ORIGINAL ARTICLE

Terbutryn toxicity to *Danio rerio*: Effects of subchronic exposure on fish growth

Lucie Plhalova, Stanislava Macova, Ivana Haluzova, Andrea Slaninova, Petra Dolezelova, Petr Marsalek, Vladimira Pistekova, Iveta Bedanova, Eva Voslarova, Zdenka Svobodova

Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Correspondence to: Mgr Lucie Plhalová, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1/3, 612 42 Brno, Czech Republic.
phone:+420-541 562 781, fax: +420-541 562 790, e-mail: lplhalova@vfu.cz

Submitted: 2009-07-23 Accepted: 2009-09-25 Published online: 2009-10-15

Key words: **zebrafish; terbutryn; acute toxicity; growth test**

Neuroendocrinol Lett 2009; **30**(Suppl 1): 242–247 **PMID:** 20027178 NEL300709A41

Abstract

OBJECTIVES: The aim of this study was to investigate effects of subchronic exposure to sublethal levels of terbutryn on growth and on histopathological changes in *Danio rerio*.
DESIGN: The acute toxicity tests were performed on the juvenile stage of *Danio rerio* according to OECD No. 203 Fish, Acute Toxicity Test. The juvenile growth tests were performed on *D. rerio* according to the OECD method No. 215. Fish at the age of 20 days were exposed to the terbutryn environmental concentration commonly detected in the Czech rivers (0.02 μg L^{-1}) and the range of sublethal concentrations of terbutryn (0.06, 0.2, 0.6 and 2 mg L^{-1}) for 28 days.
RESULTS: The 96hLC50 terbutryn mean value for the juvenile stage of *D. rerio* was 5.71 ± 0.46 mg L^{-1}. A significant decrease ($p < 0.01$) in the fish growth caused by terbutryn was observed in the concentration of 0.6 mg L^{-1}. The value of NOEC was 0.2 mg L^{-1} and LOEC was 0.6 mg L^{-1} of terbutryn. We found the damage to tubular system of kidneys in the concentration of 0.6 mg L^{-1} of terbutryn.
CONCLUSION: The environmental concentration of terbutryn in the Czech rivers did not have any effects on growth and on histopathological changes in *D. rerio* and this concentration is lower than determined LOEC and NOEC values of terbutryn.

INTRODUCTION

Triazine herbicides are divided into two groups, the asymmetrical triazines or triazinones, such as metribuzin, and the symmetrical triazines. The most commercially used symmetrial triazines are simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon and terbutryn (Stevens *et al.* 2001). Herbicidal activity of triazines was first discovered in 1952 by J. R. Geigy in Switzerland (Roberts *et al.* 1998).

Terbutryn (2-(*tert*-butylamino)-4-(ethylamino) -6-(methylthio)-*s*-triazine) belongs to substituted symmetrical triazines. Triazines form a group of similar herbicides used extensively in agriculture and non-agricultural use sites, primarily to control broadleaf and some grassy weeds, that have become ubiquitous contaminants of the environment (Moretti *et al.* 2002; Arufe *et al.* 2004a).

Terbutryn has been used worldwide for the control of most annual grasses and broadleaf weeds in many crops including winter cereals, potatoes, legumes, maize, sugarcane and citrus. Terbutryn is a selective systemic herbicide which acts as a photosynthesis inhibitor in the xylem and accumulates in the apical meristems. In plants it is metabolised by oxidation to 2-hydroxy derivates and by side-

Abbreviations & units

DMSO	dimethyl sulfoxide
GC/IT-MS	gas chromatography with ion trap mass spectrometry
NOEC	no observed effect concentration
LOEC	lowest observed effect concentration

chain de-alkylation (Roberts *et al.* 1998). Terbutryn is also used in aquatic environment as an aquatic herbicide for the control of algae, submerged and free-floating weeds and may affect many non-target organisms there (Muir, 1980; Roberts *et al.* 1998; Arufe *et al.* 2004a). Terbutryn was not only detected in surface and ground waters (Carabias-Martínez *et al.* 2003), but also it was encountered in some marine waters (in Mediterranean coastal waters) (Tolosa *et al.* 1996).

The application of terbutryn has been banned in many countries because it has the potential to bioaccumulate in organisms, but it has been still detected in water environment (Rioboo *et al.* 2007). Quednow & Puttmann (2007) reported the highest concentration of terbutryn (5.6 μg L^{-1}) in rivers in Germany. The preparations containing terbutryn have not been registered in the Czech Republic since 2005, but terbutryn can be still detected in the environment. Its highest environmental concentration was found in the Czech rivers and according to the Czech Hydrometeorological Institute report it was 0.02 μg L^{-1} in 2008.

The aim of this study was to investigate the effects of subchronic exposure to environmental and sublethal levels of terbutryn on growth and on histopathological changes in *Danio rerio*.

MATERIAL AND METHODS

Experimental fish. Tests of terbutryn toxicity were performed on *Danio rerio*, which is one of the model organisms most commonly used in toxicity tests (Lawrence, 2007; Macova *et al.* 2008). The procedure complied with OECD guidelines No. 203 and 215. Experimental procedures were in compliance with the national legislation (Act No. 246/1992 Coll., on the protection of animals against cruelty, as amended and decree No. 207/2004 Coll., on the protection, breeding and use of experimental animals, as amended).

Acute toxicity test. Acute toxicity tests on the juvenile stage of *D. rerio* were performed according to OECD No. 203 Fish, Acute Toxicity Test in series of five tests. We dissolved terbutryn in the water with the help of dimethyl sulfoxide (DMSO in quantities 0.1%). In each test series we used five ascending concentrations of the tested substance (4, 5, 6, 7 and 8 mg L^{-1}) and two control groups (one control group only in dilution water and the second control group maintained in dilution water with solvent – DMSO in quantities 0.1%). Food was withheld 72 h preceding start of the test. Ten juvenile fish (aged 2-3 months, length 30 ± 5 mm, weight 0.3 ± 0.1 g) were placed in each test aquarium (fish were randomly selected from the stock population). We used semi-static test procedure (test solutions were renewed at 24-hour intervals). During the tests, the condition of fish was checked and the number of dead fish was recorded for different concentrations every 24 hours as well as the water temperature, pH and the oxygen saturation of water. Water temperature in the tests was 23 ± 2 °C. The dissolved oxygen concentrations were above 60%, pH of the water ranged from 8.07 to 8.49. The duration of each test was 96 hours. The results of acute toxicity tests (the number of dead fish in individual test concentrations) were processed by the probit analysis (EKO-TOX 5.2 programme) to determine the 96hLC50 terbutryn values.

The subchronic toxicity test. Tests of subchronic exposure of juvenile fish (at the age of 20 days) to terbutryn were performed on *D. rerio* according to OECD No. 215 Fish, Juvenile Growth Test in series of four tests. The tests were carried out with 20 fish used for each concentration and for the control groups (one control with dilution water only and the second control with dilution water and solvent - DMSO in quantities 0.1%). The average beginning weight of fish used in the experiment was 0.009 ± 0.003 g. Fish were placed in test aquariums and exposed to a range of sublethal concentration of terbutryn (0.02 μg L^{-1} – environmental concentration in the Czech rivers, 0.06, 0.2, 0.6 and 2.0 mg L^{-1}). The duration of these semi-static tests (the solutions were renewed at 48-hour intervals) was 28 days. Fish were fed with dried *Artemia salina* without nutshells in amount of 8% of their body weight per day, the food ration was based on initial fish weight and was recalculated after 14 days. At the end of the tests fish were weighed again and their length was determined. Food was withheld from the fish 24 h prior to weighing. During the tests, the living conditions were checked at 24-hour intervals and the number of dead fish was recorded in each concentration. Water temperature in tests was 23 ± 2 °C, oxygen saturation of water was above 60 %, pH of the water ranged from 8.13 to 8.50. Tank-average specific growth rates were calculated using the following formula according to the OECD No. 215:

$$r = \frac{\overline{\log_e W_2} - \overline{\log_e W_1}}{t_1 - t_2} * 100$$

r – tank-average specific growth rate

W_1, W_2 – weight of a particular fish at times t1 and t2 respectively

$\overline{\log_e W_1}$ – average of the logarithms of the values W_1 for the fish in the tank at the start of the study period

$\overline{\log_e W_2}$ – average of the logarithms of the values W_2 for the fish in the tank at the end of the study period

t_1, t_2 – time (days) at start and end of study period

Water quality parameters. The basic physical and chemical parameters of dilution water used in toxicity tests were: COD_{Mn} (chemical oxygen demand) 1.4–1.9 mg L^{-1}; total ammonia below the limit of determination

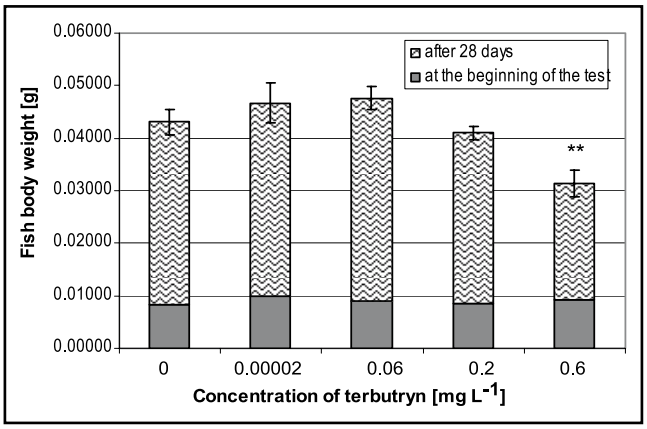

Figure 1. Comparison of body weight of control (0 mg L^{-1} terbutryn, with DMSO and water) and test fish (concentrations of terbutryn from 0.00002 to 0.6 mg L^{-1}) (** $p < 0.01$).

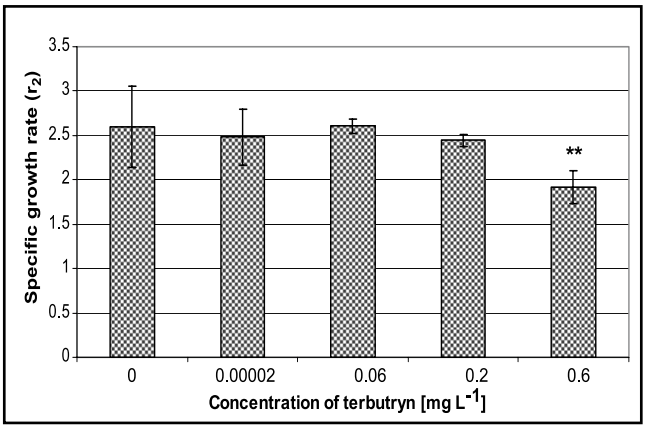

Figure 2. Comparison of specific growth rate and tested terbutryn concentrations (concentrations of terbutryn from 0.00002 to 0.6 mg L^{-1}) (** $p < 0.01$).

(< 0.04 mg L^{-1}); NO_3^- 24.5–31.4 mg L^{-1}; NO_2^- below the limit of determination (< 0.02 mg L^{-1}); Cl^- 18.9 – 19.1 mg L^{-1}; Σ Ca ± Mg 14 mg L^{-1}.

Statistical analysis. Results were analysed using the statistical programme Unistat 5.1. The data were subjected to one-way ANOVA and subsequently to Dunnett`s test in order to assess the statistical significance of differences in tank-average fish specific growth (r) between test groups with different concentrations and that of the control group. The estimation of the LOEC and NOEC values was based on ANOVA followed by Dunnett`s test for the identification of the lowest concentration for which this difference is (is not) significant at a 0.05 probability level.

Determination of terbutryn. Gas chromatography with ion trap mass spectrometry (GC/IT-MS) was used for the determination of terbutryn concentrations in aquariums. Sample preparation was based on simple liquid-liquid extraction into hexan. Separation, identification and quantification of terbutryn were based on the GC/IT-MS method described by Djozan & Ebrahimi (2008). Gas chromatograph Varian 450-GC with Varian 220-MS ion trap mass spectrometer and VF-5ms (30 m × 0.25 mm) column were used for separation of terbutryn. Detection limit (3σ) of terbutryn is 0.01 μg L^{-1}. Expanded uncertainty was 6.5% on condition that coefficient of expansion is k = 2. During the tests, the tested substance concentrations in all aquariums were above 80% (ranging from 82% to 88%) of the measured initial concentration.

Histopathological examination. Fish were fixed in 10% neutral formalin solution and processed using conventional paraffin techniques. Tissue sections were stained with haematoxylin and eosin. Histological changes in samples of skin, gill, liver and kidney were examined by light microscopy.

RESULTS

Acute toxicity test. The 96hLC50 terbutryn mean value was 5.71 ± 0.46 mg L^{-1} (ranging from 5.20 to 6.13 mg L^{-1}) for *D. rerio.*

Subchronic toxicity test. For the growth test we selected a range of 5 initial concentrations lower than 96hLC50. We selected concentrations of terbutryn 3-fold (2.0 mg L^{-1}), 10-fold (0.6 mg L^{-1}), 30-fold (0.2 mg L^{-1}) and 100-fold (0.06 mg L^{-1}) less than 96hLC50 terbutryn value and the environmental concentration of terbutryne in the Czech rivers (0.02 μg L^{-1}).

Fish behaviour and mortality. In test groups of fish exposed to 0.02 μg L^{-1}, 0.06 mg L^{-1}, 0.2 mg L^{-1} and 0.6 mg L^{-1} of terbutryn, the mortality did not exceeded 5% during the 28-day experimental period. In both control tanks the mortality was 0% during the experiments. In the test group exposed to the highest concentration of terbutryn (2 mg L^{-1}) the mortality was 100% before fish weighting in the half of the test, fish began dying on day 5 of exposure in this group. In this concentration we also noticed changes in food intake compared to the control, fish practically did not ingest the food at all.

Growth rate. The overview of the results of body weight measurements before and after the series of four tests (means ± standard deviations) is shown in ***Figure 1***. The initial body weight was not significantly different among groups, but at the end of the trial, fish weight in tanks with concentration of terbutryn 0.6 mg L^{-1} was significantly lower ($p < 0.01$) compared to the control group.

The results of specific growth rate r_2 (means ± standard deviations) of the test groups in comparison with the control group are demonstrated in ***Figure 2***. A significant decrease ($p < 0.01$) in fish growth caused by terbutryn concentration was observed in the concentration of 0.6 mg L^{-1}. The identified value of LOEC was 0.6 mg L^{-1} of terbutryn and NOEC value was 0.2 mg L^{-1} of terbutryn.

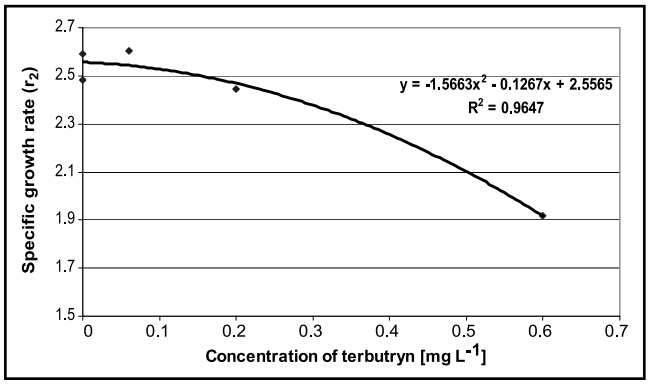

Figure 3. Relationship between concentration of terbutryn in diluton water and specific growth rate r_2 in *Danio rerio*.

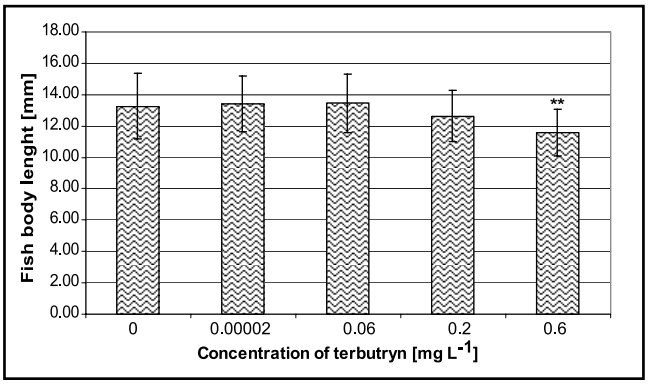

Figure 4. Comparison of the individual body length of (0 mg L^{-1} terbutryn, with DMSO and water) and test fish (concentrations of terbutryn from 0.00002 to 0.6 mg L^{-1}) (** $p < 0.01$).

The results of specific growth rates of groups in relation to different concentration of terbutryn are given in ***Figure 3***. The increased concentration of terbutryn resulted in lower fish growth. The relationship of terbutryn concentration to the fish growth is expressed by a regression equation, which demonstrates a polynomial relationship between these values. The resulting regression equation for the juvenile fish *D. rerio* is $y = -1.5663x^2 - 0.1267x + 2.5565$ ($R^2 = 0.9647$), where x represents terbutryn concentrations in dilution water and y the specific growth rate (r_2).

Body length. The results of the individual fish body length (means ± standard deviations) at the end of the experiment in comparison with control groups are presented in ***Figure 4***. A significant decrease ($p < 0.01$) in individual fish body length caused by terbutryn concentration was detected in the concentration of 0.6 mg L^{-1}.

Histopathological changes. No histopathological changes were found on skin, gills, and liver. We observed the damage to tubular system of kidneys only in fish exposed to the concentration of 0.6 mg L^{-1} of terbutryn. The foci of the renal tubular epithelial cells necrosis with dense leucocytes infiltration were found (***Fig. 5***; control group-***Fig. 6***).

Validity of the tests. Our tests met all the conditions required by OECD – the mortality in the control groups was below 10% (no fish died in the control tanks), the final weight of control fish in subchronic toxicity tests was higher than 150% of the initial weight, the dissolved oxygen concentrations were at least 60%, the water temperature did not differ by more than ± 1°C among test aquariums, test substance concentrations were above 80% (ranging from 82% to 88%) of the measured initial concentration.

DISCUSSION

Many studies have dealt with the determination of acute toxic concentrations of triazine herbicides for various fish species. Arufe *et al.* (2004b) studied acute effects of commercial formulation containing simazine on seabream (*Sparus aurata* L.), Kreutz *et al.* (2008) reported the acute effect of atrazine and atrazine with simazine on silver catfish (*Rhamdia quelen*), Velisek *et al.* (2008) studied the acute effect of metribuzin on rainbow trout (*Oncorhynchus mykiss*).

Not many authors mentioned the acute effect of terbutryn on fish. But terbutryn have been frequently detected in water environments (Tolosa *et al.* 1996; Carabias-Martinez *et al.* 2003; Quednow & Puttmann, 2007). Arufe *et al.* (2004a) found the 72hLC50 value of preparation containing terbutryn (59.4%) and triasulfuron (0.6%) for seabream (*Sparus aurata* L.) to be 1.41 mg L^{-1}, which is lower than we detected in our study (96hLC50 terbutryn mean value was 5.71 ± 0.46 mg L^{-1} for *Danio rerio*). As terbutryn is relatively insoluble in water (Rioboo *et al.* 2007) we had to use a solvent – a dimethyl sulfoxide (DMSO), which is the most commonly used delivery system for water-antisoluble chemicals in aquatic bioassays. Hallare *et al.* (2006) reported that DMSO may be used as a carrier solvent in the zebrafish embryo assay at levels below 1.5 % v/v (for stress protein analysis of the exposed embryos the solvent level should be below 0.01% v/v). We used DMSO in quantities 0.1% and we did not find out any significant differences between the control groups with dilution water only in comparison with control groups with the solvent DMSO.

It is also necessary to study sublethal effects of triazine herbicides, because many of them (including terbutryn) have the potential to bioaccumulate in organisms and act there for a long time (Rioboo *et al.* 2007). A few authors (Davies *et al.* 1994; Alvarez & Fuiman, 2005; Modra *et al.* 2008) studied effects of sublethal concentrations of triazines on fish (effects on growth, histopathological changes, haematological indices and biochemical indices). We assessed effects of sublethal concentrations of terbutryn on growth and on histopathological changes in *D. rerio.* We found out that the environmental concentration of terbutryn in the Czech rivers (0.02 μg L^{-1}) had no effects on growth and on histopathological changes in *D. rerio* and this

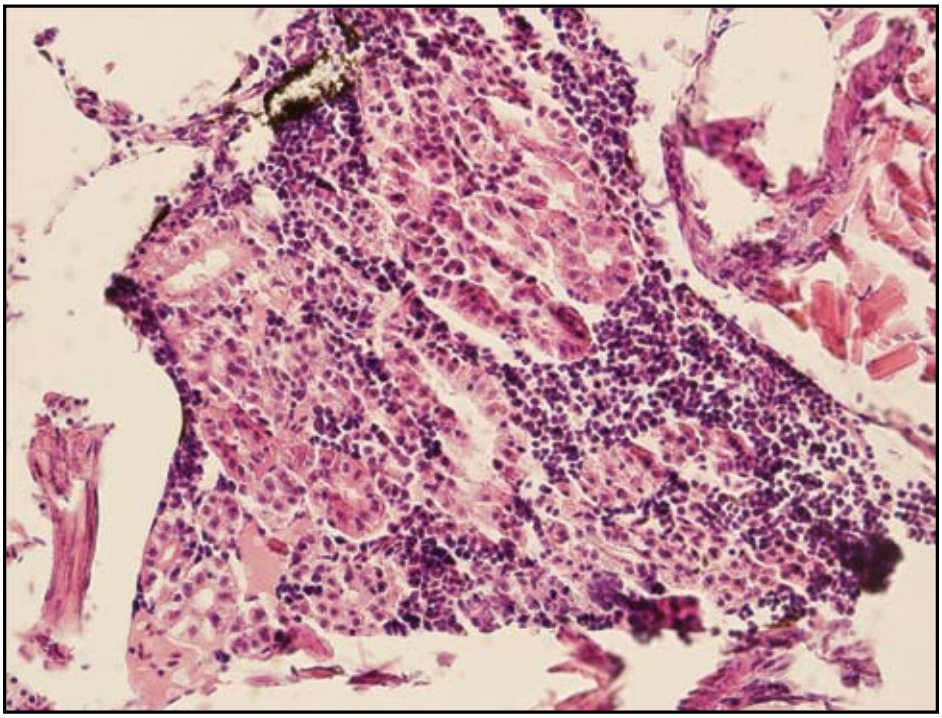

Figure 5. The damage to tubular system of kidney of *D. rerio* exposed to the concentration of 0.6 mg L^{-1} of terbutryn for 28 days (× 400).

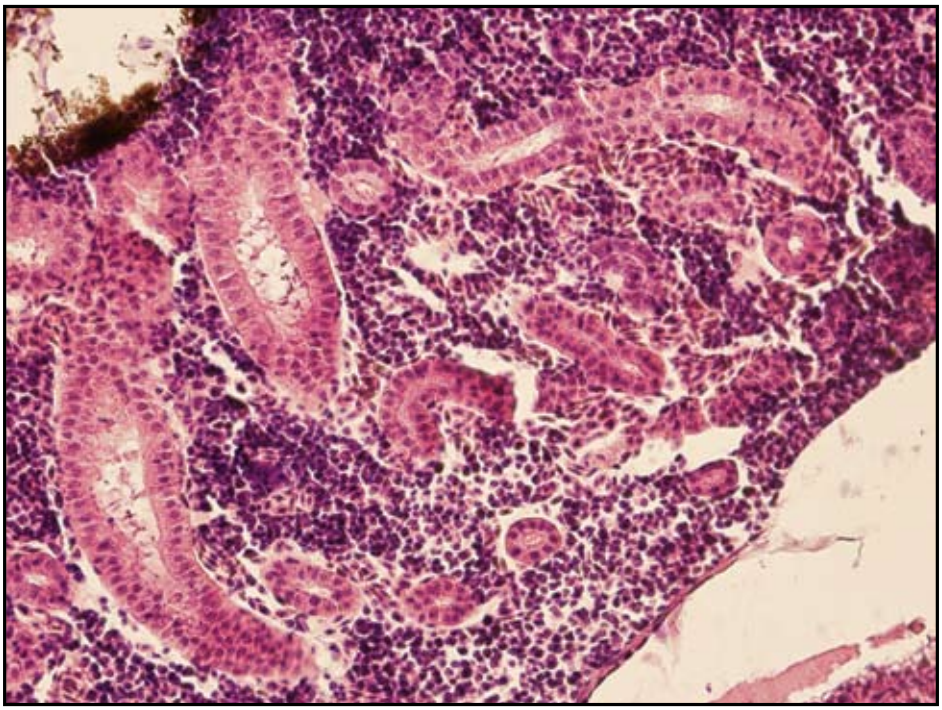

Figure 6. Section of the kidney *D. rerio* from the control group (× 400).

concentration is lower than determined terbutryn NOEC and LOEC values. Similarly, Velisek *et al.* (2009) reported no effect of the environmental concentration of terbutryn in the Czech rivers on biochemical, haematological and histopathological profiles of common carps (*Cyprinus carpio* L.), which were exposed to this concentration of terbutryn for 28 days. The concentration of 0.6 mg L^{-1} of terbutryn (LOEC) caused the decrease in the fish growth and also the histopathological changes of kidney in *D. rerio*.

Acknowledgements

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic (MSM Project No. 6215712402, IGA 199/2009/FVHE and IGA 200/2009/FVHE).

REFERENCES

1 Alvarez MC, Fuiman LA (2005). Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae. Aquat Toxicol. **74**: 229–241.
2 Arufe MI, Arellano J, Moreno MJ, Sarasquete C (2004a). Toxicity of commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. Ecotox Environ Saf. **59**: 209–216.
3 Arufe MI, Arellano J, Moreno MJ, Sarasquete C (2004b). Comparative toxic effects of formulated simazine on *Vibrio fischeri* and gilthead seabream (*Sparus aurata* L.) larvae. Chemosphere. **57**: 1725–1732.
4 Carabias-Martinez R, Rodriguez-Gonzalo E, Fernandez-Laespada ME, Calvo-Seronero L, Sanchez-San Román FJ (2003). Evolution over time of the agricultural pollution of waters in an area of Salamanca and Zamora (Spain). Water Res. **37**: 928–938.
5 Czech Hydrometeorological Institute, on-line water quality database. Available from: <http://voda.chmi.cz/ojv2/> (in Czech) (visited on-line 19.3.2009)
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 Djozan D, Ebrahimi B (2008). Preparation of new solid phase micro extraction fiber on the basis of atrazine-molecular imprinted polymer: Application for GC and GC/MS screening of triazine herbicides in water, rice and onion. Anal Chim Acta. **616**: 152-159.
8 Hallare A, Nagel K, Kohler H-R, Triebskorn R (2006). Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. Ecotox Environ Saf. **63**: 378–388.
9 Kreutz LC, Barcellos LJG, Silva TO, Anziliero D, Martins D, Lorenson M, et al (2008). Acute toxicity test of agricultural pesticides on silver catfish (*Rhamdia quelen*) fingerlings. Ciência Rura.l **38**: 1050–1055.
10 Lawrence C (2007). The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture. **269**: 1–20.
11 Macova S, Dolezelova P, Pistekova V, Svobodova Z, Bedanova I, Voslarova E (2008). Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*. Neuroendocrinol Lett. **29**: 680–684.
12 Modra H, Haluzova I, Blahova J, Havelkova M, Kruzikova K, Mikula P, et al (2008). Effects of subchronic metribuzin exposure on common carp (*Cyprinus carpio*). Neuroendocrinol Lett. **29**: 669–674.
13 Moretti M, Marcarelli M, Villarini M, Fatigoni C, Scassellati-Sforzolini G, Pasquini R (2002). In vitro testing for genotoxicity of the herbicide terbutryn: cytogenetic and primary DNA damage. Toxicol Vitro. **16**: 81–88.
14 Muir DCG (1980). Determination of terbutryn and its degradation products in water, sediments, aquatic plants, and fish. J Agric Food Chem. **28**: 714–719.
15 Quednow S, Puttmann W (2007). Monitoring terbutryn pollution in small rivers of Hesse, Germany. J Environ Monit. **9**: 1337–1343.
16 Roberts TR, Hutson DH, Lee PW, Nicholls PH, Plimmer JR (1998). Metabolic Pathways of Agrochemicals. Part 1: Herbicides and Plant growth regulators. 1st ed. Cambridge: The Royal Society of Chemistry.
17 Rioboo C, Prado R, Herrero C, Cid A (2007). Population growth study of the rotifer Brachionus sp. Fed with triazine-exposed microalgae. Aquat Toxicol. **83**: 247–253.
18 Stevens JT, Breckenridge CB, Simpkins J, Eldridge JC (2001). Symmetrical and Asymmetrical Triazine Herbicides. In: Krieger R, editor. Handbook of Pesticide Toxicology. 2nd ed. San Diego, CA: Academc Press Inc. p. 1511–1519.
19 Tolosa I, Readman JW, Blaevoet A, Ghilini S, Batrocci J, Horvat M (1996). Contamination of Mediterranean (Côte d'Azur) coastal waters by organotins and irgarol 1051 used in antifouling paints. Mar Pollut Bull. **32**: 335–341.
20 Velisek J, Svobodova Z, Piackova V, Novotny L, Blahova J, Sudova E, et al (2008). Effect of metribuzin on rainbow trout (*Oncorhynchus mykiss*). Vet Med. **53**: 324–332.
21 Velisek J, Sudova E, Machova J, Svobodova Z (2009). Effects of subchronic terbutryn exposure on some biochemical, haematological and histopathological parameters of the common carp (*Cyprinus carpio* L.). Toxicol Lett. **189S**: S208.