Effects of subchronic metribuzin exposure on common carp (*Cyprinus carpio*)

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Abstract

OBJECTIVES: Effects of metribuzin on biochemical and haematological indices, induction of specific biomarkers and impacts on biometric parameters of *Cyprinus carpio* were investigated for subchronic conditions.

METHODS: Juvenile fish were exposed to 0.175 mg.L^{-1} or 1.75 mg.L^{-1} metribuzin for 28 days. Haematological indices were assessed using unified methods of haematological examination in fish. Biochemical indices were determined by biochemical analyzer, hepatic vitellogenin content was estimated by direct sandwich ELISA. Cytochrome P450 concentration and ethoxyresorufin O-deethylase activity were measured in liver spectrophotometrically and spectrofluorimetrically, respectively.

RESULTS: Increased haematocrit and RBC count were found in fish exposed to 1.75 mg.L^{-1} metribuzin compared to control fish ($p<0.05$). Other indices contents and biomarkers levels were not significantly changed by either concentration of metribuzin.

CONCLUSION: Sublethal metribuzin pollution may have adverse impacts on haematological parameters in common carp.

Abbreviations

EROD	- ethoxyresorufin O-deethylase
VTG	- vitellogenin
CYP	- cytochrome P450
HSI	- hepatosomatic index
RBC	- red blood cell
PCV	- packed cell volume, haematocrit
Hb	- haemoglobin
MCV	- mean corpuscular volume
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
WBC	- white blood cell
TP	- total protein
TAG	- triglycerides
ALT	- alanine aminotransferase
AST	- aspartate aminotransferase
LDH	- lactate dehydrogenase

ORIGINAL ARTICLE

INTRODUCTION

Pollution of aquatic environments with contaminants of anthropogenic origin has frequently been reported. Fish exposure to both lethal and sublethal levels of pesticides can cause metabolic disturbances that can be measured as biochemical and as haematological responses (Roche & Boge, 1996; Dobsikova *et al*. 2006; El-Sayed *et al*. 2007). Sublethal exposure to several environment pollutants can be determined by endocrine disruption parameters such as vitellogenin in fish (Kime *et al*. 1999; Hiramatsu *et al*. 2006) or an increase in detoxifying parameters such as cytochrome P450 (Porte *et al*. 2002; Hartl *et al*. 2007).

Triazine herbicides are widely used pesticides frequently detected in natural watercourses (Frank *et al*. 1990; Batista *et al*. 2002; Maloschik *et al*. 2007). Adverse effects of triazine pesticide on non-target organisms have been studied (Asongalem & Akintonwa, 1998; Oropesa-Jimenez *et al*. 2005), and endocrine disruptive effects of atrazine on reproduction have been reported (Moore & Waring, 1998; Spano *et al*. 2004).

Little information exists regarding the impact of the triazine herbicide, metribuzin (4-amino-6-(1,1-dimethylethyl)-3-methylthio-1,2,4-triazin-5(4H)-one). Metribuzin herbicides are used to control a wide range of broadleaf and grass weeds infesting potatoes, alfalfa, tomatoes, peas and other crops. They are intended for pre-emergence and post-emergence application. Metribuzin disrupts the photosynthetic processes of a wide variety of plants due to inhibition of photosystem II of the Hill reaction (Pauli *et al*. 1991). Metribuzin is mildly toxic to fish (Kegley *et al*. 2007). It has been detected in surface and ground water in several countries (Lawrence *et al*. 1993; Cerejeira *et al*. 2003; Schuler & Rand, 2008). Velisek *et al*. (2008) assessed haematological, biochemical and histopathological changes in rainbow trout (Oncorhynchus mykiss) exposed to 62.51 mg.L^{-1} metribuzin for 96 h.

To our knowledge, no work has been done to address disruptive potential of sublethal concentrations of metribuzin in fish.

This study was conducted to examine the effects of sublethal concentrations of metribuzin on common carp Cyprinus carpio after 28 days subchronic exposure. Specific goals were to evaluate the effects on biochemical and haematological indices, level of hepatic vitellogenesis in male carp, biometric indices, and influence on cytochrome P450 concentration and EROD activity.

MATERIALS AND METHODS

Sixty-three juvenile common carp, Cyprinus carpio, (body weight 34–44 g, approximately 1 year old) were purchased from a fish farm. The fish were kept in six 100 L fibreglass aquaria filled with dechlorinated tap water with continuous aeration. The maximum number of fish in each aquarium was twelve. A 12h photoperiod was maintained. The fish were supplied 3 times a day with commercial feed at a total rate of 1.5% body weight. The mean values for water quality were: dissolved oxygen levels ≥60% of saturation levels; pH 8.1–8.4; temperature 21±2 °C. Fish were acclimatized for 14 days prior to experimentation.

The commercial formulation of Sencor® 70WG (Bayer CropScience AG, Germany, metribuzin 700 g.kg^{-1}) was used in the study. Carp were exposed to metribuzin at concentrations of 0.175 mg.L^{-1} (23 fish) and 1.75 mg.L^{-1} (21 fish) for 28 days. The test concentrations were chosen as 1/45 and 1/450 96LC50 reported for rainbow trout and bluegill sunfish; 76–80 mg.L^{-1} (Gangolli, 1999). The control group (19 fish) was subjected to dechlorinated tap water only. The experiment was performed under semistatic conditions, in which the water was renewed every 48 h. After the stipulated exposure period, blood was collected from the heart using a heparinized needle. An aqueous solution of heparin sodium salt at 0.01 ml.ml^{-1} blood was used for blood stabilization. The fish were killed by cutting the vertebral column at the base of the skull. Body weight was recorded and the gonads and liver were dissected. Hepatosomatic index was calculated with the following formula: HSI=liver weight/total body weight×100. The liver samples were stored at –85 °C until cytochrome P450, EROD, and vitellogenin determination. Gonads were fixed in 10% buffered formalin and examined by light microscopy for sex determination.

Haematological parameters (RBC and WBC counts, PCV, Hb, MCV, MCH, MCHC) were determined in individual blood samples according to Svobodova *et al*. (1986). The remaining heparinized blood was centrifuged (800 g, 10 min, 4 °C) to separate plasma. Assessed plasma biochemical indices (glucose, albumin, TP, TAG, sodium, calcium, phosphorus, chloride, potassium, lactate, LDH, AST and ALT) were measured with the biochemical analyzer Cobas EMira using commercial test kits (BioVendor).

Liver sample processing

Individual liver samples (n=63) were homogenized in buffer (pH=7.4) and centrifuged (10,000 g, 20 min, 4 °C). The supernatant from each was pipetted into ultracentrifugation tubes and re-centrifuged at 100,000 g for 1 h at 4 °C. Each supernatant was drained, and the pellet washed with buffer (pH 7.4) and re-suspended in the same buffer. Each suspension was put into an Eppendorf tube and stored in at –85 °C for enzymatic assay. Microsomal protein concentrations were determined by the method of Lowry *et al*. (1951) before assay.

Quantities of total CYP were measured by spectrophotometry at 400–490 nm, on the basis of the difference between absorbance readings at 450 and 490 nm. Activity of EROD was determined spectrofluorimetrically. Both methods are described in detail by Siroka *et al*. (2005).

For VTG analysis, liver was homogenized in buffer (50 mM tris-HCl, pH 8.0; 0.02% aprotinin, 0.1 mM phenylmethanesulfonyl fluoride) and centrifuged (14,000 g, 60 min, 4 °C). Supernatants were examined for VTG concentration by direct sandwich enzyme-linked immunosorbent assay using Carp Vitellogenin kit (Biosense Laboratories, Norway) according to the manufacturer's instructions. Assessment was performed spectrophotometrically at 492 nm using Multiscan RC (Labsystems, Finland).

Statistical analysis

Data were evaluated for analysis of variance using Statistica 6.0 (ANOVA–Tukey Test). EROD data, of which non-normal distribution was identified, were assessed by the Kruskal–Wallis test.

RESULTS

Biometric indices

There was no significant difference from controls in the body weight of exposed fish (control: 37.84±11.75 g; metribuzin 0.175 mg.L^{-1}: 44.09±10.03 g; metribuzin 1.75 mg.L^{-1}: 35.57±10.28 g). The values of hepatosomatic index of treated fish were unchanged compared to control group (control: 2.55±0.34; metribuzin 0.175 mg.L^{-1}: 2.49±0.39; metribuzin 1.75 mg.L^{-1}: 2.56±0.47).

Haematological profile

Haemoglobin, RBC count, PCV and MCV were found to be increased in both treated groups. However, there was significant difference ($p<0.05$) in the 1.75 mg.L^{-1} metribuzin exposure for RBC count and haematocrit only (Figure 1 and 2). Values recorded for white blood cell count were comparable to control levels (Table 1).

Biochemical profile

Although no significant differences were observed, TP, albumin, TAG, glucose levels, and ALT activity were found to diminish slightly at both exposure concentrations. Lactate content and sodium ion were elevated insignificantly with 1.75 and 0.175 mg.L^{-1} metribuzin. Plasma concentrations of calcium, phosphorus, chloride and potassium as well as LDH and AST activity in both treatment groups were comparable to the control group levels (Table 2).

Vitellogenin

Hepatic vitellogenesis in male fish was not elevated in either experimental group compared to control group. Low concentrations of VTG were measured in 3 male fish overall (one in control and both treated groups), whereas VTG was not detected in remaining liver samples.

Cytochrome P450 and EROD

Liver cytochrome P450 activity, evaluated through ethoxyresorufin O-deethylase (EROD), was not enhanced by either 0.175 mg.L^{-1} or 1.75 mg.L^{-1} metribuzin (CYP: 0.04±0.03; 0.04±0.04; 0.04±0.03 nmol.mg^{-1} of microsomal protein in control, 0.175 mg.L^{-1} and 1.75 mg.L^{-1} metribuzin, respectively. EROD: 8.18±7.78; 4.79±4.50; 2.93±3.61 pmol.min^{-1}.mg^{-1} of microsomal protein in control, 0.175 mg.L^{-1} and 1.75 mg.L^{-1} metribuzin, respectively.).

DISCUSSION

The impact of metribuzin on growth parameters in Lepomis macrochirus was studied by Fairchild and Sappington (2002). After a six week experiment in outdoor aquatic mesocosms, no significant effects were

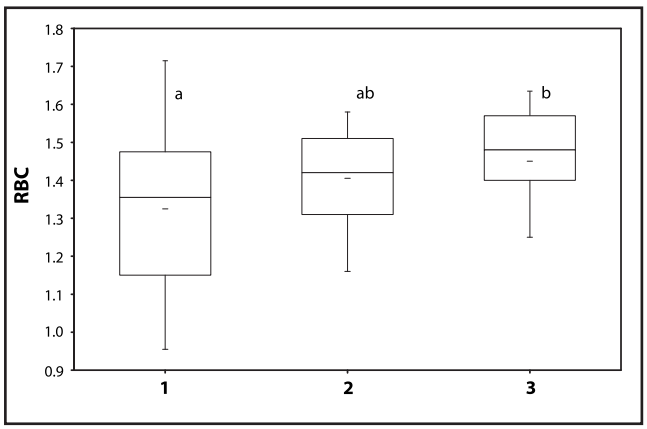

Figure 1. RBC count in test groups of carp after 28 days exposure
RBC = RBC [10^{12}.L^{-1}]; 1 = control fish (n=19); 2 = fish exposed to 0.175 mg.L^{-1} metribuzin (n=23); 3 = fish exposed to 1.75 mg.L^{-1} metribuzin (n=21)
a, b = different alphabetic letters differ significantly ($p<0.05$)
Median = Middle line of the box; Lower (Upper) Quartile = Bottom (Top) line of the box; Lower (Upper) whisker = Lower (Upper) adjacent value

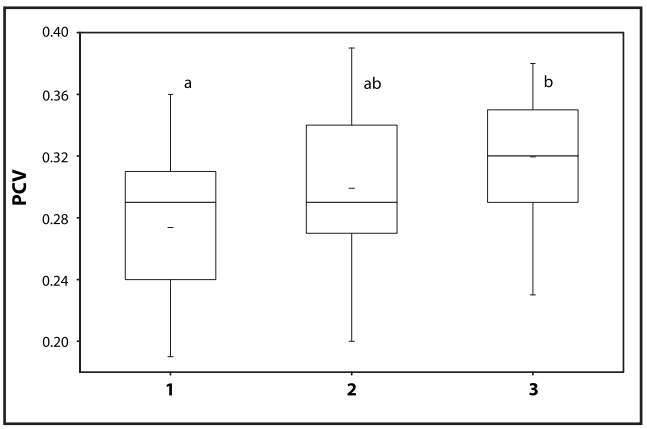

Figure 2. PCV in test groups of carp after 28 days exposure
PCV = PCV [L.L^{-1}]; 1 = control fish (n=19); 2 = fish exposed to 0.175 mg.L^{-1} metribuzin (n=23); 3 = fish exposed to 1.75 mg.L^{-1} metribuzin (n=21)
a, b = different alphabetic letters differ significantly ($p<0.05$)
Median = Middle line of the box; Lower (Upper) Quartile = Bottom (Top) line of the box; Lower (Upper) whisker = Lower (Upper) adjacent value

Table 1. Haematological indices in test groups of carp after 28 days exposure

Index	Units	Control	Metribuzin 0.175 mg.L^{-1}	Metribuzin 1.75 mg.L^{-1}
Hb	[g.L^{-1}]	44.2 ± 18.3	48.4 ± 12.8	49.2 ± 20.6
MCV	[10^{-15}.L]	209 ± 40	214 ± 36	222 ± 25
MCH	[10^{-12}.g]	33.3 ± 12.5	35.0 ± 11.7	33.8 ± 14.1
MCHC	[L.L^{-1}]	0.16 ± 0.06	0.16 ± 0.04	0.15 ± 0.06
WBC	[10^{9}.L^{-1}]	37.1 ± 14.6	31.7 ± 11.5	44.1 ± 23.8

Hb: n=19 (control); n=23 (0.175 mg.L^{-1});
n=21 (1.75 mg.L^{-1} metribuzin)
WBC: n=19 (control); n=12 (0.175 mg.L^{-1});
n=10 (1.75 mg.L^{-1} metribuzin)

Table 2. Biochemical indices in test groups of carp after 28 days exposure

Index	Units	Control	Metribuzin 0.175 mg.L^{-1}	Metribuzin 1.75 mg.L^{-1}
TP	g.L^{-1}	27.2 ± 2.9	25.3 ± 4.0	26.6 ± 2.4
albumin	g.L^{-1}	17.9 ± 3.7	15.0 ± 4.2	16.9 ± 1.6
glucose	mmol.L^{-1}	4.4 ± 1.2	3.8 ± 1.0	4.3 ± 0.6
TAG	mmol.L^{-1}	2.1 ± 0.4	1.7 ± 0.5	1.8 ± 0.4
lactate	mmol.L^{-1}	2.5 ± 0.9	4.1 ± 2.4	5.7 ± 4.2
ALT	μkat.L^{-1}	0.8 ± 0.3	0.6 ± 0.3	0.5 ± 0.1
AST	μkat.L^{-1}	4.3 ± 1.4	4.7 ± 1.5	3.6 ± 0.8
LDH	μkat.L^{-1}	11.6 ± 4.6	10.8 ± 4.2	12.3 ± 5.3
Na	mmol.L^{-1}	126.3 ± 14.3	135.5 ± 12.1	137.3 ± 7.7
Cl^{-}	mmol.L^{-1}	111.5 ± 3.7	112.2 ± 4.5	109.8 ± 2.2
Ca	mmol.L^{-1}	2.3 ± 0.2	2.5 ± 0.2	2.6 ± 0.1
P	mmol.L^{-1}	3.4 ± 1.4	3.1 ± 1.2	3.7 ± 0.7

n=10 (control); n=8 (Cl^{-}:0.175 mg.L^{-1} metribuzin);
n=13 (AST: 0.175 mg.L^{-1} metribuzin);
n=14 (remaining indices: 0.175 mg.L^{-1} metribuzin);
n=5 (remaining indices: 1.75 mg.L^{-1} metribuzin)

detected at treatment levels ranging up to and including 75 μg.L^{-1}. Effects of metribuzin on HSI have not been studied previously. HSI is considered a general indicator of overall fish health and environmental conditions. Both positive and negative associations between HSI and specific contaminants have been shown (Everaarts *et al*. 1993; Larose *et al*. 2008).

In the acute test on rainbow trout exposed to 62.51 mg.L^{-1} metribuzin, Velisek *et al*. (2008) found a significantly lower RBC count and PCV. High concentrations of toxic substances can significantly damage the haematopoetic system of fish and in some cases they can even cause an increased disintegration of erythrocytes (Svobodova *et al*. 1994). In the present subchronic study an increase in PCV and RBC count was seen in fish exposed to 1.75 mg.L^{-1} metribuzin. The increase in RBC count may be attributed to enhanced erythropoietic activity as a response to respiratory distress caused by metribuzin. Morphological changes in gill structure such as hyperplasia of gill epithelium are commonly reported symptoms of pollutant toxic effects (Oropesa-Jimenez *et al*. 2005). This can reduce the absorption of oxygen by the gills. Histological examination of gills was not performed in our study.

Red blood cell count and PCV were found to decrease up to 60 days in Tilapia mossambica exposed to atrazine (Prasad *et al*. 1991). Atamanalp and Yanik (2003) did not observe significant changes in the levels of RBC and WBC counts, PCV, MCV and MCHC in Oncorhynchus mykiss after 3 weeks exposure to mancozeb. In contrast, Cazenave *et al*. (2005) reported significantly higher values of haematological indices in fish exposed to pollutants compared to fish captured in an unpolluted area.

The slight reduction seen in total protein, albumin, glucose content, and triglyceride level in fish plasma could be the result of inhibition of synthesis in the liver and/or augmented utilisation. Total protein in plasma has been shown to decrease in fish with long-term exposure to pollutants (Svobodova *et al*. 1994). Bansal *et al*. (1979) observed significantly decreased levels of TAG in Labeo rohita plasma following 60 day chlordane treatment.

The decreased activity of ALT may be attributed to a repressor effect in its synthesis or to the direct action of metribuzin on the enzyme. Changes in sodium level indicate impairment of regulation of the osmolarity system caused by metribuzin. The content of the other major serum electrolyte, chloride, was not affected. Serum sodium and chloride elevations were observed by Bansal *et al*. (1979) in the previously cited study using chlordane, which had a more pronounced influence on ion levels.

The mechanism of metribuzin metabolism in fish has not been studied. Bleeke *et al*. (1985) demonstrated the importance of cytochrome P450 for its metabolism in mice. In our study, the concentration of CYP and EROD activity did not increase. It has been observed that single xenobiotic compounds can act as inductors of specific isoenzymes, but inhibit others. In that case the amount of total CYP may not be affected (van der Oost *et al*. 2003), which may explain our results. For detection of pollution of aquatic environments, the CYP1 family has been so far proven to comprise the most sensitive indicators. The presence of CYP1A is associated with the activity of EROD that catalyses the production of 7-hydroxyresorufin from ethoxyresorufin (Siroka & Drastichova, 2004). Induction of CYP1A levels can be prevented by a high concentration of certain contaminants or by the presence of pollutants specifically inhibiting cytochrome P450 (Schlezinger & Stegeman, 2001; Siroka *et al*. 2005).

In the present study, we aimed to evaluate xenoestrogenic potency of metribuzin in fish using vitellogenin as a biomarker of exposure to xenoestrogens (Mikula

et al. 2006). Vitellogenin is normally synthesized in the female liver; the base levels of VTG in male fish are below those reported for females (Versonnen *et al*. 2004; Korner *et al*. 2007). The lack of induction of VTG in males in the present study may be due to weak endocrine-disruptive effects of metribuzin as well as the short exposure period used. In addition, endocrine disruption caused by triazine pesticides is not solely determined by VTG induction (Spano *et al*. 2004).

In conclusion, sublethal metribuzin pollution may have adverse impacts upon haematological parameters in common carp. Total cytochrome P450 concentration in fish is not a suitable biomarker of metribuzin pollution of the aquatic environment.

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