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Histopathological and biochemical changes in goldfish kidney due to exposure to the herbicide Sencor may be related to induction of oxidative stress

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ABSTRACT

Molecular mechanisms of toxicity by the metribuzin-containing herbicide Sencor to living organisms, particularly fish, have not yet been extensively investigated. In the present work, we studied the effects of 96 h exposure to 7.14, 35.7, or 71.4 mg L^{-1} of Sencor (corresponding to 5, 25, or 50 mg L^{-1} of its herbicidal component metribuzin) on goldfish (*Carassius auratus* L.), examining the histology, levels of oxidative stress markers, and activities of antioxidant and related enzymes in kidney as well as hematological parameters and leukocyte profiles in blood. The treatment induced various histopathological changes in goldfish kidney, such as hypertrophy of intertubular hematopoietic tissue, small and multiple hemorrhages, glomerular shrinkage, a decrease in space between glomerulus and Bowman's capsule, degeneration and necrosis of the tubular epithelium. Sencor exposure also decreased activities of selected enzymes in kidney; activities of catalase decreased by 31–34%, glutathione peroxidase by 14–33%, glutathione reductase by 17–25%, and acetylcholinesterase by 31%. However, glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities increased by 25–30% and 22% in kidney after treatment with 7.14 or 35.7 mg L^{-1} and 71.4 mg L^{-1} Sencor, respectively. Kidney levels of protein carbonyls increased by 177% after exposure to 35.7 mg L^{-1} of Sencor indicating extensive damage to proteins. Lipid peroxide concentrations also increased by 25% after exposure to 7.14 mg L^{-1} of Sencor, but levels were reduced by 42% in the 71.4 mg L^{-1} exposure group. The data indicate that induction of oxidative stress is one of the mechanisms responsible for Sencor toxicity to fish.

1. Introduction

The extensive use by modern agriculture of different agrochemicals such as pesticides is emerging as a threat to the ecological balance of aquatic ecosystems. Synthetic pesticides are recognized as serious pollutants in the aquatic environment with the potential to cause deleterious effects on the biota, especially fish occupying the upper trophic level.

Triazines (a six-membered ring containing three carbon and three nitrogen atoms) have been extensively used for weed control since the early 1950s. Triazine herbicides are categorized in two groups, the asymmetrical triazines, such as metribuzin, and the symmetrical triazines, such as simazine, atrazine, terbutryn and others (Stevens et al., 2001). The herbicidal activity of triazines is believed to be mediated by inhibition of photosynthesis (Das et al., 2000) and intensification of reactive oxygen species (ROS) production through its interference with photosystem-II (Pauli et al., 1990; Nemat and Hassan, 2006). In fish, the triazine pesticides affect hematological and histopathological parameters (Velisek et al., 2008, 2012; Oropesa et al., 2009), stimulate DNA damage (Santos and Martinez, 2012), immune response (Fatima et al., 2007), induce

Abbreviations: AChE, acetylcholine esterase; CP, carbonyl protein groups; GPx, glutathione peroxidase; H-SH, high molecular mass thiols; LOOH, lipid peroxides; L-SH, low molecular mass thiols; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GST, glutathione-*S*-transferase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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oxidative stress (Elia et al., 2002; Fatima et al., 2007), and reduce growth rate and reproduction (Tillitt et al., 2010).

Metribuzin [4-amino-6-*tert*-butyl-3-(methythio)-1,2,4-triazin-5-one] is an asymmetrical triazine herbicide often used for the control of grasses and broad-leaved weeds in the production of soybeans, potatoes, tomatoes, sugar cane, alfalfa, asparagus, maize and cereals. It is extensively used as an active ingredient in multiple herbicides that are used worldwide such as Artist, Lexone 2, Sencor, Sencorex WG, Shotput and others. Runoff of metribuzin, like other triazine and triazinone herbicides, can readily contaminate surface waters due to its high water solubility 1.22 mg L^{-1} and half-life of 30 days (Pauli et al., 1990; Wauchope et al., 1992). The half-life of metribuzin in pond water is approximately seven days (Hartley and Kidd, 1983). It was shown that the concentration of metribuzin in surface water of Middle West of Brazil ranged up to 0.351 μg L^{-1} (Dores et al., 2006), but was less than 25 μg L^{-1} in Midwestern United States surface water (Battaglin et al., 2001).

Today, there are some data concerning metribuzin effects on different fish species. In particular, Velisek et al. (2008, 2009) showed histopathological changes in the caudal kidneys of rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) after acute exposure to Sencor 70 WG. Plhalova et al. (2012) found no morphological changes in the kidney, but histopathological lesions in hepatocytes of *Danio rerio* after 28-day exposure to Sencor 70 WG. Mekhed et al. (2004) observed increase in activities of basic enzymes of gluconeogenesis (glucose-6-phosphatase and fructose-1,6-bisphosphatase) and decrease in glucose levels in liver, muscle and brain of carp exposed to Sencor for 14 days. Exposure of fish *Tilapia mossambica* to sublethal concentrations of metribuzin decreased total protein, carbohydrate and cholesterol levels in liver, muscle, kidney and gills (Saradhamani and Selvarani, 2009). Unfortunately, very little is known about free radical processes in the kidneys of various fish species under acute and chronic exposure to metribuzin.

Kidney is a major route for the excretion of xenobiotics and receives the largest portion of postbranchial blood in fish. Hence, intoxication with pesticides may potentially induce histopathological changes and modify biochemical composition of fish kidney (Ortiz et al., 2003; Atamaniuk et al., 2013). The present work aimed to disclose some molecular toxicity mechanisms of the metribuzin-based pesticide, Sencor, in goldfish with a focus on blood and kidney parameters that could potentially be developed as biomarkers of pesticide intoxication.

2. Material and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), oxidized glutathione (GSSG), glucose-6-phosphate (G6P), xylenol orange, cumene hydroperoxide, ferrous sulphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Tris(hydroxymethylaminomethane), NaCl, KH_2PO_4, NADH, NADP, hydrogen peroxide (H_2O_2) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich Corporation (USA). Acetylthiocholine iodide and NADPH was purchased from Carl Roth (Germany). Sencor 70 WG was purchased from Bayer Crop Science (Germany). All other reagents were of analytical grade.

2.2. Animals and experimental conditions

Goldfish (*Carassius auratus* L.) with body masses of 80–100 g were obtained from a local fish farm (Halych district, Ivano-Frankivsk region, Ukraine) in November 2012. Fish were acclimated to laboratory conditions for 4 weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water at 19.0–20.0 °C, pH 6.9–7.1, 8.1–8.6 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) 38–40 mg L^{-1}. Fish were fed once a day with commercial Cyprinid Carp Co Excellent (Koi Grower, The Netherlands) pellets, containing 36% protein, 7% fat, 3.6% cellulose, 8.7% ash, 1% phosphorus and vitamins C, A, D_3 and E. Both, control and experimental fish groups were fed during the acclimation period (4 weeks), but were fasted for 1 day prior to and during experimentation.

Experiments were carried out in 120 L glass aquaria containing 100 L of water. Groups of seven fish were placed in aquaria with different nominal concentrations of the herbicide Sencor (Bayer, Germany): 7.14; 35.7 or 71.4 mg L^{-1}, which corresponded to 5, 25 or 50 mg L^{-1} of metribuzin, respectively, and exposed to these conditions for 96 h. No mortality occurred during exposures. Fish in the control group were maintained in the same manner, but without addition of Sencor.

Aquarium water was not changed over the 96 h experimental course in order to avoid stressing the fish. Levels of dissolved oxygen, temperature and pH were monitored every 24 h and did not change over the experimental time course. After fish exposure, blood was quickly taken from caudal vessels using a syringe rinsed with 50 mM Na_2EDTA as an anticoagulant. Fish were then quickly sacrificed by transspinal transsection without anesthesia and kidneys were dissected, rinsed in ice-cold 0.9% NaCl, dried by blotting on filter paper, frozen, and stored in liquid nitrogen until use.

All experiments were conducted in accordance with the institutional animal ethics guidelines of Precarpathian National University and were approved by the Animal Experimental Committee of Precarpathian National University.

2.3. Evaluation of hematological parameters and leukocyte formula in blood

2.3.1. Estimation of total hemoglobin concentration and hematocrit value

Total hemoglobin concentration was determined after erythrocyte hemolysis in Drabkin's solution using a commercial kit (Genesis Co, Ltd., Ukraine) following the manufacturer's instructions.

Hematocrit was determined following the procedure of Ptashynski et al. (2002). Immediately after blood sampling, small amounts of whole blood were transferred to microcapillary tubes, which were then carefully sealed on both ends and centrifuged (2000 × *g*, 20 min, 4 °C) using an OPN-8 centrifuge (USSR). Hematocrit values were calculated as the percentage of red blood cell pellet in the total blood column.

2.3.2. Examination of leukocyte content

For microscopic examination of leukocyte content, small drops of whole blood were directly smeared on slides ($n = 2$ per fish) and air-dried. Smears were fixed and stained with azure-eosin water solution as described previously (Vasylkiv et al., 2010). Cytological analysis was conducted by scoring at a 1600× magnification using a Leitz microscope (Leitz Wetzbar GmbH, Germany). Different types of leukocytes were identified according to the Fish Blood Cell Atlas (Ivanova, 1983). A total of 200 leukocyte cells were counted per smear and assigned to different leucocyte categories. Data are shown as the percentages of different leucocytes per 200 cells counted.

2.4. Histological examination of goldfish kidney

Kidney samples from control and treated fish were fixed in 10% neutral-buffered formalin, and then the samples were processed

for routine wax histological evaluation (dehydrated and embedded in paraffin). Sections of 5 μm were taken and stained with hematoxylin and eosin stains as described by Luna (1968).

2.5. Determination of oxidative stress indices in kidney

2.5.1. Assay of lipid peroxides

The lipid peroxide (LOOH) content was assayed by the xylenol orange method (Hermes-Lima et al., 1995). For this purpose, tissue samples were homogenized (1:5 w/v) using a Potter-Elvehjem glass homogenizer in 96% ice-cold (4 °C) ethanol, and centrifuged (5000 × *g*, 10 min, 4 °C). The supernatants were used for assay as described previously (Lushchak et al., 2005). The content of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass (nmol gwm^{-1}) of tissue.

2.5.2. Measurement of protein carbonyl groups

Carbonyl groups of proteins (CP) in kidney were determined as described previously (Lushchak et al., 2005). Tissue samples were homogenized (1:10 w:v) in homogenization medium (50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged (16,000 × *g*, 15 min, +4 °C). Supernatants were removed and 0.25 mL aliquots were mixed with 0.25 ml of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged (5000 × *g*, 5 min, +20 °C). Protein carbonyl (CP) content was measured in the resulting pellets by reaction with 2,4-dinitrophenylhydrazine, leading to the formation of dinitrophenylhydrozones. The amount of CP was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of $22 \pm 10^3\ M^{-1}\ cm^{-1}$. Values are expressed as nanomoles of CP per milligram of protein.

2.5.3. Estimation of thiol-containing compounds

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (Ellman, 1959). Using supernatants prepared as above for the CP assays, total thiol concentration (the sum of low and high molecular mass thiols) was measured as described previously (Lushchak and Bagnyukova, 2006). For determination of low-molecular mass thiols (L-SH), aliquots of supernatants were mixed with trichloroacetic acid (TCA) to reach a final TCA concentration of 10%, centrifuged (16,000 × *g*, 5 min, +4 °C) to remove pelleted protein and the final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram wet mass of tissue (μmol gwm^{-1}). The high-molecular mass thiol (H-SH) concentration was calculated by subtracting the L-SH concentration from total thiol concentration.

2.6. Assay of enzyme activities in kidney

2.6.1. Determination of activities of antioxidant and associated enzymes

Tissue extracts were prepared as for the CP and thiol assays. Activities of antioxidant enzymes, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), and associated enzymes, namely glutathione-*S*-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), were measured as described earlier (Lushchak et al., 2005). One unit of SOD activity is defined as the amount of enzyme (per milligram protein) that inhibited the quercetin oxidation reaction by 50% of maximal inhibition. Inhibition values for SOD activity were calculated using a Kinetics computer program (Brooks, 1992). One unit (U) of catalase, GST, GR, GPx or G6PDH activities is defined as the amount of enzyme consuming 1 μmol of substrate or generating 1 μmol of product per minute. Activities are expressed as international units (or milliunits) per milligram of soluble protein (U $mg\ protein^{-1}$).

2.6.2. Assay of lactate dehydrogenase activity

The activity of lactate dehydrogenase (LDH) was assayed spectrophotometrically using a Specol 211 spectrophotometer (Germany) by monitoring the change in NADH absorbance at 340 nm (Lushchak et al., 2001). One unit of enzyme activity is defined as the amount of enzyme consuming 1 μmol of substrate per minute. The activity is expressed as international units per milligram protein.

2.6.3. Assay of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined by the method of Ellman et al. (1961) with adaptation of the procedure to microplate determination. The reaction mixture, containing 100 mM potassium phosphate buffer, pH 8.0 and 0.3 mM DTNB was mixed with 10 μL supernatant and incubated for 10 min at 25 °C to allow the reaction of DTNB with supernatant thiols. Subsequently, the mixture was supplemented with acetylthiocholine iodide to reach a final concentration of 7.5 mM and the reaction rate was monitored spectrophotometrically at 412 nm. Enzyme activity was calculated using a molar extinction coefficient of $13.6 \times 10^3\ M^{-1}\ cm^{-1}$. One unit of AChE activity was defined as the amount of enzyme producing 1 μmol of thionitrobenzoate per minute and was expressed per milligram of soluble protein (mU $mg\ protein^{-1}$).

2.7. Protein measurements

Soluble protein concentration was measured with the Coomassie Brilliant blue G-250 (Bradford, 1976) using bovine serum albumin as a standard.

2.8. Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) followed by a Dunnett's test to compare multiple experimental treatment groups to the single control group using Mynova computer program (version 1.3); a value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

In this work we investigated the effects of Sencor, a metribuzin-containing herbicide, on blood parameters as well as the morphology and biochemical parameters of the kidney. These parameters combine broadly used vital diagnostics (e.g. hematocrit, leukocyte profile) with an analysis of multiple metabolic parameters of kidney, an organ that in fish has important roles in hematopoiesis as well as transformation and excretion of xenobiotics. Together, these analyses may provide a convenient approach to characterize the effects of pesticides on fish and provide a biomarker for pollution of the aquatic environment.

3.1. Effect of Sencor on hematological and immunological parameters

It is known that exposure to chemical pollutants can induce alterations in hematological parameters which can indicate adaptive responses to a stress agent or the direct effects of these contaminants on erythrocytes or their production (Simonato et al., 2008). In the present study, the level of total hemoglobin demonstrated a tendency to increase in fish exposed to 35.7 or 71.4 mg L^{-1} of Sencor relative to controls (Table 1). However, the difference was not statistically significant. Similarly, Oropesa et al. (2009) and

Table 1
The level of total hemoglobin (g L^{-1}), hematocrit value (%) and relative content of different leukocytes (%) in blood of goldfish, exposed to control conditions or 7.14, 35.7 or 71.4 mg L^{-1} of Sencor for 96 h.

Parameter		Fish group			
	Control	7.14 mg L^{-1}	35.7 mg L^{-1}	71.4 mg L^{-1}	
Hematological parameter	Total hemoglobin (g L^{-1})	66.3 ± 8.0	65.3 ± 6.3	71.6 ± 6.8	86.7 ± 7.7
	Hematocrit (%)	29.6 ± 1.9	30.1 ± 1.0	26.6 ± 0.9	36.7 ± 1.9*
Leukocytes type	Lymphocytes (%)	68.0 ± 9.3	71.9 ± 2.0	74.3 ± 1.5	62.0 ± 1.8
	Stab neutrophils (%)	9.52 ± 2.14	7.40 ± 1.13	4.54 ± 1.00	14.2 ± 1.5*
	Segmented neutrophils (%)	5.90 ± 0.95	4.38 ± 0.76	4.61 ± 0.92	5.71 ± 1.22
	Eosinophils (%)	NF	NF	NF	NF
	Basophils (%)	NF	NF	NF	NF
	Monocytes (%)	5.37 ± 0.94	10.2 ± 1.1*	7.71 ± 1.41	9.84 ± 0.85*
	Metamyelocytes (%)	3.86 ± 1.24	1.62 ± 0.40	1.32 ± 0.40	3.07 ± 0.57
	Myelocytes (%)	3.10 ± 0.81	1.96 ± 0.39	2.60 ± 0.78	2.09 ± 0.60
	Promyelocytes (%)	2.89 ± 0.98	2.34 ± 0.66	3.63 ± 0.69	3.01 ± 0.63
	Myeloblasts (%)	NF	NF	NF	NF
	Hemocytoblasts (%)	NF	NF	NF	NF

Data for leukocytes are presented as percentages of total leukocytes number, evaluated per 200 leukocytes cells. NF—cells were either not found or were only 0.1–0.5% in some groups. Data are expressed as means ± S.E.M., $n = 6–7$.

* Significantly different from the respective control value with $P < 0.05$ using ANOVA with a post-hoc Dunnett's test.

Velisek et al. (2012) reported no effect on total hemoglobin levels in common carp exposed to simazine over extended times.

The hematocrit measures the percentage of packed red blood cells in a volume of blood. Fish exposed to the highest concentration of Sencor (71.4 mg L^{-1}) for 96 h showed a hematocrit that was 23% higher than the control value (Table 1). Nieves-Puigdoller et al. (2007) reported a similar significant increase in hematocrit in Atlantic salmon (*Salmo salar*) exposed to atrazine. It is known that in response to pollutants, structural changes of fish gill epithelia may lead to a reduction of the respiratory surface and induction of hypoxia (Nieves-Puigdoller et al., 2007). The increase in hematocrit reflects development of general stress and may represent a compensatory response to hypoxia. Several authors reported a decrease in hemoglobin and hematocrit in metribuzin- and atrazine-treated fish, indicating pesticide-induced dysfunction of erythropoiesis (Svobodova et al., 1994; Velisek et al., 2008, 2009). Additionally, it was shown that elevated numbers of erythrocytes in the blood stream could follow from increased release from red cell depots and/or from increased synthesis by hematopoietic tissues (e.g. liver, spleen and kidney) (Svobodova et al., 1994). In the kidney of Sencor-exposed goldfish, we observed hypertrophy of intertubular hematopoietic tissue (Fig. 1). This response occurred at all dose levels of the metribuzin-containing herbicide Sencor.

In concert with hematological parameters, the leukocyte profile of fish blood is a valuable tool for nonlethal (vital) diagnostics of fish intoxication (Svoboda et al., 2001; Li et al., 2011) and has become an important means of understanding the possible mechanisms of toxin impact (Borges et al., 2007). Leukocytes are important elements of the immune system and play pivotal roles in the protection of organisms under stress and intoxication. Indeed, the leukocyte profile is a sensitive indicator of environmental stress (Cole et al., 2001). Table 1 shows that no changes in the relative amounts of most leukocytes were found in response to Sencor exposure. However, increased relative counts of stab neutrophils (a 49% increase) occurred in fish exposed to 71.4 mg L^{-1} Sencor and monocytes increased by 90 and 83% in fish exposed to 7.14 or 71.4 mg L^{-1} of Sencor, respectively. Similarly, other authors reported increased monocyte count, stab and segmented neutrophils in common carp exposed for extended times to atrazine or metribuzin (Svobodova and Pecena, 1988; Velisek et al., 2009). Generally, a decrease in lymphocyte numbers with a concurrent increase in monocytes and neutrophils occurred in response to stress (Murad and Houston, 1988). Therefore, the increase in the relative counts of stab neutrophils and monocytes in our experiments (but not lymphocytes) demonstrated a weak effect of the metribuzin-containing herbicide Sencor on the leukocyte profile of goldfish.

3.2. Histopathological examination of Sencor-induced changes in kidney

The kidney is important for the maintenance of a stable internal environment with respect to water and salt balance as well as excretion and xenobiotic metabolism. In mammals and fish, the kidney is known to be responsible for excretion of triazine pesticides and products of their biotransformation. Therefore, pathological changes to kidney might be expected to be good indicators of environmental pollution (Gunkel, 1981). Indeed, triazine pesticides were found to have a direct effect on kidney structure and function in freshwater fish (Velisek et al., 2008, 2009).

A microscopic examination of goldfish kidney showed that animal exposure to Sencor at low concentration (7.14 mg L^{-1}) induced appearance of small hemorrhages, glomerular shrinkage and degeneration of the tubular epithelium in the organ (Fig. 1B). Fish treatment with the pesticide at intermediate concentration used (35.7 mg L^{-1}) decreased the space between glomerulus and Bowman's capsule along with the abovementioned changes (Fig. 1C). Under fish exposure to the highest herbicide concentration (71.4 mg L^{-1}) we additionally observed multiple hemorrhages and necrosis of the tubular epithelium in goldfish kidney (Fig. 1D). These changes in the structure of kidney suggest that goldfish exposure to Sencor affects fish health and clearly depends on the xenobiotic concentration being more substantially influenced at the highest concentration used.

Similar degenerative changes were observed in the tubular and intertubular epithelium in kidney of different fish exposed to different triazine pesticides (Fischer-Scherl et al., 1991; Velisek et al., 2008, 2009, 2012). Velisek et al. (2008) found hyaline degeneration in epithelial cells of the renal tubules. Fischer-Scherl et al. (1991) observed the accumulation of cellular debris in the Bowman's space and increased protein levels in urine at low concentrations of atrazine suggesting changes in the renal tubular epithelium. However, Plhalova et al. (2012) found no morphological changes in the kidney of any treated groups (up to 53 mg L^{-1} of metribuzin). Light microscopy observations revealed necrotic areas in hematopoietic and excretory tissues in the kidney of simazine-exposed fish. This was visible as eosinophilic droplets within the cytoplasm of proximal tubular epithelial cells (Oropesa et al., 2009). Necrosis is a regressive morphological state of a cell which occurs after

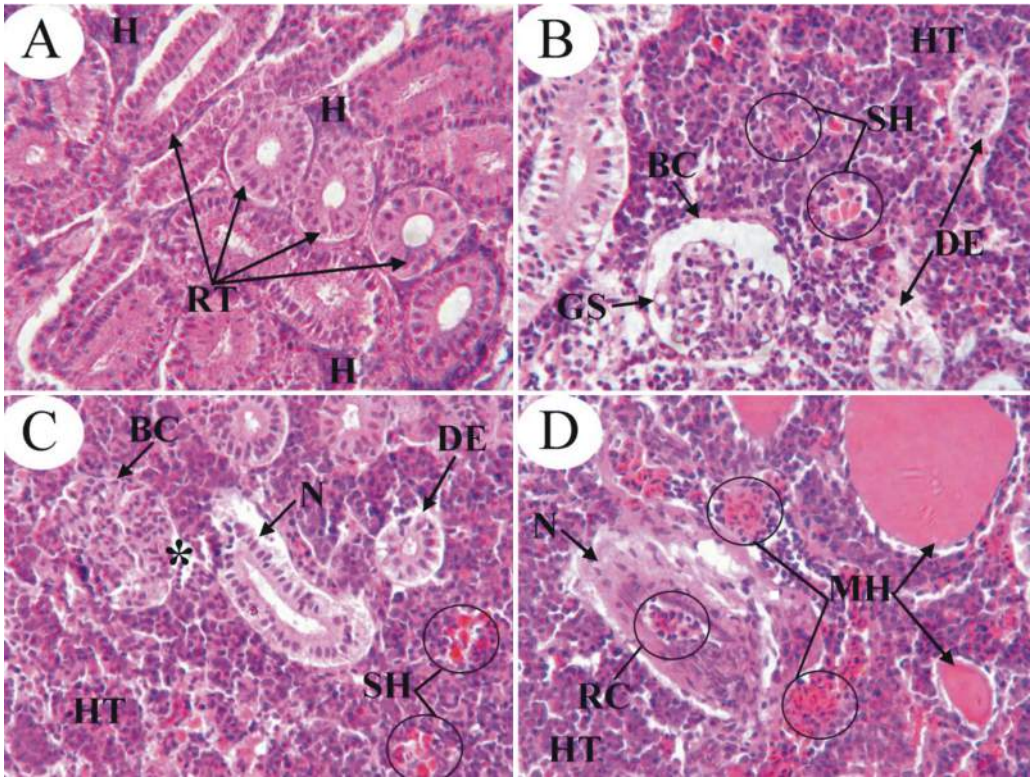

Fig. 1. Light micrographs of sections through kidney of goldfish (*C. auratus* L.) showing histological structures of the control group (A), and animals treated with 7.14 (B), 35.7 (C) or 71.4 (D) mg L^{-1} of Sencor for 96 h. Samples were stained with hematoxylin–eosin and photomicrographs were taken using 400× magnification. RT—renal tubules; H—hematopoietic tissue; BC—Bowman's capsule; GS—glomerular shrinkage; SH—small hemorrhage; DE—degeneration of tubular epithelium; N—necrotic cells and nuclei of tubular epithelium; MH—multiple hemorrhage; HT—hypertrophy of intertubular hematopoietic tissue; RC—red blood cells in necrotic tubules and Bowman's capsule; asterisk—decrease in space between glomerulus and Bowman's capsule. These are representative pictures of sections prepared from multiple fish; kidney tissue from at least four animals was sectioned, stained and examined for each of the groups (control and three experimental groups).

irreversible loss of cell function and has a marked pathological importance (Bernet et al., 1999).

3.3. Sencor effects on oxidative stress indices in kidney

An increase in protein carbonyl (CP) group levels is one of most commonly used markers of oxidative stress (Lushchak, 2011). Levels of CP were 177% higher in kidney of goldfish exposed to 35.7 mg L^{-1} Sencor as compared with control values (Fig. 2A). This could result from enhanced protein oxidation due to intensified ROS production under Sencor exposure. The formation of additional CP is irreversible in most cases and leads to protein dysfunction via

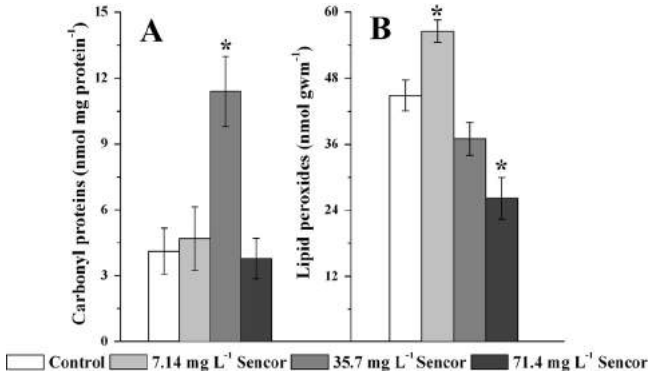

Fig. 2. Oxidative stress indices: levels of (A) carbonyl proteins (CP) and (B) lipid peroxides (LOOH) in kidney of goldfish, exposed to control conditions or 7.14, 35.7 or 71.4 mg L^{-1} of Sencor for 96 h. Data are presented as means ± S.E.M, $n = 5–7$. * Significantly different from the control group with $P < 0.05$.

conformational changes, increased susceptibility to protease action or formation of supramolecular aggregates (Lushchak, 2011).

Lipid peroxidation is one of the most important early events in cell degeneration leading to necrosis and occurs mainly in the cell membrane. Stimulation of lipid peroxidation is also generally assumed to be one of the molecular mechanisms of pesticide-induced toxicity (Lushchak, 2011; Chung et al., 2013). In kidney, we observed a 25% higher LOOH concentration after exposure to 7.14 mg L^{-1} of Sencor, but at 71.4 mg L^{-1} pesticide LOOH levels had decreased to a level that was just 42% of control values. Increased lipid hydroperoxide content indicates enhanced oxidative modification to lipids. Thus, it can be suggested that Sencor exposure enhanced ROS levels resulting in stimulated oxidation of polyunsaturated fatty acids. At the same time, it can be suggested that decreased lipid hydroperoxide levels at high Sencor levels could result from either decreased production or enhanced degradation of LOOH. Similarly, Blahova et al. (2013) reported increased levels of oxidized lipids in experimental zebrafish groups exposed to 30 or 90 μg L^{-1} atrazine. In previous studies we reported stimulation of lipid peroxidation in fish by pesticides such as Roundup (Lushchak et al., 2009) and Tattoo (Kubrak et al., 2012).

Thiol-containing components are frequently used as markers of oxidative stress because they can be easily oxidized and serve as a sink for free radicals and other reactive species (Halliwell and Gutteridge, 1989; Hermes-Lima, 2004). In animal tissues low molecular mass thiols are represented mainly by the tripeptide glutathione (GSH) and the free amino acid cysteine (Lushchak, 2012). In the present study, we did not find any changes in low or high molecular mass thiol concentrations in kidney of goldfish exposed to the herbicide Sencor (Table 2). Similarly, no effects were observed in thiol concentrations in kidney of goldfish exposed

Table 2
The concentrations of high (H-SH) and low molecular mass (L-SH) thiols, and activities of glutathione-*S*-transferase (GST) and acetylcholinesterase (AChE) in kidney of goldfish exposed to control conditions or 7.14, 35.7 or 71.4 mg L^{-1} of Sencor for 96 h.

Parameter	Fish group			
	Control	7.14 mg L^{-1}	35.7 mg L^{-1}	71.4 mg L^{-1}
H-SH (μmol gwm^{-1})	9.97 ± 0.67	9.80 ± 0.48	9.17 ± 0.37	10.9 ± 1.1
L-SH (μmol gwm^{-1})	2.21 ± 0.19	2.44 ± 0.31	2.92 ± 0.08	2.54 ± 0.10
GST (U mg protein^{-1})	0.75 ± 0.05	0.84 ± 0.07	0.71 ± 0.06	0.67 ± 0.04
AChE (mU mg protein^{-1})	12.6 ± 1.3	12.8 ± 0.9	8.7 ± 0.3*	12.6 ± 0.5

Data are presented as means ± S.E.M, $n = 5–7$.
* Significantly different from the control group with $P < 0.05$.

for extended times to the mancozeb-containing fungicide, Tattoo (Atamaniuk et al., 2013).

In conclusion, the data in this section show that changes in markers oxidative stress in kidney of goldfish exposed to different concentrations of Sencor were not linearly dose-dependent.

3.4. Sencor effects on activities of antioxidant and related enzymes in kidney

Antioxidant enzymes, such as SOD, catalase and GPx play an important role in tissue protection against ROS attack (Lushchak, 2011). In this study, the activity of the primary antioxidant enzyme, SOD, did not change after 96 h of goldfish exposure to any of the concentrations of Sencor used (Fig. 3A). Paulino et al. (2012) also reported no changes of SOD activity in gills of a neotropical freshwater fish (*Prochilodus lineatus*) during acute exposure to different atrazine concentrations. However, Xing et al. (2012) observed a significant decrease in SOD activity in kidney of common carp exposed to atrazine at concentrations 42.8 and 428 μg L^{-1}. Jin et al. (2010) reported increased SOD activity in liver of zebrafish (*Danio rerio*) exposed to atrazine for 14 days. Velisek et al. (2011) observed increased SOD activity in liver and brain of common carp after long-term exposure to terbutryn. Activity of SOD did not change in brain, gills, liver or intestine of common carp after 14, 28 or 60 days of simasine treatment, but activity increased in muscle after 14 and 28 days (Stara et al., 2012). However, the activity of SOD increased in muscle of fish groups exposed to high concentrations (2 mg L^{-1}, 4 mg L^{-1}) of simazine for 14 and 28 days, but decreased after 60 days of treatment as compared to the control group (Stara et al., 2012).

Under control conditions, the activity of catalase was 15.7 ± 0.6 U mg protein^{-1}. We observed lowered catalase activity, by 31 and 34%, in kidney of goldfish exposed to 35.7 and 71.4 mg L^{-1} of Sencor, respectively, as compared to control values (Fig. 3B). It was reported earlier that catalase is sensitive to free radicals, in particular superoxide anion and hydrogen peroxide, which can inactivate the enzyme (Kono and Fridovich, 1982; Lushchak et al., 2009; Semchyshyn and Lozinska, 2012). Triazine-induced inhibition and induction of catalase activity by xenobiotics was reported for various fish species. Xing et al. (2012) reported decreased catalase activity in kidney of common carp treated with atrazine at 450 μg L^{-1}. Blahova et al. (2013) also observed a significant decline in catalase activity in all experimental groups of zebrafish exposed to atrazine. However, Jin et al. (2010) reported increased catalase activity in liver of zebrafish after 14 day exposure to atrazine at a concentration of 1000 μg L^{-1}. The activity of catalase increased in different tissues (liver, muscle and brain) of common carp, exposed to high concentrations of simazine (2 and 4 mg L^{-1}) over 14 and 28 days, whereas it decreased after 60 days of treatment under the same conditions (Stara et al., 2012). Paulino et al. (2012) observed no changes in catalase activity in gills of a neotropical freshwater fish *P. lineatus* after acute exposure to different atrazine concentrations.

Glutathione peroxidase (GPx) is considered to be an efficient protective enzyme that prevents lipid peroxidation at the expense of glutathione (Moreno et al., 2005). The activity of GPx activity correlates with tissue efficiency to neutralize lipid peroxides. In this study, the activity of GPx in control fish was 216 ± 12 mU mg protein^{-1} (Fig. 3C). Exposure of fish to Sencor led to lower GPx activities by 14, 28 and 33% in the experimental groups treated with 7.14, 35.7 and 71.4 mg L^{-1} of Sencor, respectively (Fig. 3C). The decreased activity of GPx in our experiments clearly indicates a possible Sencor-induced inactivation of this enzyme probably due to enhanced levels of ROS (Bagnyukova et al., 2005). Similar results were observed previously in kidney of common carp injected with atrazine (Xing et al., 2012). Stara et al. (2012) also observed a decrease in GPx activity in liver of common carp at different concentrations of simazine, but no changes were found in the brain, muscle, gills, and intestine of the fish. The decreased activities of both CAT and GPx in kidney of Sencor treated fish indicate the highly reduced capacity to scavenge hydrogen peroxide produced in this tissue, with an increase in ROS and oxidative stress in response to acute intoxication with pesticide.

Glutathione-*S*-transferase (GST) is a very important enzyme involved in detoxification of many xenobiotics (Siddiqui et al., 1993). The activity of GST in kidney of control goldfish was 0.75 ± 0.05 U mg protein^{-1} and was not affected by Sencor treatment (Table 2). Hostovsky et al. (2012) investigated GST activity in early developmental stages of common carp after subchronic exposure to terbuthylazine and metribuzin and found no significant changes in GST activity at any of the tested terbuthylazine concentrations. Paulino et al. (2012) also reported no changes in GST activity in gills of *P. lineatus* after acute exposure to different atrazine concentrations. Previously, we also found no changes in GST activity in kidney of goldfish treated with different concentrations of the mancozeb-containing carbamate fungicide Tattoo (Atamaniuk et al., 2013).

Treatment of fish with 7.14–71.4 mg L^{-1} of Sencor decreased GR activity by 17–25% in kidney (Fig. 4A). Similarly, Velisek et al. (2011) found decreased activity of GR in liver and intestine of common carp after long-term exposure to terbutryn. Similar to catalase and GPx, GR is also susceptible to ROS and could be inactivated by them (Bagnyukova et al., 2005). Alternatively, decreased GR activity could lead to GSH depletion under conditions where its loss cannot be compensated for by the synthesis of new glutathione molecules (Zhang et al., 2004). No significant difference was found in GR activity in any groups of common carp under chronic exposure to simazine (Stara et al., 2012). However, Hostovsky et al. (2012) observed that triazine herbicides (terbuthylazine and metribuzin) increased GR activity at early developmental stages of common carp after subchronic exposure.

Glucose-6-phosphate dehydrogenase (G6PDH) is believed to be the main supplier of the NADPH needed for the GR-catalyzed reaction (Halliwell and Gutteridge, 1989). In kidney, a significant increase (by 30 and 25%) in G6PDH activity was observed after animal exposure to 7.14 and 35.7 mg L^{-1} of Sencor, respectively

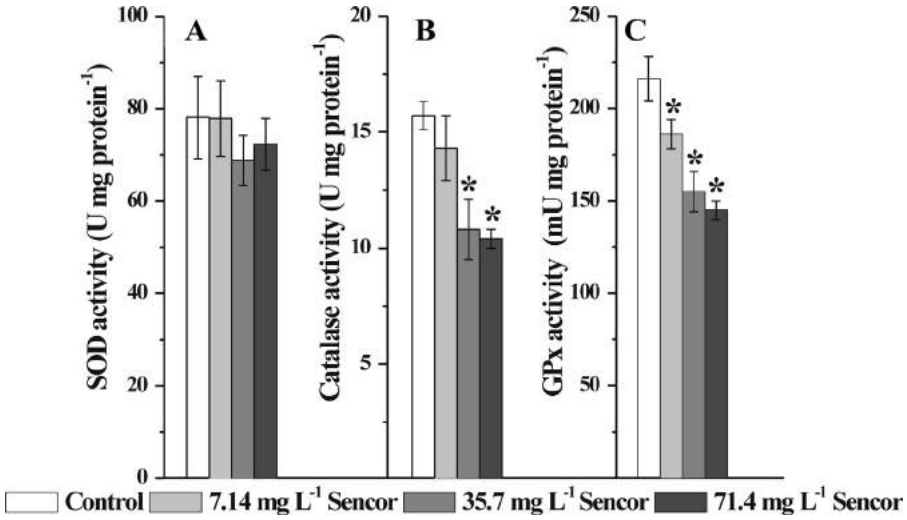

Fig. 3. The activity of antioxidant enzymes, (A) superoxide dismutase (SOD), (B) catalase and (C) glutathione peroxidase (GPx) in kidney of goldfish, exposed to control conditions or 7.14, 35.7 or 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 2.

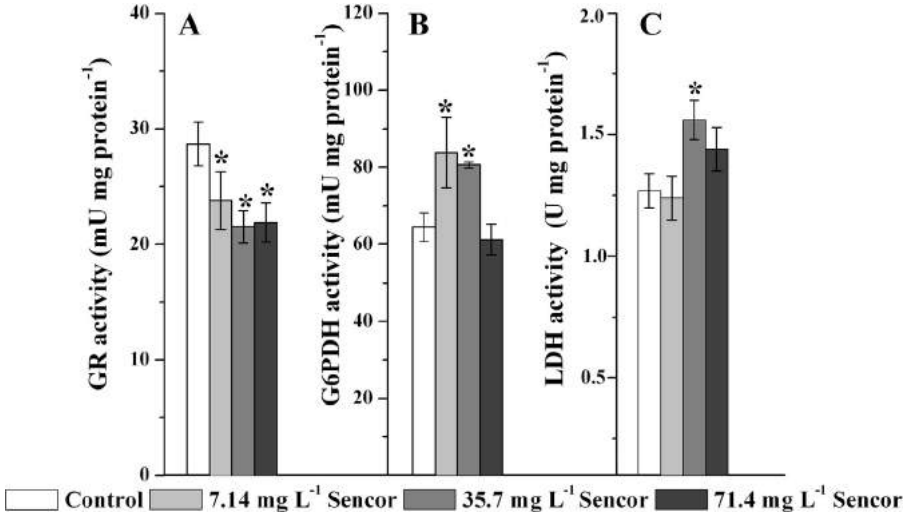

Fig. 4. The activity of (A) glutathione reductase (GR), (B) glucose-6-phosphate dehydrogenase (G6PDH) and (C) lactate dehydrogenase (LDH) in kidney of goldfish, exposed to control conditions or 7.14, 35.7 or 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 2.

(Fig. 4B). The elevated G6PDH activity could increase NADPH production in kidney to meet the needs of some antioxidants or biosynthetic processes in this tissue under Sencor exposure. Previously, we found decreased activity of this enzyme in the kidney of goldfish exposed to different concentrations of the mancozeb-containing carbamate fungicide, Tattoo (Atamaniuk et al., 2013).

LDH activity is widely used in toxicology and clinical biochemistry as a marker of cell injury at tissue and organ levels. Changes in LDH activity have been used as an indication of alterations in the pathways of cellular energy production induced by toxicants (Tietz, 1987; Gupta et al., 1991). In the present study we found that LDH activity increased by 22% in kidney of fish exposed to 35.7 mg L^{-1} Sencor. However, no changes in LDH activity occurred after exposure to other Sencor concentrations (Fig. 4C). The increase in LDH activity may indicate metabolic changes in the kidney, i.e. possible increased reliance on carbohydrate catabolism as well as formation of lactate (Simon et al., 1983). Oropesa et al. (2009) reported no effects of simazine on the activity of LDH in common carp *C. carpio* L.

The enzyme AChE has been used as a biomarker of exposure to certain groups of pesticides (Grue et al., 1997). The activity of AChE decreased by 31% in kidney of goldfish exposed to 35.7 mg L^{-1} Sencor as compared with control values (Table 2). It is known that some commonly used pesticides can inhibit AchE function by binding with this enzyme and disrupting nervous system activity (Eder et al., 2007). Atrazine is a known inhibitor of AChE and has been shown to operate in a dose-dependent manner in the freshwater fish species *Oreochromis niloticus* and *Chrysichthyes auratus* (Hussein et al., 1996). However, goldfish treatment with Tattoo did not affect AChE activity in kidney (Atamaniuk et al., 2013).

4. Conclusions

The results of this study, in particular the histopathological changes, indicate goldfish exposure to Sencor during 96 h caused severe deleterious effects which could be related to kidney dysfunction. The activity of several antioxidant and associated enzymes (GPx, catalase, LDH, GST and GR) seem to support these findings. However, the ambiguous effects of different Sencor concentrations on the level of CP and LOOH only weakly relate this severe dysfunction with oxidative stress. Using fish kidney as a biomarker of the

presence of Sencor in the aquatic environment needs further and a more detailed investigation.

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