ORIGINAL ARTICLE

Effects of subchronic exposure to Successor® 600 (pethoxamid 600 g L^{-1}) on common carp *Cyprinus carpio*

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Abstract

OBJECTIVES: We investigated the effects of the herbicide preparation Successor® 600 on biochemical and haematological indices and the histopathological parameters of common carp after 28 day exposure. Furthermore, the hepatosomatic index and induction of xenobiotic metabolizing enzymes and biomarkers were assessed.

DESIGN: Juvenile fish were exposed to sublethal concentrations of Successor® 600 (0.06 mg L^{-1}, 0.22 mg L^{-1} or 0.60 mg L^{-1}) for 28 days. Haematological indices were assessed using unified methods of haematological examination in fish. Plasma biochemical indices were measured by biochemical analyzer, the vitellogenin concentration in male fish plasma was estimated by direct sandwich ELISA. In hepatopancreas, ethoxyresorufin-O-deethylase (EROD) activity was measured spectrofluorimetrically, concentration of total cytochrome P450 (CYP), glutathion (GSH) content and glutathion-S-transferase (GST) activity were determined spectrophotometrically. Histological changes in samples of hepatopancreas, skin, gills, spleen, head kidney and trunk kidney were examined by light microscopy.

RESULTS: Haemoglobin, MCH and MCHC were significantly ($p<0.05$) reduced in fish treated with Successor® 600 of 0.22 and 0.60 mg L^{-1}. LDH was enhanced ($p<0.05$), in the highest concentration of the preparation. Vitellogenin was detected in all male fish, with no difference among groups. HSI, GSH and GST were elevated ($p<0.05$), owing to the exposure, whereas CYP and EROD were not affected. Slight histopathological changes were demonstrated in skin, gills and hepatopancreas, with steroid tissue in head kidney samples of 0.60 mg L^{-1} treated fish.

CONCLUSION: Successor® 600 affected the haematological profile of the treated fish, while the effects on biochemical indices were less expressed. Male plasma vitellogenin concentrations were not indicative of estrogen disruptive effects after 28 days. The importance of GSH and GST for the metabolisation were demonstrated. In contrast, CYP and EROD were not influenced by any concentration tested. HSI was found to reflex pollution with Successor® 600. Histopathological indices caused by the treatment were observed in various tissue samples of the treated fish.

Abbreviations & units

EROD – ethoxyresorufin O-deethylase
Vtg – vitellogenin
CYP – cytochrome P450
HSI – hepatosomatic index
GST – glutathione-S-transferase
GSH – glutathione
RBC – red blood cell count
PCV – packed cell volume, haematocrit
Hb – haemoglobin
MCV – mean corpuscular volume
MCH – mean corpuscular haemoglobin
MCHC – mean corpuscular haemoglobin concentration
WBC – white blood cell count
GLU – glucose
ALB – albumine
TP – total protein
TAG – triglycerides
ALT – alanine aminotransferase
AST – aspartate aminotransferase
LDH – lactate dehydrogenase
XME – xenobiotic metabolizing enzymes

INTRODUCTION

In order to maintain and intensify contemporary agricultural production, new crop protection compounds are developed and applied. Except for positive effects, the risk of undesired aquatic contamination still exists. The impact of "traditional" pesticides on non-target organisms have been discussed widely (Modra *et al.* 2008; Dobsikova *et al.* 2006) whereas there is insufficient information on the possible adverse influence of novel substances.

Successor® 600 (pethoxamid 600 g L^{-1}) is a herbicide formulation introduced for grass and broadleaf weed control mainly in corn and soybeans. It is supposed for pre- or early postemergent application. Pethoxamid (2-chloro-N-(2-ethoxyethyl)-N(2-methyl-1-phenyl-prop-1-enyl)acetamide) lacks 2,6-anilide disubstituted structure, thus it cannot be classified as an chloroacetanilide compound (e.g. alachlor, metolachlor). It belongs to the chloroacetamide chemical family. Pethoxamid appears to inhibit cell division in plants by interference with the biosynthesis of fatty acids.

The aim of our study was to evaluate the impact of Successor® 600 (pethoxamid 600 g L^{-1}) exposure on common carp (*Cyprinus carpio*) under subchronic conditions. We examined the effects on haematological and biochemical indices and hepatosomatic index. Induction of xenobiotic metabolizing enzymes and specific biomarkers, as well as effects on histological structure of selected tissues were investigated.

MATERIAL AND METHODS

Juvenile common carp *Cyprinus carpio* (1 year old, body weight 75±15 g) were randomly distributed into eight 100L glass aquaria. A total of 94 fish were used, the maximum number in each aquarium was 12. The fish were acclimated to laboratory conditions for 14 days. They were supplied twice a day with commercial feed at a total rate of 1,5 % body weight. The fish were weighed again and the amount of feed was recalculated in the middle of the exposure period. The commercial formulation (Stähler International GmbH & Co.) of Successor® 600 (pethoxamid 600 g L^{-1}) was applied to the tanks to reach concentrations of 0.06; 0.22 and 0.60 mg L^{-1}. The producer states the preparation contains pethoxamid, solvent naphta (petroleum), ethoxylated polyarylphenol, calcium dodecylbenzene sulphonate and 2-ethylhexan-1-ol. Control fish were subjected to dechlorinated tap water. Each treated and control group was performed in duplicate. The experiment was conducted in a semistatic system for 28 days, solutions were changed every 48 hours. The pH of the solutions varied from 7.5 to 8.2, water temperature was maintained at 21±1.5 °C, oxygen saturation was ≥ 60 %. A photoperiod of 12/12 hrs was used.

After 28 days of exposure, individual blood samples were taken by cardiac puncture and stabilized with aqueous solution of heparin (50 IU per ml of blood). The fish were sacrificed, body weight was recorded and the gonads and liver (hepatopancreas) were dissected. The hepatosomatic index was calculated using the following formula: HSI = liver weight/total body weight × 100. Gonads were fixed in 10% buffered formalin and examined by light microscopy for sex determination. The liver samples were stored at -85 °C until further analyses were performed.

All analyses were conducted in 22–24 individual samples from each group of fish, except for Vtg and histological examination.

Haematological parameters. RBC, WBC, PCV, Hb, MCV, MCH, MCHC and differential leucocyte count – leucogram were assessed according to Svobodova *et al.* (1991).

Biochemical profile. Plasma biochemical indices (glucose, albumin, TP, TAG, calcium, phosphorus, chloride, lactate, LDH, AST and ALT) were determined by the biochemical analyzer CobasEmira using commercial test kits (BioVendor).

Vitellogenin. Plasma vitellogenin concentrations were measured by a 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).

Total CYP and EROD activity. Liver samples were homogenized in buffer (pH 7.4), centrifuged (10,000 g, 20 min, 4 °C) and the supernatant was re-centrifuged again (100,000 g, 1 h at 4 °C). The final supernatant was drained and the pellet was washed and resuspended in the buffer (pH 7.4). Each suspension was put into an Eppendorf tube and stored at -85 °C until enzymatic assays.

Microsomal protein concentrations were measured before the assays by the method of Lowry *et al.* (1951). Quantities of total CYP were determined spectrophoto-

Table 1: Haematological indices in common carp after 28 days exposure to Successor® 600

Indice	Unit	Control	Successor® 600 0.06 mg L^{-1}	Successor® 600 0.22 mg L^{-1}	Successor® 600 0.60 mg L^{-1}
RBC	[10^{12} L^{-1}]	1.36±0.17^{ab}	1.39±0.16^{b}	1.35±0.14^{ab}	1.26±0.15^{a}
WBC	[10^{9} L^{-1}]	37.2±15.8	38.0±10.9	39.3±12.7	45.1±17.0
Hb	[g L^{-1}]	67.2±11.8^{a}	62.2±15.3^{a}	41.2±13.2^{b}	33.8±10.5^{b}
PCV	[L L^{-1}]	0.27±0.02	0.28±0.02	0.28±0.03	0.27±0.04
MCV	[10^{-15} L]	202±25^{a}	202±22^{a}	211±24^{ab}	221±25^{b}
MCH	[10^{-12} g]	49.3±7.8^{a}	45.6±12.8^{a}	30.9±10.7^{b}	26.7±7.6^{b}
MCHC	[L L^{-1}]	0.24±0.04^{a}	0.22±0.05^{a}	0.15±0.05^{b}	0.13±0.04^{b}

a, b = different alphabetic letters differ significantly (p<0.05)

Table 2: Biochemical indices in common carp after 28 days exposure to Successor® 600

Indice	Unit	Control	Successor® 600 0.06 mg L^{-1}	Successor® 600 0.22 mg L^{-1}	Successor® 600 0.60 mg L^{-1}
GLU	[mmol L^{-1}]	5.63±1.80^{a}	4.05±0.97^{b}	5.65±1.27^{a}	5.56±1.10^{a}
ALT	[μkat L^{-1}]	0.54±0.30	0.47±0.21	0.50±0.33	0.57±0.23
Cl^{-}	[mmol L^{-1}]	112.08±5.60	108.15±20.09	116.22±3.92	114.62±8.75
LDH	[μkat.L^{-1}]	6.08±4.71^{a}	5.41±5.18^{a}	9.20±6.92^{ab}	12.46±8.95^{b}
lactate	[mmol L^{-1}]	2.47±1.43	2.26±1.30	1.88±0.96	2.08±0.87
AST	[μkat L^{-1}]	2.27±0.74^{a}	2.48±1.47^{a}	5.10±5.45^{b}	4.20±2.28^{ab}
TP	[g L^{-1}]	26.34±2.17	26.63±2.57	27.22±2.76	26.14±2.72
ALB	[g L^{-1}]	16.51±2.46	18.35±2.25	17.93±2.51	16.95±2.65
TAG	[mmol L^{-1}]	2.06±0.34	2.28±0.50	2.23±0.52	2.37±1.01
Ca	[mmol L^{-1}]	2.56±0.40	2.55±0.20	2.73±0.27	2.61±0.20
P	[mmol L^{-1}]	2.33±0.68	2.20±0.62	2.19±0.66	2.33±0.93

a, b = different alphabetic letters differ significantly (p<0.05)

metrically at 400–490 nm, on the basis of the difference between readings at 450 and 490 nm. The activity of EROD was examined spectrofluorimetrically (Chang & Waxman, 1998; Nilsen *et al.* 1998; Rutten *et al.* 1992) for excitation/emission wavelenghts setting 535/585 nm. For details see Siroka *et al.* (2005).

GSH, GST activity. In order to analyse glutathione-S-transferase (GST) activity, glutathione (GSH) content and protein concentration in liver samples, they were individually extracted with a phosphate buffer (pH 7.2) and homogenized. Supernatants from centrifuged homogenates (10,500 g, 20 min, at 4 °C) were used for determination. The activity of GST was measured spectrophotometrically at 340 nm according to Habig *et al.* (1974). Glutathione was determined spectrophotometrically at 414 nm using Ellman's method (1959). Concentrations (nmol GSH mg^{-1} of protein) were calculated according to a standard calibration. The protein concentrations were assessed with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) using bovine serum albumin as a standard.

Histological examination. For control and each concentration used, samples of hepatopancreas, skin, gills, spleen, head kidney and trunk kidney of four fish were removed and treated for histological examination.

Statistical analysis. Data were evaluated for analysis of variance (ANOVA–Tukey Test) using Unistat 6.1 software.

RESULTS

Haematological profile. Haemoglobin content, MCH and MCHC were decreased statistically significantly (p<0.05) in 0.22 and 0.60 mg L^{-1} treated fish. MCV was enhanced (p<0.05) in fish exposed to the highest concentration of Successor® 600, while the number of red and white bloods cells was not influenced (***Table 1***). No differences in the leucocyte profile of the formulation treated fish were found when compared to the control group.

Biochemical indices. Lactate dehydrogenase activity was found to increase in Successor® 600 0.60 mg L^{-1} treated fish (p<0.05), whereas the dose-related influence of the formulation on other indices was not observed (***Table 2***).

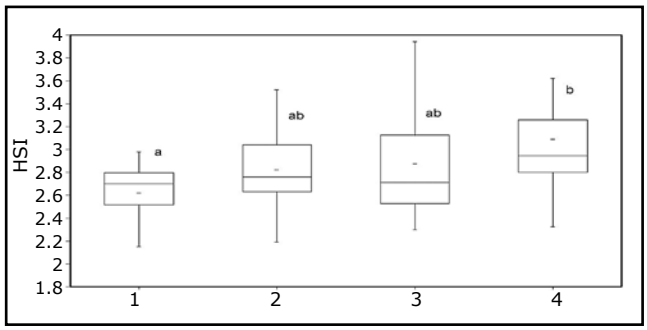

Figure 1. HSI of carp after 28 days exposure.
1 = control fish; 2–4 = fish exposed to Successor® 600 (2 = 0.06 mg L^{-1}; 3 = 0.22 mg L^{-1}; 4 = 0.60 mg L^{-1})
a, b = different alphabetic letters differ significantly (p<0.05)
Median = Middle line of the box; Lower (Upper) Quartile = Bottom (Top) line of the box; Lower (Upper) whisker = Lower (Upper) adjacent value

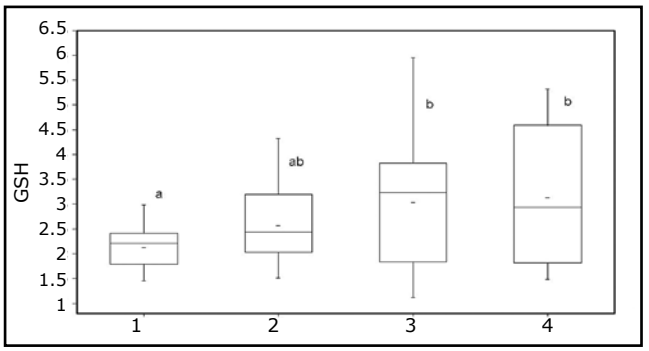

Figure 2. GSH content [nmol mg^{-1} of protein] in carp liver after 28 days exposure.
1 = control fish; 2–4 = fish exposed to Successor® 600 (2 = 0.06 mg L^{-1}; 3 = 0.22 mg L^{-1}; 4 = 0.60 mg L^{-1})
a, b = different alphabetic letters differ significantly (p<0.05)
Median = Middle line of the box; Lower (Upper) Quartile = Bottom (Top) line of the box; Lower (Upper) whisker = Lower (Upper) adjacent value

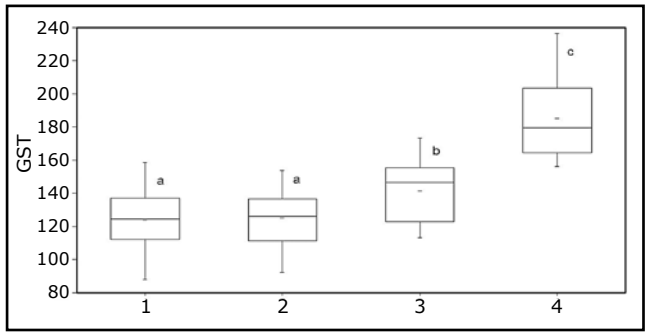

Figure 3. GST activity [nmol min^{-1} of mg^{-1} protein] in carp liver after 28 days exposure.
1 = control fish; 2–4 = fish exposed to Successor® 600 (2 = 0.06 mg L^{-1}; 3 = 0.22 mg L^{-1}; 4 = 0.60 mg L^{-1})
a, b, c = different alphabetic letters differ significantly (p<0.05)
Median = Middle line of the box; Lower (Upper) Quartile = Bottom (Top) line of the box; Lower (Upper) whisker = Lower (Upper) adjacent value

Vitellogenin. Plasma vitellogenin was detected in all tested male fish, including controls (control (n=5): 166±133 ng ml^{-1}; Successor® 600 0.06 mg L^{-1} (n=7): 440±254 ng ml^{-1}; Successor® 600 0.22 mg L^{-1} (n=12): 182±216 ng ml^{-1}; Successor® 600 0.60 mg L^{-1} (n=8): 197±251 ng ml^{-1}). No significant differences among groups were observed.

Cytochrome P450 and EROD. There was no significant difference from controls in concentration of total CYP and EROD in any treated group (CYP: 0.17±0.06; 0.13±0.06; 0.18±0.08; 0.20±0.07 nmol mg^{-1} of microsomal protein in control and preparation of 0.06; 0.22 and 0.60 mg L^{-1}, respectively. EROD: 19.78±11.99; 22.52±10.91; 17.72±11.37; 22.82±9.27 pmol min^{-1} mg^{-1} of microsomal protein in control and preparation concentrations of 0.06; 0.22 and 0.60 mg L^{-1}, respectively).

HSI. The value of HSI was elevated in all fish treated, however, the statistical significant difference (p<0.05) from control was found only in the highest concentration used (***Figure 1***).

GSH and GST. Content of glutathione and glutathione-S-transferase activity were raised (p<0.05) in two highest concentrations used (***Figure 2, 3***) when compared to the control group.

Histological examination. No pathological lesions were detected in the control fish samples. In all treated groups, increased activity of mucous cells was found in skin, the number of the cells was enhanced in the 0.60 mg L^{-1} group. Slight regressive alterations were demonstrated in gills of fish treated with either concentration of the preparation. No changes were revealed in trunk kidney and spleen of any examined sample. Extensive incidence of steroid tissue was observed in head kidney of fish exposed to the highest concentration of Successor® 600. Hepatocytes were slightly changed in fish from 0.22 and 0.60 mg L^{-1} treated groups.

DISCUSSION

Haematological profile. Pronounced reduction in haemoglobin content (p<0.05) in fish exposed to two highest concentrations of Successor® 600 caused a significant (p<0.05) decrease in MCH and MCHC, as PCV and RBC of the treated fish were not significantly affected when compared to the control group. A decrease in haemoglobin has been reported in fish after long-term exposure to pesticide compounds. However, it was usually accompanied by a similar shift in RBC and PCV values (Khalaf-Allah, 1999; Jee *et al.* 2005; Sweilum, 2006). The decline of haemoglobin concentration may originate from either a decrease in its synthesis or in an increase in the rate at which it is destroyed. As RBC, PCV and total protein levels were not significantly affected, hemolysis or bleeding and hemodilution are not probable causes of the finding. Alterations of hepatocytes found in the 0.22 and 0.60 mg L^{-1} treated groups could have contributed to the disorder of haemoglobin synthesis, as well as histological changes observed in head kidney of 0.60 mg L^{-1} treated fish. The number of

erythrocytes was found to be reduced in the 0.60 mg L^{-1} treated group, and MCV was significantly elevated ($p<0.05$) concurrently. Both impaired synthesis of haemoglobin and the incipience of erythropoiesis disturbances by a higher concentration of the formulation may explain the results.

Biochemical profile. Lactate dehydrogenase is the terminal enzyme of anaerobic glycolysis. It is regarded as a marker of tissue damage, hypoxia conditions and switch over to anaerobic metabolism (Das *et al.* 2004; Monteiro *et al.* 2007; Ahmad, 2009). Since glucose and lactate concentrations were not influenced in a dose-dependent manner, cellular harm remains the most likely reason for the elevation. However, other markers of tissue alterations were not enhanced except for AST, whose rise was not significant due to large experimental variability.

Vitellogenin. Under normal conditions, vitellogenin is detected only in the plasma of mature female fish. Its presence in male and juvenile fish indicates previous contact with xenoestrogenic compounds, since Vtg synthesis is estrogen-dependent (Kime *et al.* 1999). Circulating estrogen concentrations in male fish are too low to trigger expression of the Vtg gene (Sumpter & Jobling, 1995). Jobling *et al.* (1998) found plasma Vtg in male fish under controlled conditions at concentrations of 100 ng ml^{-1}. We observed concentrations of Vtg from 11 to 700 ng ml^{-1} in the test male carp, with a range from 27 to 325 ng ml^{-1} in the control fish. However, Successor® 600 did not further influence plasma vitellogenin levels in the exposed fish after 28 days. The carp were obtained from a commercial fish farm, where no contamination with estrogenic or estrogenic-like compounds was suspected.

XME. Determination of enzymes and cofactors involved in xenobiotics biotransformation is widely practised for assesment of exposure to pollutants. As compared with phase I systems, the induction responses of phase II enzymes are generally less pronounced (van der Oost *et al.* 2003). In our study, however, the exposure to Successor® 600 did not influence CYP concentration and EROD activity, while GSH and GST were significantly elevated. On the other hand, the response of total CYP to xenobiotics exposure is less sensitive than that in the levels or activities of selected isoenzymes (Bucheli & Fent, 1995). It was demonstrated that single xenobiotic compounds can act as inducers of specific isoenzymes, but inhibit others. This may result in a considerable alteration of isoenzyme levels, whereas the amount of total cytochrome P450 is not always affected (van der Oost *et al.* 2003). Ethoxyresorufin O-deethylase activity may be used as indicative of the cytochrome P4501A1 enzyme system function. Many compounds that induce EROD may also act as inhibitors of this enzyme activity under various conditions (e.g. at very high concentrations or in the presence of other inducing compounds) (Whyte *et al.* 2000).

The main route of pethoxamid detoxication may be the elimination of the conjugated form without the previous modification. It was suggested that the major metabolic pathway of pethoxamid in animals, plants and soil proceeds through glutathione conjugate (Kato *et al.* 2001). Tripeptide glutathione (L-γ-glutamyl-cysteinyl-glycine; GSH) is a major component of cellular antioxidant defenses (Otto & Moon, 1996) and a key conjugate of electrophilic intermediates in phase II of metabolism, the conjugation reaction is mediated principally via GST (van der Oost *et al.* 2003). Another function of the GST family is the transport of endogenous hydrophobic compounds such as steroids, bilirubin, haeme and bile salts (Blanchette *et al.* 2007) and the prevention of lipid peroxidation (Yang *et al.* 2001). GST may constitute up to 10 % of the hepatic cytosolic proteins (Leblanc, 1994). Both induction and inhibition of GST activity have been reported in fish either captured from polluted sites or treated with chemicals under laboratory condition (Frasco & Guilhermino, 2002; Monod *et al.* 1988; van der Oost *et al.* 1994).

HSI. The hepatosomatic index is regarded as a general indicator of fish health and the quality of the aquatic environment. HSI is a non-specific biomarker, influenced by factors such as sex, season, disease, nutritional level etc. (Slooff *et al.* 1983; van der Oost *et al.* 2003). In fish captured in polluted sites, an elevation of relative liver weights was accompanied both by liver cell hypertrophy (Slooff *et al.* 1983) and hyperplasia (Poels *et al.* 1980). Our results are in accordance with Stephensen *et al.* (2000), who suggested a relation between elevated HSI and increased activity of xenobiotic biotransformation enzymes. Enhanced HSI and hepatic enzyme activities were reported also by e.g. Huuskonen & Lindstrom-Seppa (1995) and Figueiredo-Fernandes *et al.* (2006). There is an association between liver weight and storing reserves (glycogen) (Svobodova, 1977). However, specific examination for their determination was not performed.

Histological examination. Changes in gills and skin are a common sign of fish from polluted areas. They indicate impairment of the organism and adaptation to the pollution situation (Oropesa-Jimenez *et al.* 2005). The incidence of steroid tissue in head kidney samples (Successor® 600 0.60 mg L^{-1}) could be in relationship with pronounced hypohaemoglobinemia in fish from the same group. Slight alterations detected in hepatopancreas could contribute to the significant enhancement of LDH, though, other biochemical parameters were not indicative of liver derogation.

In conclusion, under subchronic conditions, we have proven that the novel pesticide preparation Successor® 600 may cause significant disturbances of haemoglobin synthesis and induce production of liver xenobiotic biomarkers. Further research is necessary to elucidate the impacts and possible recovery process after a long-term exposure to the herbicide.

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