**: 607–610.
5 Desouky MMA, Abdel-Gawad H, Hegazi B (2013). Distribution, fate and histopathological effects of ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. Food Chem Toxicol. **52**: 42–52.
6 Flynn K, Spellman T (2009). Environmental levels of atrazine decrease spatial aggregation in the freshwater mussel, *Elliptio complanata*. Ecotoxicol Environ Safe. **72**: 1228–1233.
7 Frontera JL, Vatnick I, Chaulet A, Rodriguez EM (2011). Effects of glyphosate and polyoxyethylenamine on growth and energetic reserves in the freshwater crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Arch Environ Contam Toxicol. **61**: 590–598.
8 Garo K, Hamdy SAH, Soliman G (1998). Histopathological changes in the hepatopancreas of the crayfish *Procambarus clarkii* (Giard, 1853) (Crustacea, Decapoda, Cambaridae) exposed to malathion. J Union Arab Biol Cairo **10**: 65–76.

9 Heiba FN (1999). Effect of the insecticide diazinon on the hepatopanceas of the freshwater crayfish, *Procambarus clarkii*. Egypt J Aquat Biol Fish. 3: 197–213.
10 Holdich DM, Reeve ID (1988). Functional morphology and anatomy. *In*: Holdich DM, Lowry RS (Eds.), Freshwater Crayfish: Biology, Management and Exploitation. Croom Helm, London, UK, pp. 34–51.
11 Kegley SE, Hill BR, Orme S, Choi AH (2010). PAN Pesticide Database. Pesticide Action Network, San Francisco.
12 Kouba A, Petrusek A, Kozák P (2014). Continental-wide distribution of crayfish species in Europe: update and maps. Knowl Managt Aquat Ecosyst. **413**: 5.
13 Kozak P, Buric M, Kanta J, Kouba A, Hamr P, Policar T (2009). The effect of water temperature on the number of moults and growth of juvenile signal crayfish *Pacifastacus leniusculus* Dana. Czech J Anim Sci. **54**: 286–292.
14 Kozak P, Fureder L, Kouba A, Reynolds J, Souty-Grosset C (2011). Current conservation strategies for European crayfish. Knowl Managt Aquat Ecosyst. **401**: 1.
15 Kroupova H, Prokes M, Macova S, Penaz M, Barus V, Novotny L, Machova J (2010). Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.). Environ Toxicol Chem. **29**: 535–540.
16 LeBaron HM, McFarland JE, Burnside OC (2008). The Triazine Herbicides, 50 Years Revolutionizing Agriculture. Elsevier, San Diego, pp. 600.
17 Meijide FJ, Guerrero GA (2000). Embryonic and larval development of a substratebrooding cichlid *Cichlasoma dimerus* (Heckel, 1840) under laboratory conditions. J Zool. **252**: 481–493.
18 Momot WT (1995). Redefining the role of crayfish in aquatic ecosystems. Rev Fish Sci. **3**: 33–63.
19 OECD, (Organization for Economic Cooperation and Development) (2000). Guideline for Testing of Chemicals 215. Fish juvenile growth test, Paris, France, 16 pp.
20 Papadopoulou-Mourkidou E, Karpouzas DG, Patsias J, Kotopoulou A, Milothridou A, Kintzikoglou K, Vlachou P (2004). The potential of pesticides to contaminate the groundwater resources of the Axios river basin in Macedonia, Northern Greece. Part I. Monitoring study in the north part of the basin. Sci. Total Environ. **321**: 127–146.
21 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 Res Int. **2014**: Article ID 680131.
22 Stara A, Steinbach Ch, Wlasow T, Gomulka P, Ziemok E, Machova J, Velisek J (2013). Effect of zeta-cypermethrin on common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **34 S. 2**: 37–42.
23 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.). Neuroendocrinol Lett. **33 S. 3**: 130–135.
24 US EPA, (Environmental Protection Agency) (1996). R.E.D. Fact Prometryn. Available from: http://www.epa. gov/oppsrrd1/REDs/factsheets/0467fact.pdf, (visited online 12.6.2014), pp. 11.
25 Velisek J, Kouba A, Stara A (2013). Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*). Neuroendocrinology Lett. **34 S. 2**: 31–36.
26 Velisek J, Stara A, Machova J, Dvorak P, Zuskova E, Svobodova Z (2012a). Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **33 S. 3**: 90–95.
27 Velisek J, Stara A, Machova J, Dvorak P, Zuskova E, Prokes M, Svobodova Z (2012b). Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pestic Biochem Physiol. **102**: 102–108.
28 Vogt G (2002). Functional anatomy. *In:* Holdich DM (Ed), Biology of Freshwater Crayfish. Blackwell, Oxford, pp. 53–151.
29 Vogt G (2007). Exposure of the eggs to 17a-methyl testosterone reduced hatching success and growth and elicited teratogenic effects in postembryonic life stages of crayfish. Aquat Toxicol. **85**: 291–296.
30 Vogt G (2008). The marbled crayfish: a new model organism for research on development, epigenetics and evolutionary biology. J Zool. **276**: 1–13.
31 Vogt G, Tolley L, Scholtz G (2004). Life stages and reproductive components of the Marmorkrebs (marbled crayfish), the first parthenogenetic decapod crustacean. J Morphol. **261**: 286–311.
32 Vryzas Z, Alexoudisa C, Vassilioua G, Galanisa K, Papadopoulou-Mourkidoub E (2011). Determination and aquatic risk assessment of pesticide residues in riparian drainage canals in northeastern Greece. Ecotox Environ Safe. **74**: 174–181.
33 Woltering DM (1984). The growth response in fish chronic and early life stage toxicity tests: a critical review. Aquat Toxicol. **5**: 1–21.
34 Zhou J, Chen J, Cheng Y, Li D, Hu F, Li, H (2009). Determination of prometryne in water and soil by HPLC–UV using cloud-point extraction. Talanta **79**: 189–193.

CHAPTER 6

EFFECTS OF THE TERBUTHYLAZINE METABOLITE TERBUTHYLAZINE-DESETHYL ON COMMON CARP EMBRYOS AND LARVAE

Velisek, J., Koutnik, D., Zuskova, E., Stara, A., 2016. Effects of the terbuthylazine metabolite terbuthylazine-desethyl on common carp embryos and larvae. Science of the Total Environment 539: 214–220.

My share on this work was about 20%

It was allowed by publisher on 5th May, 2018 to include the paper in this Ph.D. thesis.

Science of the Total Environment 539 (2016) 214–220

Contents lists available at ScienceDirect

Science of the Total Environment

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

Effects of the terbuthylazine metabolite terbuthylazine-desethyl on common carp embryos and larvae

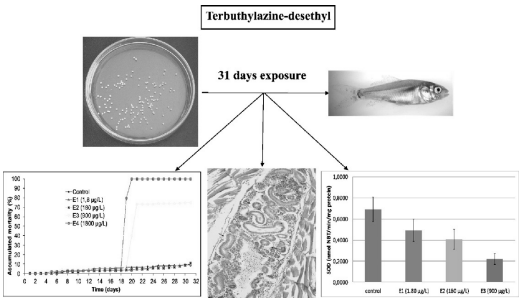
Josef Velisek *, Dalibor Koutnik, Eliska Zuskova, Alzbeta Stara

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

HIGHLIGHTS

- Terbuthylazine-desethyl influence to carp at real environmental concentration.
- Terbuthylazine-desethyl reduced growth in carp larvae and embryos.
- Terbuthylazine-desethyl caused delay in development in carp.
- Terbuthylazine-desethyl caused pathological changes in caudal kidney tubular system

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 31 August 2015
Accepted 31 August 2015
Available online xxxx

Editor: D. Barcelo

Keywords:
Triazine
Embryo
Larvae
Early development
Histopathology
Oxidative stress
Antioxidant enzymes

ABSTRACT

Toxicity of terbuthylazine-desethyl to embryos and larvae of common carp (*Cyprinus carpio*) was assessed. Based on mortality, the lethal concentration of terbuthylazine-desethyl was estimated to be 31 days LC50 = 441.6 μg/L. Carp exposed to terbuthylazine-desethyl at 1800 μg/L exhibited lower weight and length at 7 days of exposure compared to the control group. By day 20, carp exposed to 900 μg/L terbuthylazine-desethyl showed lower weight and length compared to control group. Terbuthylazine-desethyl in concentrations (180, 900, and 1800 μg/L) caused delay in ontogenetic development. Total superoxide dismutase activity was significantly lower in all exposed groups. Exposure to 180 and 900 μg/L terbuthylazine-desethyl was associated with alteration of the caudal kidney tubular system including peritubular dilatation detachment of epithelial cells from the basal lamina, and focal autolytic disintegration of the tubular epithelia. Chronic terbuthylazine-desethyl exposure affected survival, growth, ontogenetic development, and the antioxidant system and caused pathological changes to the caudal kidney.

* Corresponding author.
E-mail address: velisek@frov.jcu.cz (J. Velisek).

http://dx.doi.org/10.1016/j.scitotenv.2015.08.152
0048-9697/

1. Introduction

Pollution constitutes a problem of all over the world (Blasco and Pico, 2009; Abrantes et al., 2010; Navarro-Ortega et al., 2012). Triazines are carried into aquatic environments by surface runoff from sites of application and can negatively affect the health of aquatic organisms (Velisek et al., 2008, 2010, 2011, 2012a, 2013, 2014).

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) was issuance Commission Implementing Regulation (EU) No 820/2011 removed from the Annex to Decision 2008/934/EC. Terbuthylazine is an *s*-triazine herbicide widely used in agriculture to control grass and in Italy, it is used in maize, sorghum, and olive culture (Fait et al., 2010). The European Food Safety Authority (EFSA) has reported that terbuthylazine poses high long-term risks for organisms (EFSA, 2011) and can have genotoxic effects (Mladinic et al., 2012). Its major degradation products in ground and surface waters are terbuthylazine-2-hydroxy, terbuthylazine-desethyl-hydroxy, and terbuthylazine-desethyl (Nodler et al., 2013). In surface and ground waters of Europe were has been recorded at concentrations from 0.02 to 2.90 μg/L of terbuthylazine (Buser, 1990; Brambilla et al., 2003; CHMU, 2015) and from 0.008 to 1.80 μg/L of terbuthylazine-desethyl (Dankwardt et al., 1997; ISPRA, 2010; Benvenuto et al., 2010; Mansilha et al., 2011; Loos et al., 2013; CHMU, 2015). The real maximal concentration of terbuthylazine-desethyl detected in Czech river was 1.80 μg/L (CHMU, 2015). Terbuthylazine is slightly toxic to fish. Acute toxicity 96 h LC50, for goldfish *Carassius auratus* is 9.4 mg/L (Hartley and Kidd, 1987); for rainbow trout *Oncorhynchus mykiss* is 3.8–4.6 mg/L; for bluegill sunfish *Lepomis macrochirus*, 7.5 mg/L; and for common carp *Cyprinus carpio*, 7 mg/L (Tomlin, 2002).

The acute effects of terbuthylazine and other triazines in fish are well documented, but data on toxicity of the terbuthylazine-desethyl metabolite of terbuthylazine on embryos and larvae of fish are lacking. The aim of the present study was to describe the effects of the terbuthylazine-desethyl metabolite on early-life stages of carp, a well-established model species for testing chemical effects on early development (OECD, 2013). The effects were evaluated with respect to mortality, ontogenetic development, growth, oxidative stress biomarkers, antioxidant enzymes, occurrence of morphological anomalies, and histopathology.

2. Materials and methods

2.1. Experimental animals

Fertilized eggs of carp were obtained from a hatchery of the Faculty of Fisheries and Protection of Waters, Czech Republic. Eggs were fertilized by the methods described by Kocour et al. (2005).

2.2. Water variables

Aerated water was used with the following values: dissolved oxygen, >90%; temperature, 19.5–20.8 °C; pH, 7.4–8.2, which were monitored daily.

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 Papadopoulos et al. (2007). Water was chromatographed on a reverse phase HPLC column (Lichrosphere 100 RP18, Vertex column, pore size 100 μm, particle size 5 μm, 250 mm × 5 mm internal diameter (ID)) using solvent systems of methanol:water:ammonium:acetate 70:30:0.2 and 80:20:0.2 (v:v:v) isocratically, with a flow rate of 0.7 mL/min. Injection volumes of samples were 100 μL per injection. UV detection was recorded at 230 nm. Column eluents (1 minute 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 liquid scintillation counting (LSC). 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 5%.

2.3. Experimental protocol

The investigation was carried out using the modified No. 210 OECD test (OECD, 2013). At 24 h post fertilization, 100 fertilized eggs were placed into each of fifteen glass basins with the terbuthylazine-desethyl solution, plus three control dishes containing water only. Terbuthylazine-desethyl (chemical purity 97.4%) was obtained from Sigma-Aldrich Corporation (USA). The test was conducted in triplicate. Four concentrations of test solution were used: 1.80 μg/L (observed environmental concentration), 180 μg/L, 900 μg/L, and 1800 μg/L. Three highest groups were used as contrast groups.

The solution for each treatment was renewed daily. Behavior was monitored daily. Mortality was recorded and dead carp removed. From day 6, larvae of carp were fed ad libitum with *Artemia salina* nauplii.

On days 7, 14, 20, 27, and 31, 30 a carp in each concentration group and the control were collected for examination. Developmental periods were defined according to Penaz et al. (1983). Final evaluations included cumulative mortality, weight and total length (TL). Length was measured by stereomicroscopy using a micrometer. Mass was measured (0.1 mg) with a Mettler–Toledo scale.

2.4. Growth rate

The mean specific growth rate (SGR) for carp in exposed groups was calculated for the period from day 7 to day 31 of exposure and compared with controls using the method of Kroupova et al. (2010). 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), $\ln w_1$ = mean value of the $\ln w_1$ values, $\ln w_2$ = 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.

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

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.

Fulton's weight condition factor (FWC) was calculated for each experimental group at every sampling time:

$$\mathrm{FWC} = \frac{W \cdot 10^5}{\mathrm{TL}^3}$$

where FWC = Fulton's weight condition factor, W = mass in selected experimental group (g), and TL = total length in selected experimental group (mm).

2.5. Evaluation of 31 days LC50

Based on mortality, the 31 days LC50 for terbuthylazine-desethyl was established using Probit Analysis EKOTOX 5.1 (Ingeo) software.

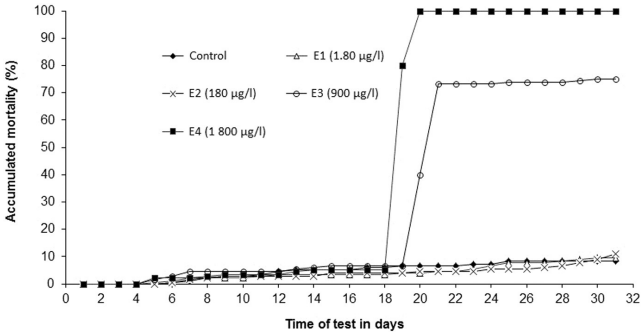


Fig. 1. Cumulative mortality of common carp larvae and juveniles following terbuthylazine-desethyl exposure.

2.6. Histology

Histological examination was conducted in groups exposed to concentrations 1.80, 180 and 900 µg/L and control after 31 days of exposure. Histology was not conducted in group exposed to concentration 1800 µg/L total mortality occurred prior to 20 days. Six carps from each experimental group and control were fixed in 10% formalin. Samples were embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy.

2.7. Oxidative stress biomarkers

Oxidative stress biomarkers were evaluated in the surviving groups and controls after 31 days exposure. 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 according to methods Stara et al. (2012a). The homogenate was divided into two portions. One for measuring thiobarbituric acid reactive substances (TBARS), and a second portion for measuring antioxidant enzymes analysis, which was centrifuged at 4 °C to obtain the post-mitochondrial supernatant.

Lipid peroxidation as TBARS was estimated spectrophotometrically according to Lushchak et al. (2005). The TBARS concentration was calculated by the absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The value was expressed as nanomoles of TBARS per mg protein.

Total superoxide dismutase (SOD) activity was estimated spectrophotometrically using 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 nanomoles of SOD per milligram of protein. The catalase (CAT) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer (1952). One unit of CAT activity was defined as the amount of the enzyme that consumes 1 µmol of H_2O_2 per min per milligram of protein. Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). One unit of GR activity was defined as the amount of the enzyme that consumes 1 nmol of NADPH per min per milligram of protein. Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

2.8. Statistical analysis

One-way ANOVA was used to compare differences among the experimental groups and controls using Statistica v. 12 for Windows (StatSoft). The differences between experimental groups and control in cumulative mortality were assessed using contingency tables (χ^2).

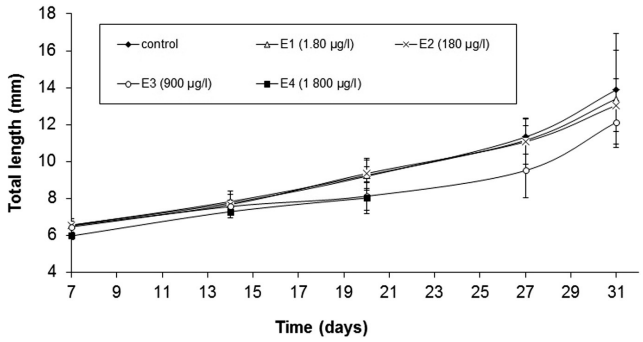


Fig. 2. Mean ± SD mass of common carp larvae and juveniles following terbuthylazine-desethyl exposure.

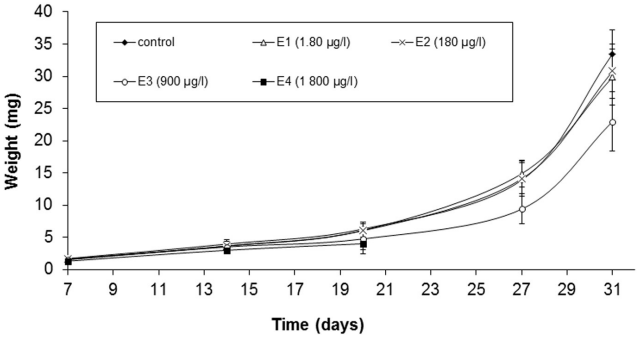


Fig. 3. Mean ± SD total length of common carp larvae and juveniles following terbuthylazine-desethyl exposure.

3. Results

3.1. Hatching and cumulative mortality

Terbuthylazine-desethyl in tested concentrations showed no negative effects on hatching and embryo viability. Significant differences ($P < 0.01$) in total accumulated mortality were found in fish exposed to 900 and 1800 µg/L terbuthylazine-desethyl compared with controls (Fig. 1). Massive mortality in these exposed groups occurred at days 18 and 19. On the basis of accumulated mortality in the experimental groups, values of lethal concentrations of terbuthylazine-desethyl was estimated at 31 days LC50 = 441.6 mg/L.

3.2. Growth, early ontogeny, and morphological anomalies

Carp in group exposed to 1800 µg/L showed significantly ($P < 0.01$) lower weight (Fig. 2) and length (Fig. 3) on day 7 of exposure compared to control. Terbuthylazine-desethyl at 900 µg/L was associated with significantly ($P < 0.01$) lower weight and length beginning at day 20 of exposure compared to control group. Terbuthylazine-desethyl had no effect on Fulton's weight condition factor. Inhibition of specific growth at 900 µg/L was 9.23% compared to controls (Table 1).

Fish exposed to terbuthylazine-desethyl at 180, 900 and 1800 µg/L exhibited delays in development compared to control group (Table 2).

Curvature of the spine and body shortening were found in <1% of all tested and control groups. These can be considered as spontaneous anomalies.

3.3. Histology

Terbuthylazine-desethyl was not associated with pathological changes in gill or liver. The primary pathology was found in caudal kidney in 180 and 900 µg/L. The severity of kidney damage was dose-dependent. Fish exposed to the highest concentration of terbuthylazine-desethyl showed alteration of the tubular system including peritubular dilatation, and detachment of epithelial cells from the basal lamina, and focal autolytic disintegration of tubular epithelia (Fig. 4).

3.4. Oxidative stress and antioxidant response

Effects of terbuthylazine-desethyl exposure on TBARS level and antioxidant responses (SOD, CAT, GR) in homogenate of carp early life stages are shown in Table 3. Significantly ($P < 0.01$) lower SOD activity was seen in all groups exposed to terbuthylazine-desethyl compared to controls. No differences were found in TBARS level or CAT and GR activity among groups.

4. Discussion

Embryo and larva toxicity tests are commonly used for assessing pesticide effects. These stages are frequently more sensitive to pesticides than are juveniles and adults (Woltering, 1984; Kristensen, 1994; McKim, 1995). Studying the toxicity of pesticides to early life stages of fish is important, not only to increase knowledge of the ontogenetic development, but also to understand species-specific adaptations and their ecological value in the course of speciation. This study provides new information on the effects of a major metabolite of terbuthylazine on embryo and larvae of carp.

Terbuthylazine-desethyl at tested concentrations showed no negative effects on hatching or embryo viability, but a significant effect was found on survival of carp larvae exposed to 900 and 1800 µg/L. Total mortality was observed at 1800 µg/L and 75% at 900 µg/L terbuthylazine-desethyl with mortality peaking on days 18 and 19 of exposure. After 18 days, only incidental mortality was observed, indicating higher tolerance of older larvae to terbuthylazine-desethyl. The

Table 1
Growth and mortality of carp after terbuthylazine-desethyl after 31 day exposure.

Carp group	Control	E1	E2	E3	E4
Terbuthylazine-desethyl (µg/L)	–	1.80	180	900	1800
m_7 (Mean ± SD, mg)	1.73 ± 0.20	1.63 ± 0.12	1.62 ± 0.20	1.56 ± 0.23	1.34 ± 0.18*
m_{31} (Mean ± SD, mg)	33.50 ± 12.66	29.00 ± 8.35	30.80 ± 9.21	23.00 ± 8.58*	–
SGR	12.35	11.99	12.27	11.21	–
I (%)	–	2.91	0.65	9.23	–
Total mortality (%)	8	9	11	75	100

[a] m_7, m_{31} = Mean carp weight in group after 7 and 31 days exposure; SGR = specific growth rate in group after 24 days exposure; I = inhibition of specific growth in selected group after 24 days exposure; SD = standard deviation.

* Significantly ($p < 0.01$) difference of experimental groups compared the control group.

Table 2
Ontogenetic development of carp after terbuthylazine-desethyl exposure.

Carp group	Control	E1	E2	E3	E4
Terbuthylazine-desethyl (μg/L)	–	1.80	180	900	1800
Times (day)					
7	E9c–L1	E9c–L1	E9c–L1	E9c–L1	E9b–E9c*
14	L2–L3b	L2–L3b	L1–L2	L1–L2	E9c–L1*
20	L4a–L4b	L4a–L4b	L3b–L4a	L3b–L4a	L3a–L3b*
27	L6–J1	L6–J1	L5–L6*	L4b–L5*	–
31	J1	J1	L5–L6*	L5–L6*	–

* Significant ($p < 0.01$) difference of experimental groups compared with the control group.

critical periods were soon after the beginning of the test, at the time of hatching, and within 10–18 days post-hatching, which includes the period of change from endogenous to exogenous nutrition (Lugowska, 2007). Our result are in agreement with studies showing no differences in carp hatching and found influence on survival of larvae following exposure to the triazine pesticides prometryn (Velisek et al., 2015), terbutryn (Velisek et al., 2012b), and simazine (Velisek et al., 2012c), and the triazine metabolite terbuthylazine-2-hydroxy (Velisek et al., 2014).

In present study, exposure to terbuthylazine-desethyl at 900 and 1800 μg/L was associated with lower weight and length of carp compared to control group. Inhibition of SRG at 900 μg/L was 9.23% compared to controls. Decrease of fish growth is a common chronic response to xenobiotics (Rosenthal and Alderdice, 1976) and can be a more sensitive parameter than mortality (Bengtsson, 1974). Reductions in growth after terbuthylazine-desethyl exposure 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. These results agree with authors reporting reduced fish growth after atrazine (Dewey, 1986; Davies et al., 1994), prometryn (Erickson and Turner, 2002; Alvarez and Fuiman, 2005; Velisek et al., 2015), terbutryn (Plhalova et al., 2009; Velisek et al., 2012b), simazine (Velisek et al., 2012c), and terbuthylazine-2-hydroxy (Velisek et al., 2014). Fulton's weight condition factors of common carp exposed to terbuthylazine-desethyl showed no differences from control fish. This is supported by another study that showed no differences in FWC after terbuthylazine-2-hydroxy and simazine exposure in carp (Velisek et al., 2012c, 2014). On the other hand, Velisek et al. (2015 and 2012b) reported a decreased of FWC in early life stages of carp after exposure of prometryn and terbutryn, respectively. Our results differ from these, as, different tested substances and different concentrations of tested substances were used in our study.

Fish exposed to 180, 900, and 1800 μg/L terbuthylazine-desethyl were delayed in development compared to control group. Developmental stage is a sensitive parameter for evaluation of effects of xenobiotics in fish (Hallare et al., 2005). The delay in carp development after exposure to terbuthylazine-desethyl can be ascribed to developmental events such as organogenesis. Delay in early ontogenetic development in carp has also been reported after exposure to prometryn (Velisek et al., 2015), terbutryn (Velisek et al., 2012b), and terbuthylazine-2-hydroxy (Velisek et al., 2014). In this study we did not observe body deformities associated with exposure. Velisek et al. (2012b, 2014, 2015) also found no malformations in carp after triazine exposure.

Some studies have demonstrated that exposure to triazine affects the antioxidant defense system in aquatic organisms, causing an imbalance between reactive oxidative system production and elimination and resulting in oxidative stress and organism damage (Stara et al., 2012a,b, 2013, 2014; Koutnik et al., 2014). In the present study, terbuthylazine-desethyl exposure has not effect on TBARS, CAT and GR activity in carp. Stara et al. (2012a) reported no significant effect of prometryne on TBARS and CAT activity in homogenates of carp early life stages. No effect of terbuthylazine-2-hydroxy on activity of CAT and GR in carp reported Velisek et al. (2014). On the other hand, Stara et al. (2012a) reported no significant effect of prometryne on TBARS and CAT activity in homogenates of carp early life stages. In this study, we found SOD activity to be significantly lower with exposure to terbuthylazine-desethyl at all tested concentrations compared to control group. Decrease in SOD activity can result from increased ROS production response to terbuthylazine-desethyl exposure. SOD and CAT are a first line of defense to oxidative stress and are used as oxidative stress

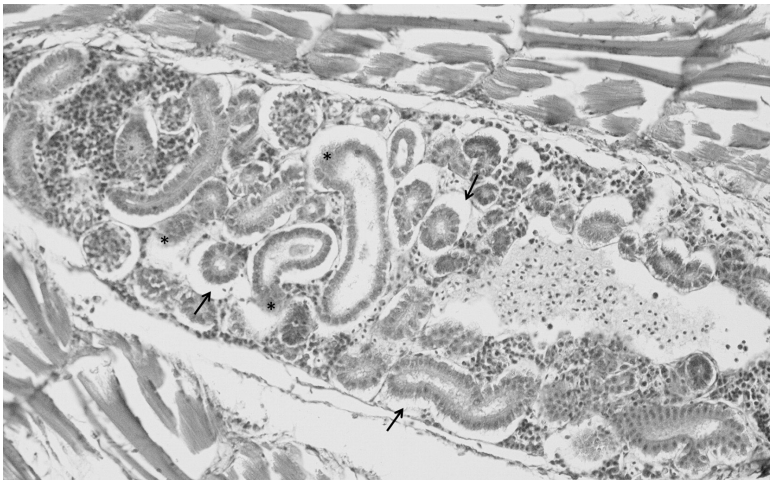


Fig. 4. Caudal kidney of common carp exposed to 900 μg/L terbuthylazine-desethyl for 31 days showing extensive peritubular dilatation (arrows) and focal disintegration of tubular epithelium (asterisks). H&E 200×.

Table 3
The effect of terbuthylazine-desethyl on oxidative stress biomarker (thiobarbituric acid reactive substances — TBARS), and antioxidant enzymes (superoxide dismutase — SOD, catalase — CAT, glutathione reductase — GR) in homogenate of carp.

Fish group	Control–	E1	E2	E3
Terbuthylazine-desethyl (μg/L)		1.80	180	900
TBARS (nmol/mg protein)	0.2091 ± 0.0292	0.2369 ± 0.0173	0.2206 ± 0.0108	0.2065 ± 0.0451
SOD (nmol NBT/min/mg protein)	0.6923 ± 0.1151	0.4931 ± 0.1063*	0.4071 ± 0.0967*	0.2209 ± 0.0511*
CAT (μmol H_2O_2/min/mg protein)	0.1742 ± 0.0283	0.1436 ± 0.0479	0.1162 ± 0.0387	0.1997 ± 0.0889
GR (nmol NADPH/min/mg protein)	0.1194 ± 0.0465	0.1390 ± 0.0487	0.1340 ± 0.0561	0.0758 ± 0.0401

* Significantly ($p < 0.01$) difference of experimental groups compared the control group.

biomarkers (Nwani et al., 2010; Ojha et al., 2011). Other authors reported similar changes of SOD after exposure to simazine (Stara et al., 2012b), prometryn (Stara et al., 2013), terbutryn (Velisek et al., 2011), and terbuthylazine-2-hydroxy (Velisek et al., 2014). These studies demonstrated that terbuthylazine-desethyl exposure can affect the antioxidant system in carp, causing imbalance of reactive oxidative system production and elimination, resulting in oxidative stress and organism damage.

Terbuthylazine-desethyl at 180 and 900 μg/L caused histhopalogical alterations in the caudal kidney. On the basis of our findings it is possible to describe terbuthylazine-desethyl as a primary nephrotoxic substance. The kidney in fish receives the largest proportion of postbranchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution (Ortiz et al., 2003). Pathological changes in cranial kidney were similar to the changes found in fish by Arufe et al., 2004; Oropesa et al., 2009; Velisek et al., 2012b,c, 2014, 2015. In our study, terbuthylazine-desethyl was not associated with pathological changes in the gill or liver. Velisek et al. (2012b, 2014) also found no changes in gill and liver of early life stages of carp after triazine exposure. On the other hand, Neskovic et al. (1993) found hyperplasia of gill epithelial cells in carp exposed to atrazine at a concentration of 1500 μg/L. Similar changes to gill epithelial cells were reported by Oropesa-Jimenaz et al. (2005) in carp following acute exposure to simazine. Arufe et al. (2004) described alterations in liver in gilthead seabream (*Sparus aurata*) after exposure to a combination of terbutryn and triasulfuron. Fischer-Scherl et al. (1991) observed changes in renal corpuscles and tubules of rainbow trout after subchronic exposure to atrazine. Our results differ from these, as, different tested substances, different concentrations of tested substances, and exposure period were used in our study. Triazine has been reported to be associated with mainly damage to fish kidney structure.

Although the effects of acute and sub-chronic exposure of fish to triazine have been well documented, there is a dearth of data on the chronic toxicity of triazine metabolite to early life stages carp at environmentally realistic concentrations. The results of this study provide further data on long-term exposure to terbuthylazine-desethyl for consideration in risk assessment. The findings contribute to knowledge of the toxic potential of metabolite terbuthylazine-desethyl to carp at actual concentrations in Czech rivers. Taken together, the data demonstrate that terbuthylazine-desethyl exhibits potential for the induction of sublethal effects on non-target organisms, such as fish, in aquatic environments. However, the aquatic environment may be polluted by many substances, the effects of which can be potentiated in concurrent exposure.

5. Conclusions

The information obtained in this study may be of use in efforts to assess the ecological risk of the major terbuthylazine metabolite terbuthylazine-desethyl in the aquatic environment. Survival, growth, ontogenetic development, and pathological changes of caudal kidney were observed only at higher exposures (180, 900 and 1800 μg/L), while changes in total superoxide dismutase activity were seen in carp exposed the environmental concentrations of terbuthylazine-desethyl (1.80 μg/L). The results showed the toxic potential of metabolite terbuthylazine-desethyl to early life stages of carp at real concentration. All parameters measured in this study displayed various dependent manners to terbuthylazine-desethyl concentrations and exposure time, possibly due to different molecular and genetic mechanisms, which need to be investigated in the future. Furthermore, in natural environment, the presence of combinations of different pesticides or other xenobiotic metabolites may complicate the exposure situation, as synergetic and/or antagonistic effects on physiology in fish can be expected. Therefore, whether the physiological reactions in fish exposed to terbuthylazine-desethyl in mixture with other xenobiotic also will occur with terbuthylazine-desethyl exposure in laboratory need to be further investigated.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The study was financially supported by the projects „CENAKVA "(No. CZ.1.05/2.1.00/01.0024), "CENAKVA II "(No. LO1205 under the NPU I program), and by the GAJU No. 018/2014/Z.

References

Abrantes, N., Pereira, R., Gonçalves, F., 2010. Occurrence of pesticides in water, sediments, and fish tissues in a lake surrounded by agricultural lands: concerning risks to humans and ecological receptors. Water Air Pollut. 212, 77–88.
Alvarez, M.C., Fuiman, L.A., 2005. Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae. Aquat. Toxicol. 74, 229–241.
Arufe, M.I., Arellano, J., Moreno, M.J., Sarasquetec, C., 2004. Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. Ecotoxicol. Environ. Saf. 59, 209–216.
Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.
Bengtsson, B.E., 1974. Effect of zinc on growth of the minnow *Phoxinus phoxinus*. Oikos 25, 370–373.
Benvenuto, F., Marin, J.M., Sancho, J.V., Canobbio, S., Mezzanotte, V., Hernandez, F., 2010. Simultaneous determination of triazines and their main transformation products in surface and urban wastewater by ultra-high-pressure liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 397, 2791–2805.
Blasco, C., Pico, Y., 2009. Prospects for combining chemical and biological methods for integrated environmental assessment. Trends Anal. Chem. 28, 745–757.
Buser, H.R., 1990. Atrazine and other s-triazine herbicides in lakes and in rain in Switzerland. Environ. Sci. Technol. 24, 1049–1058.
Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye binding. Anal. Biochem. 72, 248–254.
Brambilla, A., Rindone, B., Polesello, S., Galassi, A., Balestriniet, R., 2003. The fate of triazine pesticides in River Po water. Sci. Total. Environ. 132, 339–348.
Carlberg, I., Mannervik, B., 1975. Purification and characterization of flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.
CHMU (Czech Hydrometeorological Institute), 2015. On-line water duality database Available from: http://hydro.chmi.cz/oj/ (visited online 2.2.2015).
Dankwardt, A., Thurman, E.M., Hock, B., 1997. Terbuthylazine and desethylterbuthylazine in rain and surface water — determination by enzyme immunoassay and gas chromatography/mass spectrometry. Acta Hydrochim. Hydrobiol. 25, 1–10.
Davies, P.E., Cook, L.S.J., Goenarso, D., 1994. Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. Environ. Toxicol. Chem. 13, 1341–1354.
Dewey, S.L., 1986. Effects of the herbicide atrazine on aquatic insect community structure and emergence. Ecology 67, 148–162.

EFSA, (European Food Safety Authority), 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance terbuthylazine. EFSA J. 9, 1–133.
Erickson, W., Turner, L., 2002. Prometryn analysis of risks to endangered and threatened salmon and steelhead. Environmental Field Branch, Office of Pesticide Programs 368, p. 71.
Fait, G., Balderacchi, M., Ferrari, F., Ungaro, F., Capri, E., Trevisan, M., 2010. A field study of the impact of different irrigation practices on herbicide leaching. Eur. J. Agron. 32, 280–287.
Fischer-Scherl, T., Veseer, A., Hoffman, R.W., Kuhnhauser, C., Negele, R.D., Ewringmann, T., 1991. Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*). Arch. Environ. Contam. Toxicol. 20, 454–461.
Hallare, A.V., Schirling, M., Luckenbach, T., Kohler, H.R., Triebskorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30, 7–17.
Hartley, D., Kidd, H. (Eds.), 1987. The Agrochemicals Handbook, 2nd ed. The Royal Society of Chemistry, Lechworth, Herts, England.
ISPRA, (Istituto Superiore per la Protezione e Ricerca Ambientale), 2010a. Monitoraggio nazionale dei pesticidi nelle acque. Dati 2007–2008. Rapporti ISPRA N. 114/2010.
Kocour, M., Gela, D., Rodina, M., Linhart, O., 2005. Testing of performance in common carp *Cyprinus carpio* L. under pond husbandry conditions I: top-crossing with Northern mirror carp. Aquac. Res. 36, 1207–1215.
Koutnik, D., Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2014. The effect of long-term metribuzine exposure to signal crayfish (*Pacifastacus leniusculus* Dana). Neuroendocrinol. Lett. 35 (Supll. 2), 51–-56.
Kristensen, P., 1994. Sensitivity of embryos and larvae in relation to other stages in the life cycle of fish: a literature review. In: Muller, R., Lloyd, R. (Eds.), Sublethal and chronic effects of pollutants on freshwater fish. United Nation Organization: Fishing News Books, New Youk, USA, pp. 339–-352.
Kroupova, H., Prokes, M., Macova, S., Penaz, M., Barus, V., Novotny, L., Machova, J., 2010. Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.). Environ. Toxicol. Chem. 29, 535–540.
Loos, R., Tavazzi, S., Paracchini, B., Canuti, E., Weissteiner, C., 2013. Analysis of polar organic contaminants in surface water of the northern Adriatic Sea by solid-phase extraction followed by ultrahigh-pressure liquid chromatography–QTRAP MS using a hybrid triple-quadrupole linear ion trap instrument. Anal. Bioanal. Chem. 405, 5875–5885.
Lugowska, K., 2007. The effect of cadmium and cadmium/copper mixture during the embrionic development on deformation of common carp larvae. Electron. J. Ichthyol. 2, 46–60.
Lushchak, V.I., Bagnyukova, T.V., Husak, V.V., Luzhna, L.I., Lushchak, O.V., Storey, K.B., 2005. Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int. J. Biochem. Cell. Biol. 37, 1670–1680.
Mansilha, C., Melo, A., Ferreira, I.M., Pinho, O., Domingues, V., Pinho, C., Gameiro, P., 2011. Groundwater from infiltration galleries used for small public water supply systems: contamination with pesticides and endocrine disruptors. Bull. Environ. Contam. Toxicol. 87, 312–318.
Marklund, S., Marklund, G., 1974. Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469–474.
McKim, J.M., 1995. Early life stage toxicity tests. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology, Effects, Environmental Fate and Risk Assessment. Taylor & Francis, Washington, DC, pp. 974–1011.
Mladinic, M., Zeljezic, D., Shaposhnikov, S.A., Collins, A.R., 2012. The use of FISH-comet to detect c-Myc and TP 53 damage in extended-term lymphocyte cultures treated with terbuthylazine and carbofuran. Toxicol. Lett. 211, 62–69.
Navarro-Ortega, A., Acuna, V., Batalla, R.J., Blasco, J., Conde, C., Elorza, F.J., Elosegi, A., Frances, F., La-Roca, F., Munoz, I., Petrovic, M., Pico, Y., Sabater, S., Sanchez-Vila, X., Schuhmacher, M., Barcelo, D., 2012. Assessing and forecasting the impacts of global change on Mediterranean rivers. The SCARCE Consolider project on Iberian basins. Environ. Sci. Pollut. Res. 19, 918–933.
Neskovic, N.K., Elezovic, I., Karan, V., Poleksic, V., Budimir, M., 1993. Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.). Ecotoxicol. Environ. Saf. 25, 173–182.
Nodler, K., Licha, T., Voutsa, D.T., 2013. Twenty years later atrazine concentrations in selected coastal waters of the Mediterranean and the Baltic Sea. Mar. Pollut. Bull. 70, 112–118.
Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B., Srivastava, S.K., 2010. Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa punctatus* (Bloch). Int. J. Environ. Res. Public Health 7, 3298–3312.
OECD, (Organization for Economic Cooperation and Development), 2013,. Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems TG-No. 210: Fish, Early-Life Stage Toxicity Test (Paris, France) p. 24.
Ojha, A., Yaduvanshi, S.K., Srivastava, N., 2011. Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. Pestic. Biochem. Physiol. 99, 148–156.
Ortiz, J.B., De Canales, M.L.G., Sarasquete, C., 2003. Histopathological changes induces by lindane (gama-HCH) in various organ of fishs. Sci. Mar. 67, 53–61.
Oropesa, A.L., Garcia-Cambero, J.P., Gomez, L., Roncero, V., Soler, F., 2009. Effect of long-term exposure to simazine on histopathology, hematological, and biochemical parameters in *Cyprinus carpio*. Environ. Toxicol. 24, 187–199.
Oropesa-Jimenaz, A.L., García-Cambero, J.P., Gomez-Gordo, L., Roncero-Cordero, V., Soler-Rodríguez, F., 2005. Gill modifications in the freshwater fish *Cyprinus carpio* after subchronic exposure to simazine. Bull. Environ. Contam. Toxicol. 74, 785–792.
Papadopoulos, N., Gikas, E., Zalidis, G., Tsarbopoulos, A., 2007. Simultaneous determination of terbuthylazine and its major hydroxy and dealkylated metabolites in wetland water samples using solid-phase extraction and high-performance liquid chromatography with diode-array detection. J. Agric. Food Chem. 55, 7270–7277.
Penaz, M., Prokes, M., Kouril, J., Hamackova, J., 1983. Early development of the carp, *Cyprinus carpio*. Acta Sci. Nat. Brno 17, 1–39.
Plhalova, L., Macova, S., Haluzova, I., Slaninova, A., Dolezelova, P., Marsalek, P., Pistekova, V., Bedanova, I., Voslarova, E., Svobodova, Z., 2009. Terbutryn toxicity to *Danio rerio*: Effects of subchronic exposure on fish growth. Neuroendocrinol Lett 30, 242–365.
Rosenthal, H., Alderdice, D.F., 1976. Sub-lethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. J. Fish. Res. Board Can. 33, 2047–2065.
Tomlin, C.D.S. (Ed.), 2002. Terbuthylazine (5915-41-3)The e-Pesticide Manual, Version 2.2. British Crop Protection Council, Surrey UK.
Stara, A., Machova, J., Velisek, J., 2012a. Effect of chronic exposure to prometryne on oxidative stress and antioxidant response on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol. Lett. 33 (Supp. 3), 130–-135.
Stara, A., Machova, J., Velisek, J., 2012b. Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.). Environ. Toxicol. Pharmacol. 33, 334–343.
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.). Pestic. Biochem. Physiol. 105, 18–23.
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 Res. Int. 2014, 680131.
Velisek, J., Svobodova, Z., Piackova, V., Novotny, L., Blahova, J., Sudova, E., Maly, V., 2008. Effect of metribuzin on rainbow trout (*Oncorhynchus mykiss*). Vet. Med. Czech 5, 324–332.
Velisek, J., Sudova, E., Machova, J., Svobodova, Z., 2010. Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). Ecotoxicol. Environ. Saf. 73, 384–390.
Velisek, J., Stara, A., Kolarova, J., Svobodova, Z., 2011. Biochemical, physiological and morphological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryne in real environmental concentration. Pestic. Biochem. Physiol. 100, 305–313.
Velisek, J., Stara, A., Machova, J., Svobodova, Z., 2012a. Effects of long-term exposure to simazine in real concentration on common carp (*Cyprinus carpio* L.). Ecotoxicol. Environ. Saf. 76, 79–86.
Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Prokes, M., Svobodova, Z., 2012b. Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pestic. Biochem. Physiol. 102, 102–108.
Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Svobodova, Z., 2012c. Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol. Lett. 33 (Supp. 3), 90–95.
Velisek, J., Zuskova, E., Stara, A., Svobodova, Z., 2013. Use of biometric, hematological, and plasma biochemical variables and histopathology to assess the chronic effects of the herbicide prometryn on common carp. Vet. Clin. Pathol. 42, 508–515.
Velisek, J., Stara, A., Koutnik, D., Machova, J., 2014. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed. Res. Int. 2014, 621304.
Velisek, J., Stara, A., Koutnik, D., Machova, J., 2015. Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.). Pestic. Biochem. Physiol. 118, 58–63.
Woltering, D., 1984. The growth response in fish chronic and early life stage toxicity tests: a critical review. Aquat. Toxicol. 5, 1–21.

CHAPTER 7

EFFECT OF TERBUTHYLAZINE-2-HYDROXY AT ENVIRONMENTAL CONCENTRATIONS ON EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

Velisek, J., Stara, A., Koutnik, D., Machova, J., 2014. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed Research International, Article ID 621304.

My share on this work was about 25%

It was allowed by publisher on 23rd March, 2018 to include the paper in this Ph.D. thesis.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 621304, 7 pages
http://dx.doi.org/10.1155/2014/621304

Research Article

Effect of Terbuthylazine-2-hydroxy at Environmental Concentrations on Early Life Stages of Common Carp (*Cyprinus carpio* L.)

Josef Velisek, Alzbeta Stara, Dalibor Koutnik, and Jana Machova

Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zatisi 728/II, 389 25 Vodnany, Czech Republic

Correspondence should be addressed to Josef Velisek; velisek@frov.jcu.cz

Received 17 October 2013; Accepted 27 December 2013; Published 6 February 2014

Academic Editor: Zdenka Svobodova

The aim of the study was to investigate effects of the triazine's herbicide terbuthylazine-2-hydroxy on early life stage of common carp (*Cyprinus carpio* L.) through antioxidant indices, mortality, growth, development, and histopathology. Based on accumulated mortality in the experimental groups, lethal concentrations of terbuthylazine-2-hydroxy were estimated at 35-day LC50 = 10.9 mg/L terbuthylazine-2-hydroxy. By day 15, fish were exposed to 3.5 mg/L and by day 26, fish were exposed to 0.0029 mg/L; real environmental concentration in Czech rivers, 0.07 mg/L, 1.4 mg/L, and 3.5 mg/L terbuthylazine-2-hydroxy, showed significantly lower mass and total length compared with controls. Based on inhibition of growth in the experimental groups, lowest observed effect concentration (LOEC) = 0.002 mg/L terbuthylazine-2-hydroxy and no observed effect concentration (NOEC) = 0.0001 mg/L terbuthylazine-2-hydroxy. 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 showed a dose-related delay in development compared with the controls. Total superoxide dismutase (SOD) activity was significant lower in all groups testedly for terbuthylazine-2-hydroxy compared with the control group. At concentrations of 1.4 and 3.5 mg/L damage to caudal kidney tubules when compared to control fish was found.

1. Introduction

Triazines are selective herbicides applied before and after emergence control against annual weeds, perennial weeds, grasses, and broadleaf weeds in corn, wheat, sorghum, and many other crops. Terbuthylazine was registered in the United States in 1975 and now is the second most frequently used s-triazine. Due to the persistency, water solubility, and mobility, triazines are also detected in aquatic ecosystems. These compounds are found in rivers, lakes, and well water [1–3]. In water terbutryne and terbuthylazine undergo a variety of biotic and abiotic mechanisms of degradation such as photodegradation, oxidation, hydrolysis, and biodegradation that lead to dealkylation of alkylated amino groups, deamination, and hydroxylation at the 2 position, as well as triazine ring cleavage [4–6]. The major degradation products in ground and surface waters are the terbuthylazine-2-hydroxy, terbuthylazine-desethyl-hydroxy, and terbuthylazine-desethyl. The persistence of these metabolites ranged from moderate to high for terbuthylazine-desethyl-hydroxy, terbuthylazine-desethyl and high to very high for terbuthylazine-2-hydroxy (112–120 days) [7].

Currently 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 and are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. According to Commission Regulation (EU) number 196/2010 of 9 March 2010, amending Annex I to Regulation (EC) number 689/2008 of the European Parliament and of the Council concerning the export and import of dangerous chemicals is banned in the countries of the European Union. Although

the effects of atrazine, another s-triazine herbicide exposure to fish, have been well-documented, there is a dearth of data of their metabolites in early life stages of common carp. The aim of the present study was to describe lethal and sublethal effects of terbuthylazine-2-hydroxy on embryo and larvae of common carp using a 35-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. 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 [8–11]. The toxicity of terbuthylazine-2-hydroxy was assessed on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, Fulton's weight condition factor (FWC), and the antioxidant defenses during and at the conclusion of the test. The aim of the present study was to investigate how low concentrations can affect terbuthylazine-2-hydroxy on early life stages of carp after long-term exposure.

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. [12]. 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 19.0–22.0°C, pH 7.6–8.1, $ANC_{4.5}$ (acid neutralization capacity) 0.92 mmol/L, COD_{Mn} (chemical oxygen demand) 0.6 mg/L, total ammonia 0.02 mg/L, NO_3^- 1.50 mg/L, NO_2^- 0.05 mg/L, and sum of Ca^{2+} + Mg^{2+} 4.2 mg/L. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature were measured daily. Terbuthylazine-2-hydroxy 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, and 250 mm × 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 [13]. Measured values did not differ from the value stated for test purposes by more than 10%.

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. At 24 h after fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into fifteen crystallization basins containing the test solutions of terbuthylazine-2-hydroxy (Sigma Aldrich, Czech Republic, chemical purity 99.5%) 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: 0.0029 mg/L (reported environmental concentration in Czech rivers, Group 1-E1), 0.07 mg/L (Group 2-E2), 1.4 mg/L (Group 3-E3), and 3.5 mg/L (Group 4-E4). Terbuthylazine-2-hydroxy concentrations of 0.07 mg/L, 1.4 mg/L, and 3.5 mg/L corresponded to the 1% 96hLC50, 20% 96hLC50, 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, FWC, and the length/weight relationship. Samples were collected on days 9, 15, 22, 26, 33, and 35. Samples were fixed in 4% formalin, with 5 specimens per replicate (i.e., 15 per group).

Determination of developmental periods and stages followed Penaz et al. [14], 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 (W).

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 35, 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 9 (the first sampling

time) and ending on day 35 (end of the trial). The following formula was used:

$$\mathrm{SGR} = \frac{\overline{\ln w_2} - \overline{\ln w_1}}{t_2 - t_1} \cdot 100, \quad (1)$$

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, and t_2 = time (days)-end of exposure.

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

$$I_x\,[\%] = \frac{\mathrm{SGR}_x\,(\text{control}) - \mathrm{SGR}_x\,(\text{group})}{\mathrm{SGR}_x\,(\text{control})} \cdot 100, \quad (2)$$

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

Fulton's weight condition factor was calculated for each experimental group at every sampling time:

$$\mathrm{FWC} = \frac{W \cdot 10^5}{\mathrm{TL}^3}, \quad (3)$$

where FWC = Fulton's weight condition factor, W = mass in selected experimental group (g), and TL = total length in selected experimental group (mm).

2.6. Samples Early Life Stage of Carp and Preparation of Postmitochondrial Supernatant. Toxicity tests on terbuthylazine-2-hydroxy were ended after 35 days. At the end of the tests fish were weighed and their length was determined. 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 mmol/L potassium phosphate buffer, pH 7.0, containing 0.5 mmol/L EDTA. The homogenate was centrifuged at 4°C to obtain the postmitochondrial supernatant for antioxidant parameter analyses.

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically by the method of S. Marklund and G. Marklund [15]. 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 [16]. Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [17]. 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.

Protein levels were estimated spectrophotometrically by the method of Bradford [18] using bovine serum albumin as a standard.

2.7. Histopathology. Histopathology was evaluated in all experimental groups at the end of the experiment (day 35). Six whole fish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, stained with hematoxylin and eosin, examined by light microscopy, and photographed using a digital camera.

2.8. 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. The differences in accumulated mortality among the test groups were checked by using contingency tables (χ^2).

2.9. Evaluation of 35-Day LC50, LOEC, and NOEC. For the evaluation of 35-day LC50 values, a probit analysis was used based on mortality at different terbuthylazine-2-hydroxy concentrations. For the evaluation of LOEC and NOEC values, the probit analysis was based on inhibition of growth at different terbuthylazine-2-hydroxy concentrations; 35-day IC5 and 35-day IC10 values were used to express the NOEC and LOEC values, respectively. For evaluation, the EKOTOX 5.1 software (Ingeo, Liberec) was used.

3. Results and Discussion

3.1. Hatching. Studies have reported that hatching can be affected by exposure to chemicals [19, 20]. In present study hatching began 4 days after the onset of exposure. The majority of eggs in all treatment groups hatched by day 6. No significantly negative effects of terbuthylazine-2-hydroxy at the concentrations tested (0.0029–3.5 mg/L) on hatching and embryo viability were observed. However, a marked influence was found on survival of larvae. Our results are in accord with results of Velisek et al. [10, 11], who found no change in carp hatching following exposure to triazine pesticides terbutryn and simazine.

3.2. Accumulated Mortality. Significant ($P < 0.01$) differences in total accumulated mortality were found in fish exposed to the highest terbuthylazine-2-hydroxy concentration (3.5 mg/L), compared with controls (Figure 1). Massive mortality in this group occurred on days 23 and 26. Based on accumulated mortality in the experimental groups, values of lethal concentrations were estimated at 35-day LC50 = 10.9 mg/L terbuthylazine-2-hydroxy for early life stages of common carp. Most control larvae survived to 23 days after 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

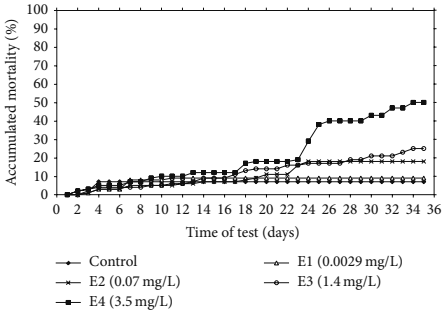


Figure 1: Accumulated percent mortality of common carp embryos, larvae, and juveniles after terbuthylazine-2-hydroxy exposure.

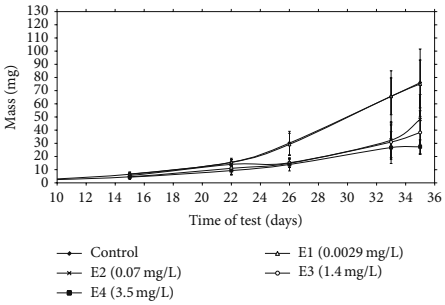


Figure 2: Mean mass ± SD of common carp larvae (juveniles) after terbuthylazine-2-hydroxy exposure.

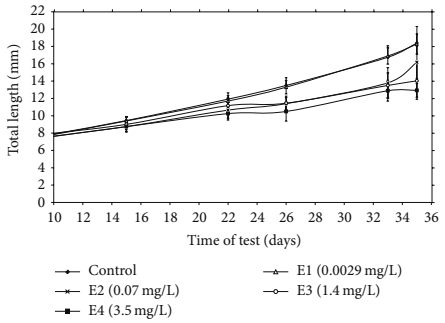


Figure 3: Total length ± SD of common carp larvae (juveniles) after terbuthylazine-2-hydroxy exposure.

occurs about day 15 after hatching [21], and starvation-induced mortality occurs after that time.

3.3. Length and Weight Growth Parameters. Growth can be considered a more sensitive measure than mortality. 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 [22]. Mass and total length of fish as related to terbuthylazine-2-hydroxy concentration in water are shown in Figures 2 and 3. In the group 4 fish beginning on day 15 of exposure showed significantly ($P < 0.01$) lower total length compared with controls. Beginning on day 26 of exposure, in groups 2, 3, and 4 fish, terbuthylazine-2-hydroxy caused significantly ($P < 0.01$) lower mass and total length compared with controls. Specific growth rates and inhibition of growth are shown in Table 1. Inhibition of growth in the group exposed to the two highest tested concentrations (1.4 and 3.5 mg/L) was 17.66% and 28.32%, respectively, compared to control. Based on inhibition of growth in the experimental groups, lowest observed effect concentration (LOEC) = 0.002 mg/L terbuthylazine-2-hydroxy and no observed effect concentration (NOEC) = 0.0001 mg/L terbuthylazine-2-hydroxy. Fulton's condition factors of common carp exposed to terbuthylazine-2-hydroxy showed no differences from untreated fish. This is supported by another study that showed a decreased growth after terbutryn and simazine exposure in carp [10, 11]. Growth reductions after terbuthylazine-2-hydroxy exposure 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.

3.4. Early Ontogeny. The developing fish embryo and early larval stages have been shown to be especially sensitive indicators of many types of aquatic pollution. A chemically induced adverse effect on embryonic stages is based on developmental events, for example, organogenesis [23, 24]. 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. The developmental stages observed at the sampling times in all tested concentrations and controls are listed in Table 2. Significant differences in early ontogeny among test groups were observed for the duration of the trial. Fish from two highest tested concentrations (1.4 and 3.5 mg/L) were delayed in development compared with the control group. The percent of individuals in larval stages (L4b or L5) increased with higher concentrations of terbuthylazine-2-hydroxy, whereas the majority of control fish reached the juvenile stage. Our results are in accord with results of Velisek et al. [10, 11], who found differences in early ontogeny of carp following exposure to triazine pesticides terbutryn and simazine.

3.5. Macroscopic Morphological Anomalies. In fish, developmental malformations have been linked to the presence of environmental pollutants such as persistent organochlorines and pesticides [25]. In the present study similar morphological anomalies were found in fish in both experimental and control groups. These included axial and/or lateral curvature

Table 1: Growth rate and fish mortality results of the 35-day embryo-larva toxicity test on common carp after terbuthylazine-2-hydroxy exposure.

	Fish group				
	Control	E1	E2	E3	E4
Terbuthylazine-2-hydroxy (mg/L)	—	0.0029	0.07	1.4	3.5
m_9 (mean ± SD, mg)	2.32 ± 0.46	2.34 ± 0.54	2.08 ± 0.43	2.11 ± 0.60	2.27 ± 0.33
m_{35} (mean ± SD, mg)	76.03 ± 25.58	75.08 ± 18.12	48.11 ± 19.93*	38.23 ± 16.55*	27.34 ± 5.28*
SGR	13.31	13.35	11.88	10.96	9.54
I (%)	—	−0.30	10.74	17.66	28.32
Total mortality (%)	7	9	18	25	50

m_9, m_{35}: mean fish mass in selected group after 9 and 35 days exposure; SGR: mean specific growth rate in selected group after 26 days exposure; I: inhibition of specific growth in selected group after 26 days exposure; SD: standard deviation. *Experimental groups significantly ($P < 0.01$) different from the control group.

Table 2: Developmental periods (DPS) during the 35-day embryo-larva toxicity test on common carp.

	Fish group				
	Control	E1	E2	E3	E4
Terbuthylazine-2-hydroxy (mg/L)	—	0.0029	0.07	1.4	3.5
Times (day)	DPS	DPS	DPS	DPS	DPS
9	Ec9-L1	Ec9-L1	Ec9-L1	Ec9-L1	Ec9-L1
15	L2-L3b	L2-L3b	L2-L3b	L2-L3a	L1-L2
22	L4a-L5	L4a-L5	L4a-L5	L4a-L4b	L3-L4a
26	L5-L6	L5-L6	L5-L6	L4a-L4b	L4a-L4b
33	L6-J1	L6-J1	L6-J1	L5-L6	L4b-L5
35	J1	J1	J1	L5-L6	L4b-L5

Table 3: Effect of terbuthylazine-2-hydroxy exposure 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 early life stages of carp.

	Fish group				
	Control	E1	E2	E3	E4
Terbuthylazine-2-hydroxy (mg/L)	—	0.0029	0.07	1.4	3.5
SOD	0.3819 ± 0.1073	0.1726 ± 0.0268*	0.1880 ± 0.0447*	0.1182 ± 0.0626*	0.0434 ± 0.0136*
CAT	0.1254 ± 0.0826	0.1296 ± 0.0678	0.1477 ± 0.0641	0.1640 ± 0.0512	0.1825 ± 0.0696
GR	0.1968 ± 0.1726	0.1823 ± 0.1346	0.1037 ± 0.0998	0.1789 ± 0.1276	0.1932 ± 0.1470

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

of the spine (lordosis and scoliosis), yolk sac deformity, and body shortening. The incidence of these anomalies was 0.2%, which could be considered a spontaneous appearance. 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 [26–28].

3.6. Antioxidant Response. 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 [9, 29, 30]. The first line of defense against oxidative stress consists of the antioxidant enzymes SOD, CAT, and GPx, which convert superoxide anions (O_2^-) into H_2O_2 and then into H_2O and O_2 [31]. Effect of chronic exposure to terbuthylazine-2-hydroxy on antioxidants responses SOD, CAT, and GR in homogenate on early life stages of carp are in Table 3. Significant differences from the control value ($P < 0.01$) were seen in Groups E1, E2, E3, and E4 on homogenate early life stages of carp in SOD activity. SOD activity was significantly lower in all groups tested terbuthylazine-2-hydroxy compared with the control group. In CAT and GR activities changes were not observed in tested groups. Superoxide dismutase is an antioxidant enzyme important in inhibiting oxyradical formation and is used as a biomarker to indicate oxidative stress [32, 33]. In our test, decrease in SOD activity may be due to increased generation of ROS induced by terbuthylazine-2-hydroxy exposure. Similar changed activities of SOD in carp tissues after pesticides exposure have also been reported by other authors: Oruc et al. [34], Oruç and Usta [35], Velisek et al. [36], and Stara et al. [29, 30].

3.7. Histopathology. Generally, triazine pesticides have a direct effect on kidney structure and function in freshwater fish [10, 11, 36–38]. No histopathological changes were demonstrated in gills and liver following exposure to terbuthylazine-2-hydroxy. The majority of histological changes were observed in caudal kidney in groups E3 (1.4 mg/L) and E4 (3.5 mg/L) compared to control fish. Fish exposed to highest tested levels of terbuthylazine-2-hydroxy showed alteration of tubular system that included destruction of tubular epithelium with casts, vacuolization of tubular epithelia, and disintegration of glomeruli. The kidney is important for the maintenance of a stable internal environment with respect to water and sodium chloride, for excretion, and, partially, for the metabolism of xenobiotic [39]. It is evident that renal alteration was related to terbuthylazine-2-hydroxy exposition, while liver and gill were not affected. On the basis of our findings it is possible to describe terbuthylazine-2-hydroxy as a primary nephrotoxic substance.

4. Conclusions

Chronic terbuthylazine-2-hydroxy exposure of early-life stages of common carp affected their growth rate, early ontogeny, antioxidant enzyme, and histology. Some of the changes (early ontogeny, histology) were observed only at two higher exposures (1.4 and 3.5 mg/L), but changes founded in growth rate and antioxidant enzyme were affected in fish exposed to the lowest concentration tested (i.e., 0.0029 mg/L), which is that reported in Czech rivers in recent years. Aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. For detailed elucidation of terbuthylazine-2-hydroxy effects further research is necessary. This research should be focused not only on the studies of effects of terbuthylazine-2-hydroxy alone but also in view of possible synergic or potentiation effect.

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 Project CENAQUA CZ.1.05/2.1.00/01.0024 and the project LO1205 with a financial support from the MEYS of the CR under the NPU I program and by the Strengthening of Excellence of the Scientific Teams in USB FFPW (Project no. CZ.1.07/2.3.00/20.0024), and by the Grant Agency of the University of South Bohemia (Project no. 018/2014/Z).

References

[1] A. Huber, M. Bach, and H. G. Frede, "Pollution of surface waters with pesticides in Germany: modeling non-point source inputs," *Agriculture, Ecosystems and Environment*, vol. 80, no. 3, pp. 191–204, 2000.

[2] M. J. Cerejeira, P. Viana, S. Batista et al., "Pesticides in Portuguese surface and ground waters," *Water Research*, vol. 37, no. 5, pp. 1055–1063, 2003.

[3] R. F. Spalding, M. E. Exner, D. D. Snow, D. A. Cassada, M. E. Burbach, and S. J. Monson, "Herbicides in ground water beneath Nebraska's management systems evaluation area," *Journal of Environmental Quality*, vol. 32, no. 1, pp. 92–99, 2003.

[4] S. Otto, L. Altissimo, and G. Zanin, "Terbuthylazine contamination of the aquifer North of Vicenza (North-East Italy)," *Environmental Science and Pollution Research*, vol. 14, no. 2, pp. 109–113, 2007.

[5] N. G. Papadopoulos, E. Gikasb, G. Zalidis, and A. Tsarbopoulos, "Determination of herbicide terbuthylazine and its major hydroxy and dealkylated metabolites in constructed wetland sediments using solid phase extraction and high performance liquid chromatography-diode array detection," *International Journal of Environmental Analytical Chemistry*, vol. 92, no. 12, pp. 1429–1442, 2012.

[6] N. G. Papadopoulos, V. Takavakoglou, E. Gikas, A. Tsarbopoulos, and G. Zalidis, "Transport and dissipation study of the herbicide terbuthylazine and its major metabolites in wetland sediment substrates planted with *Typha latifolia* L.," *Desalination and Water Treatment*, vol. 39, no. 1–3, pp. 209–214, 2012.

[7] K. Nodler, T. Licha, and D. T. Voutsa, "Twenty years later—atrazine concentrations in selected coastal waters of the Mediterranean and the Baltic Sea," *Marine Pollution Bulletin*, vol. 70, no. 1-2, pp. 112–118, 2013.

[8] J. M. McKim, "Early life stage toxicity tests," in *Fundamentals of Aquatic Toxicology, Effects, Environmental Fate and Risk Assessment*, G. M. Rand, Ed., Taylor & Francis, Washington, DC, USA, 1995.

[9] A. Stara, J. Machova, and J. Velisek, "Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in early life stages of common carp (*Cyprinus carpio* L.)," *Neuroendocrinology Letters*, vol. 33, supplement 3, pp. 130–135, 2012.

[10] J. Velisek, A. Stara, J. Machova et al., "Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.)," *Pesticide Biochemistry and Physiology*, vol. 102, no. 1, pp. 102–108, 2012.

[11] J. Velisek, A. Stara, J. Machova, P. Dvorak, E. Zuskova, and Z. Svobodova, "Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.)," *Neuroendocrinology Letters*, vol. 33, supplement 3, pp. 90–95, 2012.

[12] M. Kocour, D. Gela, M. Rodina, and O. Linhart, "Testing of performance in common carp *Cyprinus carpio* L. under pond husbandry conditions I: top-crossing with Northern mirror carp," *Aquaculture Research*, vol. 36, no. 12, pp. 1207–1215, 2005.

[13] S. Richter and R. Nagel, "Bioconcentration, biomagnification and metabolism of 14C-terbutryn and 14C-benzo[a]pyrene in Gammarus fossarum and Asellus aquaticus," *Chemosphere*, vol. 66, no. 4, pp. 603–610, 2007.

[14] M. Penaz, M. Prokes, J. Kouril, and J. Hamackova, "Early development of the carp, *Cyprinus carpio*," *Acta Scientiarum Naturalium Universita*, vol. 17, pp. 1–39, 1983.

[15] S. Marklund and G. Marklund, "Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase," *European Journal of Biochemistry*, vol. 47, no. 3, pp. 469–474, 1974.

[16] R. F. Beers Jr. and I. W. Sizer, "A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase," *The Journal of Biological Chemistry*, vol. 195, no. 1, pp. 133–140, 1952.

[17] I. Carlberg and B. Mannervik, "Purification and characterization of the flavoenzyme glutathione reductase from rat liver," *Journal of Biological Chemistry*, vol. 250, no. 14, pp. 5475–5480, 1975.

[18] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[19] M. Strmac and T. Braunbeck, "Effects of triphenyltin acetate on survival, hatching success, and liver ultrastructure of early life stages of zebrafish (*Danio rerio*)," *Ecotoxicology and Environmental Safety*, vol. 44, no. 1, pp. 25–39, 1999.

[20] S. A. Villalobos, J. T. Hamm, S. J. Teh, and D. E. Hinton, "Thiobencarb-induced embryotoxicity in medaka (*Oryzias latipes*): stage-specific toxicity and the protective role of chorion," *Aquatic Toxicology*, vol. 48, no. 2-3, pp. 309–326, 2000.

[21] K. Lugowska, "The effect of cadmium and cadmium/copper mixture during the embrionic development on deformation of common carp larvae," *Electronic Journal of Polish Agricultural Universities*, vol. 10, no. 4, article 11, 2007.

[22] B. E. Bengtsson, "Effect of zinc on growth of the minnow Phoxinus phoxinus," *Oikos*, vol. 25, no. 3, pp. 370–373, 1974.

[23] Q. H. Pickering and J. M. Lazorchak, "Evaluation of the robustness of the fathead minnow, Pimephales promelas, larval survival and growth test, U.S. EPA method 1000.0," *Environmental Toxicology and Chemistry*, vol. 14, no. 4, pp. 653–659, 1995.

[24] A. V. Hallare, M. Schirling, T. Luckenbach, H.-R. Köhler, and R. Triebskorn, "Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos," *Journal of Thermal Biology*, vol. 30, no. 1, pp. 7–17, 2005.

[25] H. von Westernhagen, "Sublethal effects of pollutants on fish eggs and larvae," in *Fish Physiology*, W. S. Hoar and D. J. Randall, Eds., vol. 6, part A, pp. 253–347, Academic Press, San Diego, Calif, USA, 1988.

[26] I. D. McCarthy and L. A. Fuiman, "Growth and protein metabolism in red drum (*Sciaenops ocellatus*) larvae exposed to environmental levels of atrazine and malathion," *Aquatic Toxicology*, vol. 88, no. 4, pp. 220–229, 2008.

[27] J. T. Hamm and D. E. Hinton, "The role of development and duration of exposure to the embryotoxicity of diazinon," *Aquatic Toxicology*, vol. 48, no. 4, pp. 403–418, 2000.

[28] A. Demicco, K. R. Cooper, J. R. Richardson, and L. A. White, "Developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos," *Toxicological Sciences*, vol. 113, no. 1, pp. 177–186, 2009.

[29] A. Stara, J. Kristan, E. Zuskova, and J. Velisek, "Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.)," *Pesticide Biochemistry and Physiology*, vol. 105, no. 1, pp. 18–23, 2013.

[30] A. Stara, J. Machova, and J. Velisek, "Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.)," *Environmental Toxicology and Pharmacology*, vol. 33, no. 2, pp. 334–343, 2012.

[31] A. Ojha, S. K. Yaduvanshi, and N. Srivastava, "Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues," *Pesticide Biochemistry and Physiology*, vol. 99, no. 2, pp. 148–156, 2011.

[32] J. Zhang, H. Shen, X. Wang, J. Wu, and Y. Xue, "Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*," *Chemosphere*, vol. 55, no. 2, pp. 167–174, 2004.

[33] C. C. De Menezes, M. B. Da Fonseca, V. L. Loro et al., "Roundup effects on oxidative stress parameters and recovery pattern of *Rhamdia quelen*," *Archives of Environmental Contamination and Toxicology*, vol. 60, no. 4, pp. 665–671, 2011.

[34] E. Ozcan Oruc, Y. Sevgiler, and N. Uner, "Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl," *Comparative Biochemistry and Physiology C*, vol. 137, no. 1, pp. 43–51, 2004.

[35] E. Ö. Oruç and D. Usta, "Evaluation of oxidative stress responses and neurotoxicity potential of diazinon in different tissues of *Cyprinus carpio*," *Environmental Toxicology and Pharmacology*, vol. 23, no. 1, pp. 48–55, 2007.

[36] J. Velisek, A. Stara, J. Kolarova, and Z. Svobodova, "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*, vol. 100, no. 3, pp. 305–313, 2011.

[37] J. Velisek, K. Stastna, E. Sudova, J. Turek, and Z. Svobodova, "Effects of subchronic simazine exposure on some biometric, biochemical, hematological and histopathological parameters of common carp (*Cyprinus carpio* L.)," *Neuroendocrinology Letters*, vol. 30, supplement 1, pp. 236–241, 2009.

[38] J. Velisek, E. Sudova, J. Machova, and Z. Svobodova, "Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.)," *Ecotoxicology and Environmental Safety*, vol. 73, no. 3, pp. 384–390, 2010.

[39] J. B. Ortiz, M. L. González de Canales, and C. Sarasquete, "Histopathological changes induced by lindane (γ-HCH) in various organs of fishes," *Scientia Marina*, vol. 67, no. 1, pp. 53–61, 2003.

CHAPTER 8

THE CHRONIC EFFECTS OF TERBUTHYLAZINE-2-HYDROXY ON EARLY LIFE STAGES OF MARBLED CRAYFISH (*PROCAMBARUS FALLAX* F. *VIRGINALIS*)

Koutnik, D., Stara, A., Zuskova E., Kouba, A., Velisek, J., 2017. The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Pesticide Biochemistry and Physiology 136: 29–33.

My share on this work was about 30%

It was allowed by publisher on 4th May, 2018 to include the paper in this Ph.D. thesis.

Pesticide Biochemistry and Physiology 136 (2017) 29–33

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*)

Dalibor Koutnik, Alzbeta Stara, Eliska Zuskova, Antonin Kouba, Josef Velisek *

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form 22 August 2016
Accepted 24 August 2016
Available online 26 August 2016

Keywords:
Triazine
Early development
Histopathology
Oxidative stress
Antioxidant enzymes

ABSTRACT

This study assessed the chronic effects of terbuthylazine-2-hydroxy (T2H), one of the main terbuthylazine degradation products, on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) by means of mortality, growth rate, early ontogeny, oxidative stress, antioxidant defence and histopathology. The crayfish were exposed to four concentrations of the tested substance as follows: 0.75 μg/l (environmental concentration), 75, 375 and 750 μg/l for 62 days. Concentrations over 75 μg/l caused lower weight compared to the control group. T2H at 750 μg/l caused delay in ontogenetic development. Levels of thiobarbituric acid reactive substances and total superoxide dismutase activity were significantly ($p < 0.01$) lower in groups exposed to 375 and 750 μg/l T2H. Crayfish in these treatments also showed alteration of tubular system including disintegration of tubular epithelium with complete loss of structure in some places of hepatopancreas and wall thinning up to disintegration of branchial filaments with focal infiltrations of hemocytes. In conclusion, chronic terbuthylazine-2-hydroxy exposure in concentrations up 75 μg/l (100 times higher than environmental concentration) affected growth, ontogenetic development, antioxidant system, caused oxidative stress and pathological changes in hepatopancreas of early life stages of marbled crayfish.

1. Introduction

During the last few decades there is a global problem with increasing concentrations of pollutants in both surface and ground waters [1]. Pesticide compounds cause physicochemical changes in water environment which may directly or indirectly lead to impacts on non-target aquatic organisms, as well as humans [1,2]. Some pesticides may accumulate in aquatic ecosystems and their degradation products can occasionally be more toxic than the parent compound [3].

Triazines are one of the most commonly found pesticides and their degradation products in water [1,4]. They inhibit photosynthesis [5]. Although most of them were banned in Europe in last years, these substances and their metabolites in residual concentrations are still present in water, soil, food and various components of the environment [6]. Currently in Europe, some triazine herbicides (atrazine and simazine) are considered hazardous for the ground water. As a result, they are ranked on the list of priority substances for testing in EU [7].

Terbuthylazine-2-hydroxy ($C_9H_{17}N_5O$; T2H) is the main metabolite of terbuthylazine, which has been used for control of weeds, aquatic plants and algae since 2006 as a substitute for the herbicide atrazine. The half-life of T2H is between 112 and 120 days in water at 20–25 °C, and 456 days at 10 °C [8]. The highest concentrations of terbuthylazine and T2H in rivers of the Czech Republic were found to be 2.9 and 0.75 μg/l respectively [6]. Masia et al. [9] report concentration of T2H in basin of Llobregat River (Catania, Spain) to be 9.24 μg/l.

Crayfish are important benthic invertebrates in the ecosystem, which are considered appropriate model organisms for assessment of environmental pollution [10,11]. There is a dearth of data on effects of triazine herbicides on crayfish following chronic exposure. Considering conservation status of European native crayfish [12], non-native and according to law non-protected species marbled crayfish (*Procambarus fallax* f. *virginalis*) were utilized as a model organism in this trial. The aim of the present study was to investigate chronic effects of long-term exposure to the T2H in a range of concentrations, including environmentally realistic exposure, on crayfish survival, growth, ontogeny, oxidative balance, antioxidant defence, and histology.

2. Materials and methods

2.1. Experimental animals

Eggs from a single marbled crayfish (*Procambarus fallax* f. *virginalis*) female (carapace length 28.94 mm, postorbital carapace length 22.69 mm, and weight 6.68 g) were gently stripped with tweezers from pleopods. The female originated from own laboratory culture.

* Corresponding author.
E-mail address: velisek@frov.jcu.cz (J. Velisek).

http://dx.doi.org/10.1016/j.pestbp.2016.08.008
0048-3575/

Marbled crayfish is a fast growing organism that frequently reproduces via parthenogenesis with high fecundity, has an early maturation and low culture requirements making it a useful model organism [13]. It is also widely available in the pet trade and non-native in Europe [14,15].

2.2. Experimental protocol

Two hundred and ten eggs (mean weight of 2.18 mg) in X. stage of embryonic development (embryo with maxilla) were put into plastic macroplates where they were held separated as single individuals. The eggs were exposed to the T2H (Sigma Aldrich, Czech Republic, chemical purity 99.5%, CAS: 66753-07-9) concentrations while the water without tested substance served as a control. Forty-two individuals were tested per group and replicate. The tested T2H concentrations were as follows: 0.75 μg/l (environmentally relevant concentrations in Czech rivers – group 1 – E1), 75 μg/l (group 2 – E2), 375 μg/l (group 3 – E3), and 750 μg/l (group 4 – E4). Groups E1–E4 were used as contrast groups.

2.3. Water quality parameters

Aerated tap water was used, with the following parameters: dissolved oxygen > 75%, temperature 19.8–20.1 °C, pH 7.4–7.9, $ANC_{4.5}$ (acid neutralization capacity) 0.95 mmol/l, COD_{Mn} (chemical oxygen demand) 1.1 mg/l, total ammonia 0.05 mg/L, NO_3^- 4.10 mg/l, NO_2^- 0.04 mg/l, Cl^- 8.9 mg/l and sum of $Ca^{2+} + Mg^{2+}$ 1.0 mg/l. Temperature was measured hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). All T2H concentrations were checked daily by high performance liquid chromatography (HPLC). Water was chromatographed on a reverse phase HPLC column (Lichrosphere 100 RP_{18}, Vertex column, pore size 100 μm, particle size 5 μm, 250 mm × 5 mm ID) using solvent systems of methanol:water:ammonium:acetate 70:30:0.2 and 80:20:0.2 (v:v:v) isocratically, with a flow rate of 0.7 ml min^{-1}. Injection volume of samples was 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]. Water samples were collected from all aquaria immediately before (24 h after application) and after renewing the test solutions (0 h). The values measured did not differ from the value stated for test purposes by >9%.

2.4. Test conditions

The macroplates were placed in a laboratory (open-air conditions) with the light regime (11:13 h light:dark). The exposure water for each treatment was renewed three times weekly. Water was gently drained from each chamber, and a new solution was slowly added. Survival was evaluated daily and dead individuals (eggs or juveniles) were removed. From the third development stage, juveniles were fed by freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii ad libitum once a day. During and at the end of the experiment, early development stages were observed to monitor development, occurrence of morphological anomalies and body weight of particular stages (always a day after moulting to allow at least partial calcification of the animal). Determination of developmental periods and stages followed Vogt et al. [17].

The toxicity test was ended after 62 days. At the end of the tests crayfish were sacrificed on ice anaesthesia, weighed, measured and stored for further processing. Weight to the nearest 0.1 mg, was measured using a Mettler-Toledo (Greifensee, Switzerland) analytical balance after removing excess water on a filter paper. The mean specific growth rate (SGR) for crayfish in each of the experimental groups was calculated for the period beginning at day 9 (the first sampling time) and ending at day 62 (end of the trial) using the method described by Kroupova et al. [18].

2.5. Oxidative stress and antioxidants biomarkers

At the end the trial surviving crayfish were immediately frozen and stored at −80 °C until the analysis. Frozen samples were homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika Staufen, 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 the second centrifuged at 4 °C to obtain the post-mitochondrial supernatant for antioxidants parameters analyses – total superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). The TBARS method described by Lushchak et al. [19] was used to evaluate lipid peroxidation.

Total superoxide dismutase activity was determined spectrophotometrically by the method of Marklund and Marklund [20]. The catalase activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer [21]. Glutathione reductase activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [22]. Protein levels were estimated spectrophotometrically by the method of Bradford [23] using bovine serum albumin as a standard.

2.6. Histopathology examination

For histological investigations crayfish were fixed in neutral buffered 10% formalin at the end of the experiment. Later they were decalcified for 4 h (slow decalcifier DC1; containing formic acid and formaldehyde, Labonord SAS, Germany), embedded by using of tissue processor Histomaster 2052/1.5. Samples were circumfused with paraffin and sections from paraffin blocks were made on rotary microtome (4 μm), stained with hematoxylin-eosin (H&E) in automatic slide staining system (TISSUE-TEK DR 2000, SEKURA Mars, USA). The most visible tissues – hepatopancreas and gills were examined under the light microscope combined with camera system (MOTIC Wetzlar, Germany).

2.7. Statistical analysis

Data are expressed as mean ± SD. STATISTICA version 12.0 for Windows (StatSoft, Inc.) was used to perform the statistical analysis. To compare difference in means, two way analysis of variance (ANOVA). The significance levels for tests were $p < 0.05$ and $p < 0.01$.

Table 1
Mean body weight (mean ± SD), specific growth rate, inhibition of specific growth, and crayfish mortality on *early life stages of* marbled crayfish (*Procambarus fallax* f. *virginalis*) after terbuthylazine-2-hydroxy (T2H) exposure.

T2H (μg/l)	–	0.75	75	375	750
M_9 (mg)	5.93 ± 0.19	5.57 ± 0.39	5.61 ± 0.40	5.57 ± 0.45	5.70 ± 0.42
M_{62} (mg)	85.27 ± 14.48	78.06 ± 11.49	65.00 ± 12.32*	55.79 ± 9.11*	48.39 ± 6.97*
SGR (%)	5.00	4.98	4.58	4.33	4.01
I (%)	–	0.40	8.40	13.40	19.80
Cumulative mortality (%)	14.2	16.7	19.0	16.7	19.0

M_9, M_{62} = Mean body weight in selected group after 9 and 62 days exposure; SGR = mean specific growth rate in selected group; I = inhibition of specific growth in selected group; SD = standard deviation.

* Experimental groups significantly ($p < 0.01$, Tukey's test) different from the control group.

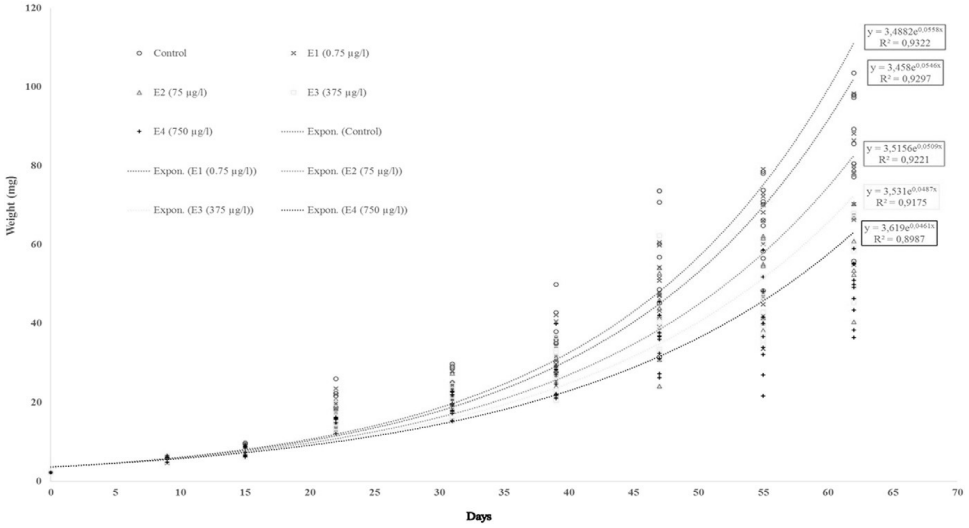


Fig. 1. Mean body weight of early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) after terbuthylazine-2-hydroxy (T2H) exposure.

3. Results

3.1. Cumulative mortality

No apparent differences were found in total cumulative mortality among groups. Mortality was below 20% among all tested groups (Table 1).

3.2. Growth parameters

Mean body weight of crayfish related to T2H concentration in water as well as a control group is depicted in Fig. 1. No significantly negative effects on body weight and specific growth rate were observed when environmentally relevant concentration of T2H (E1; 0.75 µg/l) was compared with control. From 22 days of exposure, crayfish exposed to the highest tested groups 750 µg/l (E4) T2H showed significantly ($p < 0.01$) lower weight compared with control. Similarly, such a trend was apparent on day 47 and 55 of exposure in crayfish exposed to the tested groups 375 µg/l (E3) and 75 µg/l (E2) T2H, respectively ($p < 0.01$ in both cases). Specific growth rates and inhibition of growth were calculated for 62 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to T2H in concentrations 375 (E3) and 750 µg/l (E4) was 13.40 and 19.80% compared to control, respectively.

3.3. Early ontogeny

Crayfish exposed to the highest concentration 750 µg/l T2H showed delay in ontogeny development compared with the control group. At the end of the trial 75% of individuals remained in VIII. stage of juvenile development at this concentration of T2H, whereas the majority of control crayfish reached the X. stage. During the experiment there were no apparent morphological abnormalities in crayfish.

3.4. Oxidative stress and antioxidant response

Effects of T2H exposure on oxidative stress (TBARS) and antioxidant responses (SOD, CAT, GR) in homogenate of crayfish early life stages are shown in Table 2. Crayfish exposed to the tested groups 375 µg/l (E3) and 750 µg/l (E4) T2H showed significantly ($p < 0.01$) lower values of TBARS level and SOD activity compared to controls. No differences were found in CAT and GR activity among groups.

3.5. Histopathology

There were no apparent differences in hepatopancreas tissue between control and E1 group. The structure of tubules seemed physiologically with the presence of all structural cell types. The slight alterations of this tissue were observed in groups E2, where indistinct intercellular

Table 2
Effect of chronic exposure to terbuthylazine-2-hydroxy (T2H) on level of thiobarbituric acid reactive substances (TBARS) activity and superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) activity in homogenate on early life stages of marbled crayfish (*Procambarus fallax f. virginalis*).

T2H (µg/l)	–	0.75	75	375	750
TBARS (nmol/mg protein)	0.447 ± 0.044	0.439 ± 0.029	0.417 ± 0.071	0.325 ± 0.060*	0.302 ± 0.058*
SOD (nmol NBT/min/mg protein)	0.298 ± 0.140	0.215 ± 0.072	0.165 ± 0.045	0.048 ± 0.062*	0.068 ± 0.111*
CAT (µmol H_2O_2/min/mg protein)	0.428 ± 0.262	0.491 ± 0.313	0.447 ± 0.250	0.357 ± 0.270	0.350 ± 0.276
GR (nmol NADPH/min/mg protein)	0.091 ± 0.068	0.065 ± 0.047	0.044 ± 0.034	0.111 ± 0.044	0.078 ± 0.108

* Experimental groups significantly ($p < 0.01$, Tukey's test) different from the control group.

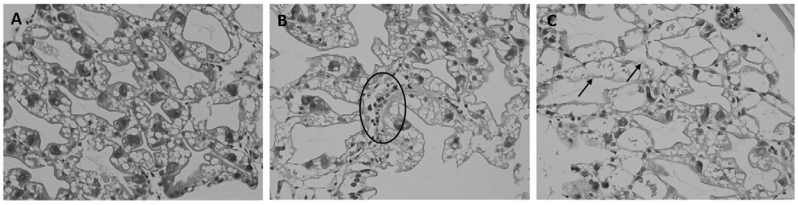


Fig. 2. Histological appearance of hepatopankreas after T2H exposition; (A) control group with typical structure of tubular epithelial cells; (B) group E2 exposed to 75 μg/l (oval indicates intertubular infiltration of hemocytes); (C) group E4 exposed to 750 μg/l (arrows show total disintegration of cell walls, asterisk shows congestion of hemolymph cells along the border of the tissue), ×400; H&E.

borders with intertubular infiltration of hemocytes were recorded. The most histological changes were observed in groups 375 μg/l (E3) and 750 μg/l (E4) compared to control. Crayfish exposed to higher levels of T2H showed alteration of the tubular system including disintegration of tubular epithelium with complete loss of structure in some places of hepatopancreas (Fig. 2).

The morphology of gills showed normal histological structure in control, 0.75 μg/l and 75 μg/l groups, while marked alterations were apparent in 375 μg/l and 750 μg/l treatments. The changes included wall thinning up to disintegration of branchial filaments with focal infiltrations of hemocytes. Described changes were more frequent and substantial in the group exposed to the highest concentration of T2H. The results highlighted the potential impact of higher concentrations of T2H on crayfish hepatopancreas and gills.

4. Discussion

The results of our experiment show that triazine decomposition product, T2H caused alterations in all monitored parameters except catalase and glutathione reductase activity. All of the changes were observed only at exposures (75, 350 and 750 μg/l). Only a limited number of studies have focused on effects of triazine herbicides degradation products e.g. T2H. This study is the first one monitoring the impact of T2H on early life stages of marbled crayfish, respectively early life stages of crayfish species in general.

Crayfish were subjected to prolonged exposure of the T2H concentration range including the environmental one. In this study were no significant differences in mortality, at any concentration tested from controls. The level of accumulated mortality was lower than 20% of all tested individuals of crayfish included control crayfish. Triazine herbicides can result in mortality of crayfish at much higher concentrations (>10 mg/L) [24]. Reported 96 h LC_{50} values for signal crayfish are 12.1 mg/l for atrazine, 13.9 mg/l for terbutryn, 14.4 mg/l for prometryn; 19.5 mg/l for hexazinone, 30.6 mg/l for metribuzin and 77.9 mg/l for simazine [25]. In surface waters triazines have been recorded at concentrations much below these levels ranging from 0.1 to 10 μg/l [6]. Environmental concentrations of triazines do not reach lethal concentrations for crayfish.

Growth can be routinely measured in all early life ecotoxicological tests. Growth represents an integration of a variety of physiological and environmental factors [26]. In our test, T2H in concentrations higher than or equal to 75 μg/l caused decrease of body weight and inhibition of growth in early life stages of marbled crayfish. The greatest difference was in the concentration of 750 μg/l T2H, where the inhibition of growth was 19.8% compared to control. The impact of reductions of growth may be the delay of reproduction as well as increased susceptibility of young crayfish to predation and disease. The results presented here are also in agreement with earlier studies on fish exposed to triazines [27–29].

In embyrolarval toxicity tests, delayed early development is commonly found after exposure of xenobiotics [29–31] and can be used as a relatively sensitive parameter for the evaluation of impacts of pesticides on aquatic organisms. Crayfish exposed the highest concentration 750 μg/l T2H showed delay in ontogeny development. According to many studies triazine herbicides cause delay of early ontogeny in aquatic organisms [24,29,32,33].

Triazine herbicides cause oxidative stress and change activity of antioxidant enzymes in aquatic organisms [24]. Changes in antioxidant enzyme activity in aquatic organisms can depend on the intensity (concentration) and the duration of the exposure [34,35]. In our study, the two highest T2H concentrations caused a decrease of TBARS level and SOD activity in crayfish. The changes in activity of SOD demonstrate a T2H induced adaptive response in crayfish, and an attempt to neutralize reactive oxygen species (ROS) [36]. Induction of SOD systems provides the main line of defence against oxidative stress and is the major enzyme in eliminating oxidative stress formed during bio activation of T2H. Similar changes were described by Koutnik et al. [37] in signal crayfish (*Pacifastacus leniusculus*) exposed to low concentrations (0.52 μg/l) of metribuzin. The reported study shows also that transfer of crayfish to metribuzin-free water leads to improvement of the physiological homeostasis. Hostovsky et al. [35] also confirmed changes in antioxidant enzymes in fish exposed to triazine herbicides, including terbuthylazine.

The liver is the primary organ of metabolism and excretion of xenobiotics. However, degradation of xenobiotics might be overwhelmed by higher concentrations of these substances leading to failure of regulatory mechanisms resulting in histopathological damage [38]. T2H in higher concentrations (375 and 750 μg/l) caused histopathological changes in hepatopancreas and gills of crayfish. This result is in concordance with Koutnik et al. [37] and Velisek et al. [32]. Both these studies demonstrated that triazine herbicides have impacts on hepatopankreas of crayfish, especially at high concentrations. Biagianti-Risbourg and Bastide [39] reported that atrazine affect the liver in fish, which shows a substantial increase in the size of lipid inclusions, followed by lipoid degeneration, enlargement of the secondary lysosomes, mitochondrial malformation and vacuolization. Short-term exposure to terbuthylazine in high concentrations in common carp (*Cyprinus carpio*) leads to irritation and injury to the gills, which negatively affects respiration, ammonia excretion and causes perturbations in ion homeostasis [40].

5. Conclusion

The exposure of marbled crayfish to T2H at concentrations similar to those encountered in the environment had no effect on early life stages. However, exposure at much higher concentrations (75 to 750 μg/l) of T2H resulted in aberrations in all monitored parameters, except catalase and glutathione reductase activities. Exposures at these high concentrations of T2H would result in changes in metabolism of marbled crayfish.

Some of these changes are likely the result of oxidative stress; however, the mechanisms remain to be elucidated.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program), and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 012/2016/Z).

References

[1] N. Abrantes, R. Pereira, F. Gonçalves, Occurrence of pesticides in water, sediments and fish tissues in a lake surrounded by agricultural lands: concerning risks to humans and ecological reports, Water Air Pollut. 212 (2010) 77–88.
[2] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, M.J. Medina-Hernández, R.M. Villanueva-Camañas, S. Sagrado, Chromatographic evaluation of the toxicity in fish of pesticides, J. Chromatogr. B 814 (2005) 115–125.
[3] S.B. Ceyhun, M. Senturk, O. Erdogan, O.I. Kufrevioglu, *In vitro* and *in vivo* effect of some pesticides on carbonic anhydrase enzyme from rainbow trout (*Oncorhynchus mykiss*) gills, Pestic. Biochem. Physiol. 97 (2010) 177–181.
[4] I.R. Bonansea, M.V. Ame, D.A. Wunderlinn, Determination of priority pesticides in water samples combining SPE and SPME coupled to GC–MS. A case study: Suquia River basin (Argentina), Chemosphere 90 (2013) 1860–1869.
[5] K. Bowmer, S. Jacobs, G. Sainty, Identification, biology, and management of *Elodea canadensis*, Hydrocharitaceae, J. Aquat. Plant Manag. 33 (1995) 13–19.
[6] CHMU (Czech Hydrometeorological Institute), On-line water duality database, 2015 Available from: http://hydro.chmi.cz/oj/ (visited online 15.5.2015).
[7] European Commission, Directive 2008/105/EC of the European Parliament and the Council on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC, Official Journal, 2008.
[8] K. Nodler, T. Licha, D.T. Voutsa, Twenty years later atrazine concentrations in selected coastal waters of the Mediterranean and the Baltic Sea, Mar. Pollut. Bull. 70 (2013) 112–118.
[9] A. Masia, J. Campo, A. Navarro-Ortega, D. Barcelo, Y. Pivo, Pesticide monitoring in the basin of Llobregat River (Catalonia, Spain) and comparison with historical data, Sci. Total Environ. 503-504 (2015) 58–68.
[10] A. Kouba, M. Buric, P. Kozak, Bioaccumulation and effects of heavy metals in crayfish: a review, Water Air Soil Pollut. 211 (2010) 5–16.
[11] A. Stara, A. Kouba, J. Velisek, Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in red swamp crayfish (*Procambarus clarkii*), Biomed. Res. Int. 2014 (2014) 680131.
[12] P. Kozak, L. Fureder, A. Kouba, J. Reynolds, C. Souty-Grosset, Current conservation strategies for European crayfish, Knowl. Manag. Aquat. Ecosyst. 401 (2011) 1.
[13] G. Vogt, The marbled crayfish: a new model organism for research on development, epigenetics and evolutionary biology, J. Zool. 276 (2008) 1–13.
[14] A. Kouba, A. Petrusek, P. Kozak, Continental-wide distribution of crayfish species in Europe: update and maps, Knowl. Manag. Aquat. Ecosyst. 413 (2014) 05.
[15] J. Patoka, L. Kalous, O. Kopecky, Risk assessment of the crayfish pet trade based on data from the Czech Republic, Biol. Invasions 16 (2014) 2489–2494.
[16] S. Richter, R. Nagel, Bioconcentration, biomagnification and metabolism of 14C-terbutryn and 14C-benzo[a]pyrene in *Gammarus fossarum* and *Asellus aquaticus*, Chemosphere 66 (2007) 603–610.
[17] G. Vogt, L. Tolley, G. Scholtz, Life stages and reproductive components of the Marmocrebs (marbled crayfish), the first pathogenetic decapod crustacean, J. Morphol. 261 (2004) 286–311.
[18] H. Kroupova, M. Prokes, S. Macova, M. Penaz, V. Barus, L. Novotny, J. Machova, Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.), Environ. Toxicol. Chem. 29 (2010) 535–540.
[19] V.I. Lushchak, T.V. Bagnyukova, V.V. Husak, L.I. Luzhna, O.V. Lushchak, K.B. Storey, Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues, Int. J. Biochem. Cell Biol. 37 (2005) 1670–1680.
[20] S. Marklund, G. Marklund, Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[21] R.F. Beers, I.W. Sizer, A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, J. Biol. Chem. 195 (1952) 133–140.
[22] I. Carlberg, B. Mannervik, Purification and characterization of flavoenzyme glutathione reductase from rat liver, J. Biol. Chem. 250 (1975) 5475–5480.
[23] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] D. Koutnik, A. Stara, J. Velisek, The effect of selected triazines on fish: a review, Slov. Vet. Res. 52 (2015) 107–131.
[25] J. Velisek, A. Kouba, A. Stara, Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*), Neuroendocrinol. Lett. 34 (Suppl. 2) (2013) 31–36.
[26] J.B. Sprague, Measurement of pollutant toxicity to fish. III. Sub-lethal effects and "safe" concentrations, Water Res. 5 (1971) 245–266.
[27] J. Velisek, A. Stara, D. Koutnik, J. Machova, Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.), Pestic. Biochem. Physiol. 118 (2015) 58–63.
[28] J. Velisek, A. Stara, D. Koutnik, E. Zuskova, Effects of terbuthylazine on early life stages of common carp, Neuroendocrinol. Lett. 36 (Suppl. 1) (2015) 120–125.
[29] J. Velisek, D. Koutnik, E. Zuskova, A. Stara, Effects of the terbuthylazine metabolite terbuthylazine-desethyl on common carp embryos and larvae, Sci. Total Environ. 539 (2016) 214–220.
[30] J. Velisek, A. Stara, J. Machova, P. Dvorak, E. Zuskova, M. Prokes, Z. Svobodova, Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.), Pestic. Biochem. Physiol. 102 (2012) 102–108.
[31] J. Velisek, A. Stara, J. Machova, P. Dvorak, E. Zuskova, Z. Svobodova, Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.), Neuroendocrinol. Lett. 33 (Suppl. 3) (2012) 90–95.
[32] J. Velisek, A. Stara, D. Koutnik, E. Zuskova, A. Kouba, Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*), Neuroendocrinol. Lett. 35 (Suppl. 2) (2014) 93–98.
[33] J. Velisek, A. Stara, D. Koutnik, J. Machova, Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.), Biomed. Res. Int. 2014 (2014) 621304.
[34] A. Slaninova, M. Smutna, H. Modra, Z. Svobodova, A review: oxidative stress in fish induced by pesticides, Neuroendocrinol. Lett. 30 (2009) 2–12.
[35] M. Hostovsky, J. Blahova, L. Plhalova, V. Kopriva, Z. Svobodova, Effects of the exposure of fish to triazine herbicides, Neuroendocrinol. Lett. 35 (Suppl. 2) (2014) 3–25.
[36] A. Stara, J. Kristan, E. Zuskova, J. Velisek, Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.), Pestic. Biochem. Physiol. 105 (2013) 18–23.
[37] D. Koutnik, A. Stara, E. Zuskova, A. Kouba, J. Velisek, The effect of subchronic metribuzine exposure to signal crayfish (*Pacifastacus leniusculus* Dana 1852), Neuroendocrinol. Lett. 35 (Suppl. 2) (2014) 51–56.
[38] B. Velmurugan, M. Selvayagam, E.I. Cengiz, E. Ulmu, The effects of fenvalerate on different tissues of freshwater fish *Cirrhinus mrigala*, J. Environ. Sci. Health B 42 (2007) 157–163.
[39] S. Biagianti-Risbourg, J. Bastide, Hepatic perturbations induced by a herbicide (atrazine) in juvenile grey mullet *Liza ramada* (Mugilidae, teleostei): an ultrastructural study, Aquat. Toxicol. 31 (1995) 217–229.
[40] I. Mikulikova, H. Modra, J. Blahova, K. Kruzikova, P. Marsalek, I. Bedanova, Z. Svobodova, Recovery ability of common carp (*Cyprinus carpio*) after a short-term exposure to terbuthylazine, Pol. J. Vet. Sci. 16 (2013) 17–23.

CHAPTER 9

THE EFFECT OF LONG-TERM METRIBUZINE EXPOSURE TO SIGNAL CRAYFISH (*PACIFASTACUS LENIUSCULUS* DANA)

Koutnik, D., Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2014. The effect of long-term metribuzine exposure to signal crayfish (*Pacifastacus leniusculus* Dana). Neuroendocrinology Letters 35: 51–56.

My share on this work was about 30%

It was allowed by publisher on 17th April, 2018 to include the paper in this Ph.D. thesis.

Neuroendocrinology Letters *Volume 35 Suppl. 2 2014*

ORIGINAL ARTICLE

The effect of subchronic metribuzin exposure to signal crayfish (*Pacifastacus leniusculus* Dana 1852)

Dalibor Koutnik, Alzbeta Stara, Eliska Zuskova, Antonin Kouba, Josef Velisek

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic

Correspondence to: Dipl.-Ing. Dalibor Koutnik
University of South Bohemia in Ceske Budejovice,
Faculty of Fisheries and Protection of Waters,
Research Institute of Fish Culture and Hydrobiology
Zatisi 728/II CZ-389 25 Vodnany, Czech Republic.
tel: +420 383 382 402; fax: +420 383 382 396; e-mail: dkoutnik@frov.jcu.cz

Submitted: 2014-09-23 Accepted: 2014-11-08 Published online: 2014-11-30

Key words: **triazine; crayfish; oxidative stress; antioxidant enzymes; histopathology**

Neuroendocrinol Lett 2014; **35**(Suppl. 2):51–56 **PMID:** 25638366 NEL351014A04

Abstract

OBJECTIVES: The aim of the study was to investigate effects of the triazine herbicide metribuzin on signal crayfish *Pacifastacus leniusculus* Dana by determining oxidative stress (thiobarbituric acid reactive substances) and antioxidant indices (total superoxide dismutase, catalase, glutathione reductase) in hepatopancreas, muscle, and gill as well as assessing their histopathology.

DESIGN: Crayfish were exposed to metribuzin concentrations of 0.52 μg.l^{-1} (realistic environmental concentration) and 3.06 mg.l^{-1} (10% 96hLC50) for 10 and 30 days followed by a 30-day depuration period without exposure to metribuzin.

RESULTS: In the thiobarbituric acid reactive substances, superoxide dismutase, and catalase were observed differences in all examined tissues compared to the control group. Differences from control were observed in glutathione reductase activity in hepatopancreas after 10 days for both exposure concentrations and after 30 days at 3.06 mg.l^{-1}. Histological examination revealed extensive focal autolytic disintegration of tubular epithelium in hepatopancreas of crayfish exposed to metribuzin for 30 days.

CONCLUSIONS: Chronic exposure of metribuzin resulted in oxidative damage to cell lipids, in changes of antioxidant activity in crayfish tissue, and pathological changes in hepatopancreas. The results suggest that selected oxidative stress biomarkers, antioxidant enzymes, and pathologies of hepatopancreas may have potential as biomarkers for monitoring residual triazine herbicides in the aquatic environment.

Abbreviations:

ANOVA	- analysis of variance
CAT	- catalase
LC50	- lethal concentration
LPO	- lipid peroxidation
GR	- glutathione reductase
SOD	- superoxide dismutase
TBARS	- thiobarbituric acid reactive substances

INTRODUCTION

The increasing worldwide contamination of surface and groundwater systems with thousands of industrial and natural chemical compounds is a critical environmental problem (Schwarzenbach *et al.* 2006). Pesticides make a major contribution to the pollution of aquatic ecosystems. There is compelling evidence that use of agricultural pesticides

has a strong impact on water quality and is a factor in extensive pollution of rivers, lakes, and estuaries, affecting non-target aquatic organisms (Velisek *et al.* 2012; Stara *et al.* 2012, 2013).

Triazines herbicides are among the most commonly used pesticides worldwide. 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 ground and surface water as well as in other environmental compartments (Chapadense *et al.* 2009). Therefore it is prudent to study the long-term effects of these substances on non-target organisms.

Metribuzin (4-amino-6-tert-butyl-3-(methythio)-1,2,4-triazin-5-one) is an asymmetrical triazine herbicide. It was first registered as a pesticide in the USA in 1973. Metribuzin is used to selectively control certain broadleaf and grassy weeds in a wide range of sites including vegetable and field crops, turf grasses in recreational areas, and non-crop areas (Fairchild & Sappington 2002). The contamination of water may result from spray and vapor drift, runoff and leaching from treated land, or from accidental spills (Fairchild & Sappington 2002).

Crayfish are important benthic invertebrates in the ecosystem, and they are considered an appropriate model organism for pollution of water (Kouba *et al.* 2010; Stara *et al.* 2014). There is a dearth of data on effects on crayfish of chronic exposure to metribuzin at environmentally realistic concentrations. European native crayfish are facing distribution losses across their range (Kouba *et al.* 2014), are endangered, and often protected by both European and national laws (Kozak *et al.* 2011). Hence, we selected adults of the invasive and widely-spread signal crayfish *Pacifastacus leniusculus* as a model non-target aquatic organism. The aim of the present study was to investigate effects of long-term exposure to low metribuzin concentrations on oxidative stress, antioxidant defense, and histopathology in signal crayfish *Pacifastacus leniusculus* L.

MATERIALS AND METHODS

Chemicals

Metribuzin (chemical purity 99.3%) and other chemicals were purchased from Sigma–Aldrich Corporation (USA).

Experimental animals

Trap-caught crayfish originated from the natural population in the Horni Kozlov Pond, Vysocina region, Czech Republic. The mean carapace length was 46.4 mm, and mean weight was 38.5 g.

Experiment design

Crayfish were held in aquaria containing 100 L of freshwater. Water temperature ranged from 18.5 to 20.8 °C, pH 7.4–8.03, and oxygen saturation 72–99%, with fotoperiod light:dark 12:12. Aquaria were equipped with plastic shelters to deter cannibalism (Kouba *et al.* 2012). Crayfish were acclimatized for 10 days before the beginning of the experiment.

Experimental protocol

The trial was a semistatic design conducted over 60 days. Crayfish were exposed for 30 days to metribuzin followed by a 30-day depuration period in water without the herbicide. Signal crayfish (n=108) were allocated, in groups of 12, to one of two experimental metribuzin concentrations or to an untreated control group. Each treatment was tested in triplicate. The selected metribuzin concentrations were: 0.52 $\mu g.l^{-1}$ (the reported environmental concentration in Czech rivers) and 3.06 $mg.l^{-1}$. The latter concentration corresponds to 10% of the 96 h LC50 value of metribuzin to this species (Velisek *et al.* 2013). Crayfish were fed once daily on a commercial diet for fish, SteCo Pre Grower-14 2.0 mm (Coppens International, Netherlands), at 1% body weight per day.

The solution was renewed daily 2 h after feeding to maintain water quality and the appropriate concentration of metribuzin. To ensure comparability between nominal and actual compound concentrations, water in the aquaria was analyzed throughout the experimental period by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Barcelo & Hennion 1997). The mean concentration of metribuzin in the water samples was consistently within 8% of the intended concentration.

Tissue samples and preparation of post-mitochondrial supernatant

At the completion of each exposure period, 10, 30, and 60 (30 days depuration) days, three crayfish from each group were randomly selected, anesthetized on melting ice and killed. The gills, hepatopancreas, and abdominal muscle were quickly removed, immediately frozen, and stored 20 days at −80 °C until analysis. Frozen tissue samples were weighed and homogenized using an Ultra Turrax homogenizer (Ika, Germany) with 50 mM potassium phosphate buffer (1:10, w/v), 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 post-mitochondrial supernatant for further analyses of antioxidant parameters.

Indices of oxidative stress and antioxidant parameters

The TBARS method described by Lushchak (2005) was used to evaluate lipid peroxidation (LPO). Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund (1974). The catalase (CAT; EC 1.11.1.6) activity was performed following the method of Beers and Sizer (1952). Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH

oxidation at 340 nm (Carlberg & Mannervik 1975). Protein levels were estimated spectrophotometrically by the Bradford (1976) method, using bovine serum albumin as standard.

Histopathology

Histopathology was evaluated in all experimental groups on the sampling days. The samples of gill and hepatopancreas were immediately fixed in 10% formalin, drained, and embedded in paraffin. Sections were cut from the paraffin blocks, stained with hematoxylin-eosin, examined by light microscopy, and photographed using a digital camera.

Statistical analysis

One-way ANOVA was conducted to compare differences among the test groups using the software program Statistica, version 12.0 for Windows (StatSoft).

RESULTS

Crayfish behavior

There were no observed differences in feed intake, sheltering, escaping, and rate of movement among crayfish treatment groups during the trial. No mortality was observed.

Oxidative stress indices

The level of TBARS in gill of all experimental groups was significantly increased ($p<0.05$) after 10 days exposure, but decreased ($p<0.01$) compared to the control group after 30 days exposure. The level of TBARS was significantly increased ($p<0.01$) in muscle of crayfish exposed 30 days to metribuzin at 3.06 mg.l^{-1} compared to control. Higher TBARS levels were observed in hepatopancreas of crayfish in both metribuzin exposure groups compared to control after 10 days. There were no differences between the exposed groups and control in any examined tissues after 30-day depuration (Table 1).

Antioxidant enzymes

The SOD activity in gill, muscle, and hepatopancreas of all groups is summarized in Table 2. The SOD activity in gill was significantly ($p<0.01$) decreased in the group exposed to 3.06 mg.l^{-1} metribuzin after 10 days, but values were higher ($p<0.01$) at both exposure levels compared to control after 30 days and 60 days. In muscle, the SOD activity was significantly ($p<0.01$) lower than in controls in the group exposed to the 3.06 mg.l^{-1} metribuzin after 10 days, and higher with both concentrations after 30 days ($p<0.01$) exposure. The SOD activity in hepatopancreas at both tested concentrations was significantly lower ($p<0.01$) compared to the control after 10 days.

Effects of chronic exposure to metribuzin on activity of CAT are shown in Table 3. The CAT activity in gill was significantly ($p<0.01$) increased in both metribuzin exposure groups at 10 days, and in liver was increased after 30 days exposure. The CAT activity in muscle was significantly ($p<0.01$) decreased in the group exposed to 3.06 mg.l^{-1} metribuzin after 10 and 30 days. Lower values than control were observed after 30 days depuration for both tested metribuzin concentrations.

Glutathione reductase activity is shown in Table 4. The GR activity in hepatopancreas was increased ($p<0.05$) in both experimental groups after 10 days, and, after 30 days, was decreased ($p<0.05$) compared to control in the group receiving 0.52 µg.l^{-1} metribuzin.

Histopathology

There were no apparent differences in hepatopancreas tissue between all crayfish groups sampled 10th day of test. The morphology of examined hepatopancreas in this sampling time was normal and all different cell types were comparatively uniform in size and shape and

Tab. 1. Effect of chronic exposure to metribuzin on level of thiobarbituric acid reactive substances (TBARS, nmol mg^{-1} protein) in signal crayfish (*Pacifastacus leniusculus* Dana) tissues.

Tissue	Exposure time (days)	Test groups		
		Control	E 1 (0.52 µg.l^{-1})	E 2 (3.06 mg.l^{-1})
Gill	10	0.0844±0.0078	0.0995±0.0083*	0.1066±0.0131*
	30	0.1045±0.0229	0.0890±0.0211**	0.0687±0.0203**
	recovery (30)	0.0661±0.0161	0.0782±0.0078	0.0756±0.0141
Muscle	10	0.1025±0.0333	0.1087±0.0116	0.0946±0.0256
	30	0.0526±0.0192	0.0424±0.0145	0.1018±0.0286**
	recovery (30)	0.1417±0.0214	0.1198±0.0370	0.1059±0.0222
Hepatopancreas	10	0.2397±0.0470	0.3528±0.0703*	0.3644±0.1136*
	30	0.3926±0.1517	0.4028±0.1336	0.3432±0.1075
	recovery (30)	0.2952±0.0528	0.2995±0.0699	0.3954±0.1129

Data are means ± S.D., n=9. Significant differences compared with control value, *$p<0.05$; **$p<0.01$.

Tab. 2. Effect of chronic exposure to metribuzin on superoxide dismutase (SOD, nmol NBT min^{-1} mg^{-1} protein) activity in signal crayfish (*Pacifastacus leniusculus* Dana) tissues.

Tissue	Exposure time (days)	Test groups		
		Control	E 1 (0.52 µg.l^{-1})	E 2 (3.06 mg.l^{-1})
Gill	10	0.0886±0.0368	0.0612±0.0219	0.0149±0.0046**
	30	0.0486±0.0169	0.1652±0.0505**	0.0603±0.0261**
	recovery (30)	0.0857±0.0514	0.1569±0.0371**	0.1809±0.0399**
Muscle	10	0.2894±0.0700	0.2220±0.0685	0.1341±0.0675**
	30	0.1127±0.0801	0.3816±0.1630**	0.3360±0.0952**
	recovery (30)	0.1547±0.0466	0.2113±0.2481	0.1061±0.0375
Hepatopancreas	10	0.4782±0.1663	0.2318±0.0669**	0.2542±0.0594**
	30	0.2607±0.0721	0.2153±0.0573	0.2974±0.0767
	recovery (30)	0.3030±0.0477	0.3798±0.0713	0.382±0.0732

Data are means ± S.D., n=9. Significant differences compared with control value, *p<0.05; **p<0.01.

Tab. 3. Effect of chronic exposure to metribuzin on catalase (CAT, µmol H_2O_2 min^{-1} mg^{-1} protein) activity in signal crayfish (*Pacifastacus leniusculus* Dana) tissues.

Tissue	Exposure time (days)	Test groups		
		Control	E 1 (0.52 µg.l^{-1})	E 2 (3.06 mg.l^{-1})
Gill	10	0.0500±0.0256	0.0969±0.0477**	0.1318±0.0720**
	30	0.1526±0.1030	0.1413±0.0803	0.1567±0.0882
	recovery (30)	0.1798±0.1312	0.2035±0.1248	0.3364±0.1269
Muscle	10	0.0925±0.0690	0.0800±0.0436	0.0405±0.0205**
	30	0.1162±0.0875	0.1021±0.0812	0.0798±0.6065**
	recovery (30)	0.2152±0.0729	0.1378±0.0483**	0.0893±0.0450**
Hepatopancreas	10	1.1588±0.1456	1.4438±0.4888	0.8957±0.3653
	30	0.9138±0.3769	1.3464±0.5726**	1.2972±0.3467**
	recovery (30)	1.0773±1.4706	0.7639±0.3575	0.8531±0.4467

Data are means ± S.D., n=9. Significant differences compared with control value, *p<0.05; **p<0.01.

Tab. 4. Effect of chronic exposure to metribuzin on glutathione reductase (GR, nmol NADPH/min/mg protein) activity in signal crayfish (*Pacifastacus leniusculus* Dana) tissues.

Tissue	Exposure time (days)	Test groups		
		Control	E 1 (0.52 µg.l^{-1})	E 2 (3.06 mg.l^{-1})
Gill	10	0.0406±0.0121	0.0339±0.0117	0.0363±0.1428
	30	0.0475±0.0155	0.0329±0.0140	0.0740±0.0513
	recovery (30)	0.0590±0.0333	0.0846±0.0714	0.0809±0.0398
Muscle	10	0.1436±0.1391	0.0930±0.0511	0.0757±0.0369
	30	0.0950±0.0797	0.0356±0.0278	0.0557±0.0276
	recovery (30)	0.0895±0.0801	0.0758±0.0556	0.1330±0.0750
Hepatopancreas	10	0.2171±0.0649	0.3950±0.1895*	0.3556±0.1428*
	30	0.2601±0.1273	0.1771±0.1047*	0.2478±0.1431
	recovery (30)	0.2751±0.1854	0.2551±0.1783	0.1719±0.0978

Data are means ± S.D., n = 9. Significant differences compared with control value, *p<0.05.

easily recognized. The apparent changes of hepatopancreas were observed in groups exposed to metribuzin for 30 days (Figure 1). The main histopathological findings revealed extensive focal autolytic disintegration of tubular epithelium. The intensity of changes and the alteration of tissue was more pronounced in group exposed to 3.06 mg.l^{-1} of metribuzin compared with control. On the basis of the examination performed 30 days after metribuzin exposition, where no analogous histopathological changes were observed, we could suppose, that all changes are reversible. This meaning is supported by the finding of higher occurrence of mononuclear cells in interstitial hemolymph space. No pathological changes were observed in gill of signal crayfish following chronic exposure to metribuzin.

DISCUSSION

Many classes of environmental pollutants or their metabolites exert toxicity related to oxidative stress and can cause oxidative damage in aquatic organisms (Lushchak *et al.* 2005; Stara *et al.* 2013, 2014). The main objective of this study was to determine the influence of metribuzin on signal crayfish oxidative stress, antioxidant parameters, and histology. The assessment of oxidative stress markers is critical to the investigation of oxidative stress in organisms. Pro-oxidant activity can be used to assess water pollution (Slaninova *et al.* 2009). The steady-state concentration of the markers of oxidative stress is a balance between production and elimination, producing a steady-state ROS level.

The TBARS assay quantifies oxidative stress and damage in fish tissue through assessment of levels of the lipid peroxidation that occurs with free radical generation (Oakes & van der Kraak 2003). Our data demonstrated that chronic exposure to metribuzin affected TBARS levels in tissue of signal crayfish. Stara *et al.* (2014) did not observe significant differences from controls in TBARS levels in tissue of adult red swamp crayfish *Procambarus clarkii* following prometryne exposure. Responses to oxidative stress may differ depending on species, age, duration of exposure, tissue/organ, and concentration of the herbicide tested.

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.* 2011). These antioxidants scavenge free radicals to prevent oxidative damage. Superoxide dismutase and CAT systems provide a first line of defense against ROS (Nwani *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). In our study, chronic exposure to metribuzin affected SOD and CAT activity in signal crayfish. Overall, results indicated disruption of the normal oxidation process, suggesting a failure in antioxidant defense systems as indicated by SOD and CAT levels. These results concur with Stara *et al.* (2014), who found changes in SOD and CAT activity in red swamp crayfish *Procambarus clarkii* following prometryne exposure.

Glutathione reductase plays an essential role in cell defense against reactive oxygen metabolites. Glutathione reductase maintains the reduced status of glutathione, which is necessary for gluthatione peroxidase activity; hence GR regulates homeostatic oxido-reductive balance in the living cell (Djordjevic *et al.* 2010). In our study, we found difference from control in GR activity in liver after 10 and 30 days exposure to metribuzin. Generally, elevated GR activity reflects the oxidation of reduced glutathione, which is converted to glutathione, the substrate of GR activity (Elia *et al.* 2006). Stara *et al.* (2014) found significantly increased activity of GR in red swamp crayfish after prometryne exposure.

The effect of chronic exposure to low concentrations of metribuzin on histology of crayfish has not yet been investigated. In our study, crayfish exposed to metribuzin at both 0.52 µg.l^{-1} and 3.06 mg.l^{-1} demonstrated changes in hepatopancreas. The crustacean

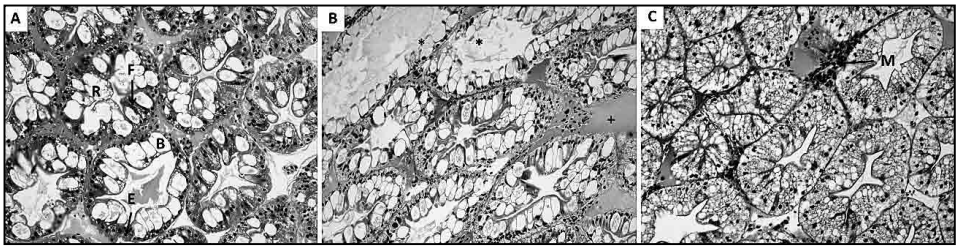


Fig. 1. Transversal sections of hepatopankreatic tubules of signal crayfish (*Pacifastacus leniusculus* Dana). A - control group; B - group exposed to 3.06 mg.l^{-1} metribuzin for 30 days; C – group examined 30 days after metribuzin exposure (depuration period) (100×). Transversal sections of tubules show four different types of cells. R (resorptive) cells are consist of multiple lipid vacuoles variable in size; B (blisterlike) cells contain one large secretory vesicle; E (embryonic) cells are undifferentiated precursors of other cell types typically located in the distal tip; F (fibrillar) cells have basophilic cytoplasm with large amounts of ribosomes and endoplasmic reticulum. Asterisks (*) mark autolytic disintegration of tubular epithelium; small cross (+) marks visible interstitial edema; and letter (M) marks mononuclear cells.

hepatopancreas is the main organ for the detoxification of pollutants. Similar pathological changes in hepatopancreas were reported in red swamp crayfish after exposure to insecticides (Heiba 1999; Desouky *et al.* 2013). On the other hand, Stara *et al.* (2014) observed no pathological changes in hepatopancreas of adult red swamp crayfish with prometryne exposure. Observed changes were probably due to accumulation of the metribuzin in the cells of the hepatopancreas or to increasing activity of lysosomal enzymes, which are capable of destroying cell organelles.

CONCLUSION

This is the first report of the chronic effects of metribuzin on oxidative stress, antioxidant enzymes and histology in crayfish. The present study demonstrated difference in oxidative stress and antioxidant defence systems in tissues, as well as pathological changes in hepatopancreas, following long-term exposure to metribuzin. Our long-term toxicity test demonstrates that metribuzin can cause differences in crayfish metabolism and disturb homeostasis even at the environmental concentrations. The information presented in this study aids in understanding the mechanisms of metribuzin's effect on this animal group. Indices applied in this study may potentially be used as indicators in monitoring residual metribuzin in the aquatic environment.

ACKNOWLEDGMENTS

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects „CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 018/2014/Z). We would like to thank the Lucidus Consultancy for manuscript improvement and English correction.

REFERENCES

1 Barcelo D, Hennion MC (1997). Trace determination of pesticides and their degradation products in water, mass spectrometric methods, LC–MS. Elsevier, Amsterdam, pp. 225–234.
2 Beers RF, Sizer IW (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem. **195**: 133–140.
3 Bradford MM (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye binding. Anal Biochem. **72**: 248–54.
4 Carlberg I, Mannervik B (1975). Purification and characterization of flavoenzyme glutathione reductase from rat liver. J Biol Chem. **250**: 5475–80.
5 Djordjevic J, Djordjevic A, Adzic M, Niciforovic AM, Radojcic B (2010). Chronic stress differentially affects antioxidant enzymes and modifies the acute stress response in liver of wistar rats. Physiol Res. **59**: 729–36.
6 Desouky MMA, Abdel-Gawad H, Hegazi B (2013). Distribution, fate and histopathological effects of ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. Food Chem Toxicol. **52**: 42–52.
7 Elia AC, Anastasi V, Dorr AJM (2006). Hepatic antioxidant enzymes and total glutathione of *Cyprinus carpio* exposed to three disinfectants, chlorine dioxide, sodium hypochlorite and peracetic acid, for superficial water potabilization. Chemosphere **64**: 1633–41.
8 Fairchild JF, Sappington LC (2002). Fate and effects of the triazinone herbicide metribuzin in experimental pond mesocosms. Arch Environ Contam Toxicol. **43**: 198–202.
9 Heiba FN (1999). Effect of the insecticide diazinon on the hepatopancreas of the freshwater crayfish, *Procambarus clarkii*. Egypt J Aquat Biol Fish. **3**: 197–213.
10 Chapadense PFG, Castro FJ, Almeina JA, Moron SE (2009). Toxicity of atrazine herbicide in *Colossoma macropomum*. Rev Bras Saúde Prod Anim. **10**: 398–405.
11 Kouba A, Kuklina I, Niksirat H, Machova J, Kozak P (2012). Tolerance of signal crayfish (*Pacifastacus leniusculus*) to Persteril 36 supports use of peracetic acid in astaciculture. Aquacult. **350–353**: 71–74.
12 Kouba A, Petrusek A, Kozak P (2014). Continental-wide distribution of crayfish species in Europe: update and maps. Knowl. Managt. Aquat. Ecosyst. **413**: 5.
13 Kozak P, Fureder L, Kouba A, Reynolds J, Souty-Grosset C (2011). Current conservation strategies for European crayfish. Knowl. Managt. Aquat. Ecosyst. **401**: 1.
14 Lushchak VI, Bagnyukova TV, Husak VV, Luzhna LI, Lushchak OV, Storey KB (2005). Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int J Biochem Cell Biol. **37**: 1670–1680.
15 Marklund S, Marklund G (1974). Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. **47**: 469–474.
16 Menezes CC, Loro VL, Fonseca MB, Cattaneo R, Pretto A, Miron DS, Santi A (2011). Oxidative parameters of *Rhamdia quelen* in response to commercial herbicide containing clomazone and recovery pattern. Pest Biochem Physiol. **100**: 145–150.
17 Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, Srivastava SK (2010). Toxicity of the herbicide atrazine: Effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa Punctatus* (Bloch). Int J Environ Res Public Health. **7**: 3298–3312.
18 Oakes KD, van der Kraak GJ (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat Toxicol. **63**: 447–463.
19 Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006). The challenge of micropollutants in aquatic systems. Science **313**: 1072–1077.
20 Slaninova A, Smutna M, Modra H, Svobodova Z (2009). Oxidative stress in fish induced by pesticides. Neuroendocrinol Lett. **30**: 2–12.
21 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 Res Int. **2014**: Article ID 680131.
22 Stara A, Steinbach Ch, Wlasow T, Gomulka P, Ziemok E, Machova J, Velisek J (2013). Effect of zeta-cypermethrin on common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **34**(Suppl. 2): 37–42.
23 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.). Neuroendocrinol Lett. **33**(Suppl. 3): 130–135.
24 Velisek J, Kouba A, Stara A (2013). Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*). Neuroendocrinology Lett. **34**(Suppl. 2): 31–36.
25 Velisek J, Stara A, Machova J, Dvorak P, Zuskova E, Svobodova Z (2012). Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **33**(Suppl. 3): 90–95.
26 Zhang JF, Shen H, Wang XR, Wu JC, Xue YQ (2004). Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere **55**: 167–174.

CHAPTER 10

GENERAL DISCUSSION

Triazines are most frequently detected pollutants in aquatic environment (Kodesova et al., 2011; Bottoni et al., 2013; Bonansea et al., 2013). As a consequence, residual amounts of triazines and their metabolites have been found in drinking water and foods (Van der Oost et al., 2003), increasing concern for the possible threats to human health posed by exposure to these pollutants (Baynes and Riviere, 2009; Boffetta et al., 2013; Kumar et al., 2013). Moreover, some of triazine pesticides are prohibited in European country. S-triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the European Community and they are included in the EU Priority Pollutants List and the U.S. EPA List. There is no data on the long-term exposure of triazines and their metabolites to early life stage of crayfish and fish at environmentally realistic concentrations. Newly hatched larvae constitute a particularly critical and sensitive life stage, because at hatching the embryos lose their protective membrane and are fully exposed to xenobiotics.

Behaviour

Changes in behavioural (for example, social interactions, and dominance hierarchies) after xenobiotics exposure can have serious implications for organisms (Shinn et al., 2015). Triazines alters chemical perception of natural substances and critical olfactory-mediated behaviours in crayfish (Belanger et al., 2015). We observed no behaviour (moving and food intake) changes in tests with all tested triazines and their metabolites. Our result agree with studies showing no differences in behaviour of early life stages, juveniles and adult of fish and crayfish following exposure to triazines or their metabolites at environmentally relevant concentrations (Stara et al., 2012, 2014, 2016). Velisek et al. (2013) reported behaviour changes as increased of moving backward, lost equilibrium, loss of claws and walking legs in juvenile signal crayfish (*Pacifastacus leniusculus* Dana) after acute exposure (higher than 1 mg/L) to triazine (atrazine, hexazinone, metribuzine, prometryne, simazine, and terbutryne). These results indicate that behaviour is impaired by only exposure to acute, but not by environmentally relevant levels of triazine or their metabolites, but some studies on mammals showed that exposures to xenobiotic during early ontogenesis result in behavioural alterations in adult (Weis, 2014).

Growth

Growth can be routinely measured in all early life ecotoxicological tests. Growth is generally considered a more sensitive parameter than mortality (Bengtsson, 1974), however, this difference in sensitivity to xenobiotics can be species-specific (Woltering, 1984). We observed important differences in growth in tests on early life stages of carp after longer exposure of prometryne (Velisek et al., 2015a), terbuthylazine (Velisek et al., 2015b), terbuthylazine-desethyl (Velisek et al., 2016a), tebuthylazine-2-hydroxy (Velisek et al., 2014a) and in test on early life stages of marble crayfish after longer exposure of tebuthylazine-2-hydroxy (Koutnik et al., 2017). The impact of reductions of growth may be the delay of reproduction as well as increased susceptibility of young carp or crayfish to predation and disease. Their ability to obtain food and to compete for suitable habitats might also be reduced.The results presented here are also in agreement with earlier studies on fish and crayfish exposed to triazines and their metabolite (Velisek et al., 2012a, b). Plhalova et al. (2009) reported growth reduction of zebrafish (*Danio rerio*) after 28 days of exposure to terbutryne. Erickson and Turner (2002) reported adverse effects on growth of fathead minnows (*Pimephales promelas*) exposed to prometryne.

Ontogenetic development

The early developing of fish and crayfish have been shown to be especially sensitive indicators of xenobiotics. A chemically induced adverse effect on embryonic stages is based on developmental events, for example, organogenesis (Pickering and Lazorchak, 1995; Hallare et al., 2005). In embryolarval toxicity tests, delayed of early development is commonly found after exposure to pesticides (Woltering, 1984; Velisek et al., 2016b). Data from the literature indicate that in embryolarval toxicity tests with aquatic organisms early development is sensitive parameter for evaluation of impacts of xenobiotics. We observed delay in early ontogenetic development in carp after longer exposure of prometryne (Velisek et al., 2015a), terbuthylazine (Velisek et al., 2015b), terbuthylazine-desethyl (Velisek et al., 2016a), tebuthylazine-2-hydroxy (Velisek et al., 2014a) and in test on early life stages of marble crayfish after longer exposure of prometryne (Velisek et al., 2014b) and tebuthylazine-2-hydroxy (Koutnik et al., 2017), but these dealy in early ontogenetic development were only in highest concentrations of triazines. These findings are in accord with other studies which describe delay of ontogenetic development in common carp after exposure to terbuthylazine (Stepanova et al., 2012), and terbutryne (Velisek et al., 2012a). Early development of carp and crayfish is not a sensitive biomarker for monitoring of triazines and their metabolites in aquatic environment.

Histopathology

Triazines has been reported to be associated with damage to aquatic organism's kidney structure (Gunkel, 1981; Neskovic et al., 2003; Velisek et al., 2009, 2010, 2011). We observed in our study histopathological changes of gills and hepatopancreas of crayfish and carp. Gills are a vital organ as they play an important role in diffusion and transport of respiratory gases and regulation of osmotic and ionic balance. Gills are a primary target organ for most pollutant, uptake from water is the most important route, and may be one of the first organs to exhibit symptoms of toxicity (Desouky et al., 2013). Changes in our studies caused of tested triaznes and their metabolites in gills may affect osmoregulatory gills function and gases exchange. The hepatopancreas of aquatic organisms is likely the main organ for the detoxification of pollutants (Vogt, 2002) and produces and secretes all digestive enzymes involved in carbohydrate metabolism, production of emulsifiers, excretion, and calcium, and heavy metal storage (Holdich and Reeve, 1988). Changes of hepatopancreas in our experiments are probably due to accumulation of the triaznes and their metabolites in the cells of hepatopancreas or due to increasing the activity of lysosomal enzymes which are capable of destroying cell organelles. Observed pathological changes are in accord with those described in Desouky et al. (2013) in red swamp crayfish (*Procambarus clarkii*) gills after exposure of ethion. Histopathological tissue changes in cranial kidney were similar to the changes found in fish by other authors (Fischer-Scherl et al., 1991; Arufe et al., 2004; Oropesa et al., 2009; Velisek et al., 2012a, 2014b, 2015a). The histopathology of fish and crayfish a sensitive biomarker for monitoring of triazine and their metabolites in aquatic environment.

Oxidative stress and antioxidant parameters

Xenobiotics can affect many physiological and biochemical processes in tissues (Durmaz et al., 2006), leading to the production of reactive oxygen species (Stara et al., 2016). The antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase) scavenge free radicals to prevent oxidative damage (Menezes et al., 2011). Superoxide dismutase and catalase systems provide a first line of defense against reactive oxygen species (Nwani 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). In my studies, I found changes in antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase) for exposure of the tested triazines and theit metabolites at environmentally realistic exposures. Overall, results indicated disruption of the normal oxidation process, suggesting a failure in antioxidant defense systems. These results concur with Stara et al. (2014), who found changes in superoxide dismutase and catalase activity in red swamp crayfish (*Procambarus clarkii*) following prometryne exposure. Hostovsky et al. (2014) also confirmed changes in antioxidant enzymes in fish exposed to triazine herbicides, including terbuthylazine. Antioxidant defence enzymes and oxidative damage appear to be reliable biomarker of xenobiotic to non-target species.

Conclusion

Triazines and their metabolites are most frequently detected pesticides in aquatic environment. S-triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and they are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. Aqautic organisms are widely used as biological monitors of xenobiotics. The present dissertation is a contribution to the assessment of effects of triazines and their metabolites on early life stages of common carp (*Cyprinus carpio* L.) and marbled crayfish (*Procambarus fallax* f. *virginalis*). The common carp was selected as a model fish due to its worldwide economic importance. The decapod crustaceans belong to the most conspicuous aquatic invertebrates, often playing ecologically and economically important roles. These species, typically crayfish, fulfil criteria described for bioindicators. Crayfish are considered as keystone species due their general ecological importance and their role as productive ecosystem engineers in the aquatic ecosystems. They can be usually easily identified, their populations can be abundant and widespread, having small home range, hence migrations do not influence the level of xenobiotic accumulated in their tissues. Specimens are therefore representatives of the locations in which they occur. They are easily captured in natural conditions as well as cultured and the body size usually provides sufficient amount of tissues for individual biochemical and chemical analyses. Another advantage of using invertebrates and early life stages of fish as bioindicators is fulfilling of 3R (Replace, Reduce, Refine) concept.

The our results of studies provide data on chronic exposure to triazines and their metabolites for consideration in risk assessment. For testing we selected three active substances of triazines (prometryne, terbuthylazine and metribuzine) and their metabolites (terbuthylazine-desethyl and tebuthylazine-2-hydroxy), which are most frequently detected in surface waters. The findings contribute to knowledge of the toxic potential of triazine herbicides and their metabolites to common carp and crayfish at real concentrations in the Czech rivers.

REFERENCES

Arufe, M.I., Arellano, J., Moreno, M.J., Sarasquetec, C., 2004. Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. Ecotoxicology and Environmental Safety 59: 209–216.

Baynes, R.E., Riviere, J.E., 2009. Risks Associated with melamine and related triazine contamination of food. Emerging Health Threats Journal 3: 314–345.

Belanger, R.M., Peters, T.J., Sabhapathy, G.S., Khan, S., Katta, J., Abraham, N.K., 2015. Atrazine exposure affects the ability of crayfish (*Orconectes rusticus*) to localize a food odor source. Archives of Environmental Contamination and Toxicology 68: 636–645.

Bengtsson, B.E., 1974. Effect of zinc on growth of the minnow *Phoxinus phoxinus*. Oikos 25: 370–373.

Boffetta, P., Colin, B., Mandel, J.S., 2013. Atrazine and cancer: a review of the epidemiologic evidence. European Journal of Cancer Prevention 22: 169–180.

Bonansea, R.I., Ame, M.V., Wunderlin, D.A., 2013. Determination of priority pesticides in water samples combining SPE and SPME coupled to GC-MS. A case study: Suquia River basin (Argentina). Chemosphere 90: 1860–1869.

Bottoni, P., Grenni, P., Caracciolo, A.B., 2013. Terbuthylazine and other triazines in Italian water resources. Microchemical Journal 107: 136–142.

Desouky, M.M.A., Abdel-Gawad, H., Hegazi, B., 2013. Distribution, fate and histopathological effects of ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. Food Chemistry and Toxicology 52: 42–52.

Durmaz, H., Sevgiler, Y., Uner, N., 2006. Tissue-specific antioxidative and neurotoxic responses to diazinon in *Oreochromis niloticus*. Pestide Biochemistry and Physiology 84: 215–226.

Erickson, W., Turner, L., 2002. Prometryn analysis of risks to endangered and threatened salmon and steelhead. Environmental Field Branch, Office of Pesticide Programs 368, p. 71.

Fischer-Scherl, T., Veseer, A., Hoffman, R.W., Kuhnhauser, C., Negele, R.D., Ewringmann, T., 1991. Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*). Archives of Environmental Contamination and Toxicology 20: 454–461.

Gunkel, V.G., 1981. Bioaccumulation of a herbicide (atrazine, s-triazine) in the whitefish (*Coregonusfera* J.): uptake and distribution of the residue in fish. Archiv für Hydrobiologie, Supplement 59: 252–287.

Hallare, A.V., Schirling, M., Luckenbach, T., Kohler, H.R., Triebskorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. Journal of Thermal Biology 30: 7–17.

Holdich, D.M., Reeve, I.D., 1988. Functional morphology and anatomy. In: Holdich, D.M., Lowry, R.S., (Eds.), Freshwater Crayfish: Biology, Management and Exploitation. Croom Helm, London, UK, pp. 34–51.

Hostovsky, M., Blahova, J., Plhalova, L., Kopriva, V., Svobodova, Z., 2014. Effects of the exposure of fish to triazine herbicides. Neuroendocrinology Letter 35 (Suppl. 2): 3–25.

Kodesova, R., Kocarek, M., Kodes, V., Drabek, O., Kozak, J., Hejtmankova, K., 2011. Pesticide adsorption in relation to soil properties and soil type distribution in regional scale. Journal of Hazardous Materials 186: 540–550.

Koutnik, D., Stara, A., Zuskova E., Kouba, A., Velisek, J., 2017. The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Pesticide Biochemistry and Physiology, 136: 29–33.

Kumar, S., Bhat, H.R., Kumawat, M.K., Singh, U.P., 2013. Design and one-pot synthesis of hybrid thiazolidin-4-one-1,3,5-triazines as potent antibacterial agents against human disease-causing pathogens. New Journal of Chemistry 37: 581–584.

Menezes, C.C., Loro, V.L., Fonseca, M.B., Cattaneo, R., Pretto, A., Miron, D.S., Santi, A., 2011. Oxidative parameters of *Rhamdia quelen* in response to commercial herbicide containing clomazone and recovery pattern. Pesticide Biochemistry and Physiology 100: 145–150.

Neskovic, N.K., Elezovic, I., Karan, V., Poleksic, V., Budimir, M., 1993. Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.). Ecotoxicology and Environmental Safety 25: 173–182.

Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B., Srivastava, S.K., 2010. Toxicity of the herbicide atrazine: Effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa Punctatus* (Bloch). International Journal of Environmental Research and Public Health 7: 3298–3312.

Oropesa, A.L., Garcia-Cambero, J.P., Gomez, L., Roncero, V., Soler, F., 2009. Effect of longterm exposure to simazine on histopathology, hematological, and biochemical parameters in *Cyprinus carpio*. Environmental Toxicology 24: 187–199.

Pickering, Q.H., Lazorchak, J.M., 1995. Evaluation of the robustness of the fathead minnow, *Pimephales promelas*, larval survival and growth test, U.S. EPA method 1000.0. Environmental Toxicology and Chemistry 14: 653–659.

Plhalova, L., Macova, S., Haluzova, I., Slaninova, A., Dolezelova, P., Marsalek, P., et al., 2009. Terbutryn toxicity to *Danio rerio*: Effects of subchronic exposure on fish growth. Neuroendocrinology Letter 30 (Suppl. 1): 242–365.

Shinn, C., Santos, M.M., Lek, S., Grenouillet, G., 2015. Behavioral response of juvenile rainbow trout exposed to an herbicide mixture. Ecotoxicology and Environmetnal Safety 12: 15–21.

Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2016. Effects of terbuthylazine-desethyl, a terbuthylazine degradation product, on red swamp crayfish (*Procambarus clarkii*). Science of the Total Environment 566/567: 733–740.

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 2014: Article ID 680131.

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 Letter 33 (Suppl. 3): 130–135.

Stepanova, S., Plhalova, L., Dolezelova, P., Prokes, M., Marsalek, P., Skoric, M., et al., 2012. The effects of subchronic exposure to terbuthylazine on early developmental stages of common carp. Science World Journal 2012: Article ID 615920.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13: 57–149.

Velisek, J., Koutnik, D., Zuskova, E., Stara, A., 2016a. Effects of the terbuthylazine metabolite terbuthylazine-desethyl on common carp embryos and larvae. Science of the Total Environment 539: 214–220.

Velisek, J., Stara, A, Zuskova, E., 2016b. Effect of single and combination of three triazine metabolites at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Environmental Science and Pollution Research 23 (23): 24289–24297.

Velisek, J., Stara, A., Koutnik, D., Machova, J., 2015a. Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.). Pesticide Biochemistry and Physiology 118: 58–63.

Velisek, J., Stara, A., Koutnik, D., Zuskova, E., 2015b. Effects of terbuthylazine on early life stages of common carp. Neuroendocrinology Letters 36 (Supll. 1): 120–125.

Velisek, J., Stara, A., Koutnik, D., Machova, J., 2014a. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed Research International 2014: Article ID 621304.

Velisek, J., Stara, A., Koutnik, D., Zuskova, E., Kouba, A., 2014b. Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Neuroendocrinology Letter 35 (Suppl. 2): 93–98.

Velisek, J., Kouba, A., Stara, A., 2013. Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*). Neuroendocrinology Letter 34 (Suppl. 2): 31–36.

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

Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Z. Svobodova, Z., 2012b. Simazin toxicity in environmental concentration on early life stages of common carp (*Cyprinus carpio*L.), Neuroendocrinology Letter 33 (Suppl. 3): 90–95.

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

Velisek, J., Sudova, E., Machova, J., Svobodova, Z., 2010. Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). Ecotoxicology and Environmental Safety 73: 384–390.

Velisek, J., Svobodova, Z., Piackova, V., Sudova, E., 2009. Effects of acute exposure of metribuzin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Bulletin of Environmental Contamination and Toxicology 82: 492–495.

Vogt, G., 2002. Functional anatomy. In: Holdich, D.M., (Ed), Biology of Freshwater Crayfish. Blackwell, Oxford, pp. 53–151.

Weis, J.S., 2014. Delayed behavioral effects of early life toxicant exposures in aquatic biota. Toxics 2: 165–187.

Woltering, D., 1984. The growth response in fish chronic and early life stage toxicity tests: A critical review. Aquatic Toxicology 5: 1–21.

Zhang, J.F., Shen, H., Wang, X.R., Wu, J.C., Xue, Y.Q., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere 55: 167–174.

ENGLISH SUMMARY

The effect of triazine based pesticides and their metabolites on no-target aquatic organisms

Dalibor Koutnik

The aquatic environment continues to be under threat by the use of pesticides, resulting in high risk to non-target organisms. Pesticides used in agro-ecosystems and forests enter aquatic environments such as streams, rivers, and lakes if applied in adjacent areas or if an accidental spill occurs. Such pesticides are carried into aquatic environments by surface runoff fromsites of application and can negatively affect the health of aquatic organisms. Triazines and their metabolites are frequently detected in rivers, due to persistency, water solubility, and mobility. Metabolites of triazines are more polar than the parent substance and thus pose a greater potential risk for aquatic animals. Suitable example is atrazine and its metabolites.

Therefore, the main goal of our thesis was to observe the impact of triaiznes and their matabolites on non-target aquatic organisms. For the studies, we chose the early life stages of common carp (*Cyprinus carpio* L.) and marbled crayfish (*Procambarus fallax* f. *virginalis*). In selected organisms, we observed long-term effect of triazine herbicides (prometryne, terbuthylazine, metribuzine) and their metabolites (terbuthylazine-desethyl and tebuthylazine-2-hydroxy) in concentrations commonly occurring in Czech rivers. Effects of selscted substances have been assessed through observation of behavior, growth, ontogenetic development, histopathological examination and antioxidant parameters and oxidative tissue damage on fish and crayfish.

The first part is about monitoring the effects of triazines (prometryne, terbuthylazine) on the early development stages of carp and marble crayfish. Subchronic prometryne exposure of early-life stages of common carp at concentrations of 1 200 and 4 000 μg/L affected their survival, growth rate, early ontogeny, and histology. Terbuthylazine in concentration 1 400 and 3 000 μg/L caused significant decrease of mass, total length, delayed in development and cause of alternation of tubular system of caudal kidney of carp. Prometryne in concentration 144, 1 444 and 4 320 μg/L caused decrease of weight, specific growth rates and caused histopathological changes in gill of crayfish. Moreover concentration 4 320 μg/L of prometryne caused delay in ontogenetic development of crafysh.

Second part of the work has included effects of low concentrations of metabolites (terbuthylazine-desethyl and tebuthylazine-2-hydroxy) on the early development stages of carp and marble crayfish. Chronic terbuthylazine-desethyl exposure in concetrations 180, 900, and 1 800 μg/L affected growth, ontogenetic development, and the antioxidant system and caused pathological changes in the caudal kidney of early life stages of carp. Chronic terbuthylazine-2-hydroxy exposure in concetrations 2.9, 70, 1 400 and 3 500 μg/L caused decreased of total superoxide dismutase activity of early life stages of carp. Moreover concentration 1 400 and 3 500 μg/L of terbuthylazine-2-hydroxy caused delay in ontogenetic development and pathological changes in the caudal kidney of carp. Chronic terbuthylazine-2-hydroxy exposure in concentrations up 75 μg/l affected growth, ontogenetic development, antioxidant system, caused oxidative stress and pathological changes in hepatopancreas of early life stages of marbled crayfish.

The last part of our study examined the effect of metribuzine on signal crayfish (*Pacifastacus leniusculus* Dana). Crayfish were exposed to metribuzine concentrations of 0.52 μg/L and 3.06 mg/L for 30 days and a 30-day depuration period without exposure to metribuzin. In the thiobarbituric acid reactive substances, superoxide dismutase, and catalase were observed differences in all examined tissues (gill, muscle, hepatopankreas) compared to the

control group. Differences from control were observed in glutathione reductase activity in hepatopancreas after 10 days for both exposure concentrations and after 30 days at 3.06 mg/L. Histological examination revealed extensive focal autolytic disintegration of tubular epithelium in hepatopancreas of crayfish exposed to metribuzin.

These studies provided important results for the evaluation of long-term impact and effect of (prometryne, terbuthylazine and metribuzine) and their metabolites (terbuthylazine-desethyl and tebuthylazine-2-hydroxy) on the water non-target organisms, even in real concentrations founded in surface waters. Effects of triazines and their metabolites were observed on early life stafes of 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 xenobiotics, the effects of which can be potentiated with combined exposures.

CZECH SUMMARY

Vliv triazinových pesticidů a jejich metabolitů na necílové vodní organizmy

Dalibor Koutník

Vodní prostředí je stále ohroženo použitím pesticidů, což vede k vysokému riziku pro necílové vodní organizmy. Pesticidy používané v zemědělství a lesnictví vstupují do vodního prostředí, pokud se používají v přilehlých oblastech nebo pokud dojde k jejich úniku. Tyto látky se dostávají do vodního prostředí povrchovým odtokem z místa aplikace a mohou negativně ovlivnit metabolizmus vodních organizmů. Triaziny a jejich metabolity jsou často detekovány v řekách v důsledku jejich perzistence, rozpustnosti ve vodě a mobility. Metabolity triazinů jsou polárnější než původně aplikované produkty, a proto představují větší riziko pro vodní živočichy. Vhodným příkladem je atrazin a produkty jeho rozkladu.

Proto bylo hlavním cílem naší práce sledovat vliv triaiznů a jejich metabolitů na necílové vodní organizmy. Pro naše studie jsme zvolili raná vývojová stadia kapra obecného (Cyprinus carpio L.) a raka mramorovaného (Procambarus fallax f. virginalis). U zvolených organizmů jsme sledovali dlouhodobý vliv triazinů (prometrynu, terbutylazinu, metribuzinu) a jejich metabolitů (terbuthylazin-desethyl a tebuthylazinu-2-hydroxy) v koncentracích běžně se vyskytujících v povrchových vodách České republiky. Vliv vybraných látek na ryby a raky jsme hodnotili pomocí sledování chování, růstu, ontogenetického vývoje, histopatologického vyšetření, antioxidačních parametrů a oxidačního poškození tkání.

První část práce se zabývala sledováním vlivu triazinů (prometrynu, terbuthylazinu) na raná vývojová stadia kapra a raka mramorovaného. Subchronická expozice raných vývojových stadií kapra prometrynu v koncentracích 1200 a 4000 μg/L způsobila snížení růstu, zpoždění ontogenetického vývoje a histopatologické změny v tkáních. Expozice terbuthylazinu v koncentracích 1400 a 3000 μg/L způsobila významné snížení hmotnosti, celkové délky těla, zpoždění vývoje a alternaci tubulárního systému kaudální ledviny kapra. Prometryn v koncentracích 144, 1444 a 4320 μg/L způsobil snížení hmotnosti, specifické rychlosti růstu a histopatologické změny v žábrech raků. Navíc prometryn v koncentraci 4320 μg/L způsobil zpoždění ontogenetického vývoje.

Druhá část práce se zabývala vlivem nízkých koncentrací triazinových metabolitů (terbuthylazin-desethylu a tebuhylazin-2-hydroxy) na raná vývojová stadia kaprů a raků mramorovaných. Chronická expozice terbuthylazin-udesethylu v koncentracích 180, 900 a 1 800 μg/L způsobila snížení růstu, zpomalení ontogenetického vývoje a poškození antioxidačního systému a rovněž způsobila patologické změny v kaudální ledvině raných vývojových stadií u kaprů. Chronická expozice terbuthylazinu-2-hydroxy v koncentracích 2, 9, 70, 1400 a 3500 μg/L zapříčinila snížení celkové aktivity superoxide dismutázy raných vývojových stadií kaprů. Mimo to koncentrace 1400 a 3500 μg/L terbuthylazinu-2-hydroxy způsobily zpoždění ontogenetického vývoje a patologické změny v kaudální ledvině kaprů. Chronická expozice terbuthylazinu-2-hydroxy v koncentracích 75 μg/L a vyšších ovlivnila růst, ontogenetický vývoj, antioxidační systémy a způsobila oxidační stres a patologické změny hepatopankreatu raných vývojových stadií raků.

Poslední část naší studie se zabývala dlouhodobým vlivem metribuzinu na raka signálního (*Pacifastacus leniusculus* Dana). Raci byli vystaveni koncentraci metribuzinu 0,52 μg/L a 3,06 mg/L po dobu 30 dnů a 30 denní fázi depurace (bez expozice metribuzinu). Statisticky významné rozdíly byly zjištěny ve všech tkáních (žábry, sval, hepatopankreas) v reaktivních látkách kyseliny thiobarbiturové, v aktivitě superoxid dismutázy a katalázy ve srovnání s kontrolní skupinou. Statisticky významné rozdíly byly zjištěny v aktivitě glutation reduktázy

v hepatopankreatu po 10 dnech v obou testovaných koncentracích a po 30 dnech v koncentraci 3,06 mg/L. Metribuzin způsobil rozsáhlou fokální autolytickou degeneraci tubulárního epitelu hepatopankreatu raků vystavených metribuzinu.

Výsledky této práce shrnují a poskytují důležité informace o hodnocení dlouhodobého dopadu a vlivu triazinů (prometrynu, terbuthylainu a metribuzinu) a jejich metabolitů (terbuthylazin-desethyl a tebuthylazinu-2-hydroxy) na vodní prostředí, v koncentracích běžně se vyskytujících v povrchových vodách. Účinky triazinů a jejich metabolitů na raná vývojová stadia kaprů a raků byly hodnoceny pomocí vybraných parametrů. Tyto parametry se zdají být dobrými indikátory při hodnocení poškození necílových vodních organizmů a vodního prostředí. Nicméně vodní prostředí je znečištěno mnoha látkami a jejich souhrnný společný toxický vliv může být rozdílný.

ACKNOWLEDGEMENTS

To my heartfelt thanks belongs to all my collegues who contributed to the creation of this work and to develop of my personality skills.

However, my thanks belong especially to my supervisor´s Assoc. Prof. Josef Velíšek 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 from the Laborotory of Aquatic Toxicology and Ichthyopathology, Laboratory of Environmental Chemistry and Biochemistry and other collegues of the Faculty of Fisheries and Protection of Waters and from the Research Institute of Fish Culture and Hydrobiology in Vodňany. I also deeply appreciate the assistance of the Lucidus Consultancy for English correction.

The Ph.D. work was supported by the following financial source:

- Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No CZ.1.05/2.1.00/01.0024.
- Ministry of Education, Youth and Sports of the Czech Republic "CENAKVA II" (No. LO1205 under the NPU I program)
- The Grant Agency of the University of South Bohemia No. 018/2014/Z.
- The Grant Agency of the University of South Bohemia No. 016/2016/Z.

LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Koutnik, D., Stara, A., Zuskova E., Kouba, A., Velisek, J., 2017. The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Pesticide Biochemistry and Physiology 136: 29–33. (IF 2016 = 2.590)

Velisek, J., **Koutnik, D.,** Zuskova, E., Stara, A., 2016. Effects of the terbuthylazine metabolite terbuthylazine-desethyl on common carp embryos and larvae. Science of the Total Environment 539: 214–220. (IF 2015 = 3.976)

Koutnik, D., Stara, A., Velisek, J., 2015. The effect of selected triazine on fish: a review. Slovenian Veterinary Research 52: 107–131. (IF 2014 = 0.222)

Velisek, J., Stara, A., **Koutnik, D.,** Machova, J., 2015. Effect of prometryne on early life stages of common carp (*Cyprinus carpio* L.). Pesticide Biochemistry and Physiology 118: 58–63. (IF 2014 = 2.014)

Velisek, J., Stara, A., **Koutnik, D.,** Zuskova, E., 2015. Effects of terbuthylazine on early life stages of common carp. Neuroendocrinology Letters 36: 120–125. (IF 2014 = 0.799)

Koutnik, D., Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2014. The effect of long-term metribuzine exposure to signal crayfish (*Pacifastacus leniusculus* Dana). Neuroendocrinology Letters 35: 51–56. (IF 2013 = 0.935)

Velisek, J., Stara, A., **Koutnik, D.,** Machova, J., 2014. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). BioMed Research International, Article ID 621304. (IF 2013 = 2.706).

Velisek, J., Stara, A., **Koutnik, D.,** Zuskova, E., Kouba, A., 2014. Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). Neuroendocrinology Letters 35: 93–98. (IF 2013 = 0.935)

International abstracts and conference proceedings

Velíšek, J., Stará, A., **Koutnik, D.,** Zusková, E., 2015. Effects of terbuthylazine on early life stages of common carp (*Cyprinus carpio* L.). In: Book of abstracts. 20th Interdisciplinary Toxicology Conference TOXCON 2015. 27–29 May 2015, Brno, Czech Republic, p. 125.

Koutnik, D., Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2014. Effect of chronick exposure to meteribuzine on signal crayfish (*Pacifastacus Leniusculus*). In: Abstracts book of the 19th Interdisciplinary Toxicological Conference TOXCON. 24–26. September 2014, Stara Lesná, Slovensko, Interdisciplinary Toxicology 7 (Supll. 1): 54.

Velisek, J., Lidova, J., Stara, A., **Koutnik, D.,** Machova, J., 2014. Effects of prometryne on early life stages of common. In: Abstracts of the 50th Congress of the European Societies of Toxicology (EUROTOX 2014). 7–10. September 2014, Edimburg, Scotland, UK, Toxicology Letters 229: S117.

National abstracts and conference proceedings

Koutník, D., Stará, A., Lidová, J., Zusková, E., Kouba, A., Velíšek, J., 2015. Vliv metribuzinu na raka signálního (*Pacifastacus leniusculus* Dana 1852). In: Sborník abstraktů XVI. Toxikologická konference – Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí, 26–28.8. 2015, Vodňany, s. 20.

Velíšek, J., Zusková, E., Stará, A., **Koutník, D.,** Kolářová, J., 2015. Využití pesticidů na bázi pyrethroidů k léčení parazitárních onemocnění kapra obecného. Ochrana zdraví ryb 2015, 1.–2.4. 2015, Vodňany, Sborník abstraktů, s. 62–65.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Dalibor Koutník
Research department	2013–2018 Laboratory of Aquatic Toxicology and Ichthyopathology
Daily supervisor	Assoc. Prof. Velíšek Josef
Supervisor	Assoc. Prof. Velíšek Josef
Period	1st October 2013 until September 2018

Ph.D. courses	**Year**
Pond aquacultures	2014
Applied hydrobiology	2014
Basic of scientific communication	2014
Ichthyology and systematics of fish	2014
English language	2018

Scientific seminars	**Year**
Seminar days of RIFCH	2014 2015 2016

International conferences	**Year**
Koutnik, D., Stara, A., Zuskova, E., Kouba, A., Velisek, J., 2014. Effect of chronick exposure to meteribuzine on signal crayfish (Pacifastacus Leniusculus). In: Abstracts book of the 19th Interdisciplinary Toxicological Conference TOXCON. 24–26. September 2014, Stara Lesná, Slovensko, Interdisciplinary Toxicology 7: 54. (Poster presentation)	2014

Foreign stays during Ph.D.	**Year**
Prof.dr.sc. Anđelko Opačak, Josip Juraj Strossmayer University of Osijek, Faculty of Agriculture, department of Ichthyology, Croatia (2 month)	2014
Dr. Martin Oberle, Institut für Fischerei, Bayerische Landesanstalt für Landwirtschaft, Greiendorfer Weg, Höchstadt an der Aisch, Germany (1 month)	2015

Summer school students supervision	**Year**
Filipa Beça Effects of acute toxicity of triazines on crayfish	2016

CURRICULUM VITAE

PERSONAL INFORMATION

Surname: Koutnik
First name: Dalibor
Title: Dipl.-Ing.
Born: 5.1.1984, Jindřichův Hradec, Czech republic
Nationality: Czech
Contact: dkoutnik@frov.jcu.cz

PRESENT POSITION

External Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, www.frov.jcu.cz), Research Institute of Fish Culture and Hydrobiology (RIFCH), South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA), Laboratory of Aquatic Toxicology and Ichthyopathology, Vodňany, Czech Republic.
From 2016 – present – Water Management officer, City Authority České Budějovice, Environmental Department, České Budějovice, Czech Republic.

EDUCATION

2005–2010: University of South Bohemia in České Budějovice, Agriculture Faculty, bachelor study, specialization: Fishery – Bc., Czech Republic. Bc. thesis: "Species variability of bentic organisms in Dračice river classicifed by PERLA system".

2010–2013: University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, specialization: Fishery – Dipl.-Ing. (= M.Sc. equivalent), Czech Republic. Dipl.-Ing. thesis: "Verification of success of previous noble crayfish introduction and revision of occurrence of spiny cheek crayfish in the CHKO Třeboňsko".

2014–2018: University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Watesr – Ph.D. student, Czech Republic. Ph.D. thesis: "The effect of triazine based pesticides and their metabolites on no-target aquatic organisms".

Ph.D. COURSES

Pond Aquaculture, Applied hydrobiology, Basic of scientific communication, Ichthyology and systematics of fish, English language

KNOWLEDGE OF LANGUAGES

English in a word and writing

FOREIGN STAY DURING Ph.D. STUDY

October – November 2014: Prof.dr.sc. Anđelko Opačak, Josip Juraj Strossmayer University of Osijek, Faculty of Agriculture, department of Ichthyology, Croatia

October 2015: Dr. Martin Oberle, Institut für Fischerei, Bayerische Landesanstalt für Landwirtschaft, Greiendorfer Weg, Höchstadt an der Aisch, Germany