ORIGINAL ARTICLE

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

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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 $\mu g.l^{-1}$ (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

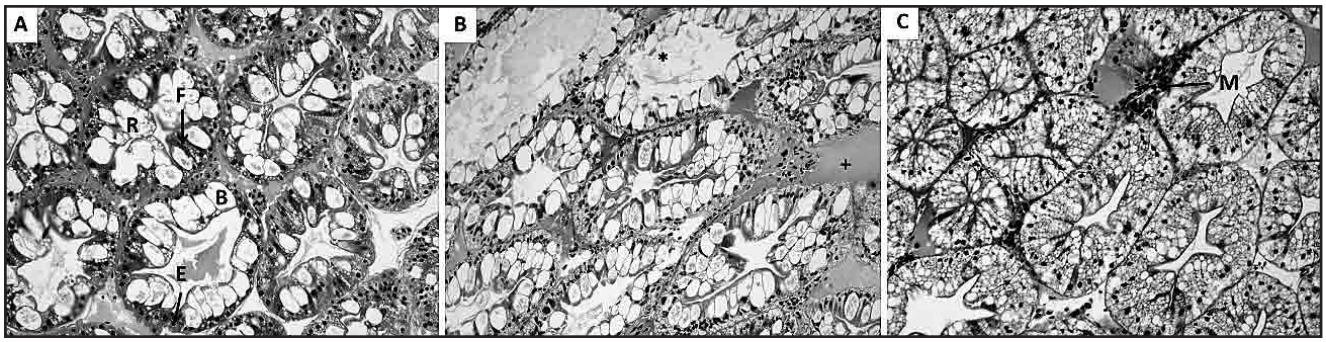

Fig. 1. Transversal sections of hepatopankreatic tubules of signal crayfish (*Pacifastacus leniusculus* Dana). A - control group; B - group exposed to 3.06 mg.l^{-1} metribuzin for 30 days; C – group examined 30 days after metribuzin exposure (depuration period) (100×). Transversal sections of tubules 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.

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