doi: 10.17221/8417-VETMED

# Effects of a cypermethrin-based pesticide on early life stages of common carp (*Cyprinus carpio* L.)

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**ABSTRACT**: The aim of this study was to assess the effects of Cyperkill 25 EC (a.i. cypermethrin 250 g/l) on cumulative mortality, growth indices, and ontogenetic development of embryos and larvae of common carp (*Cyprinus carpio* L.). An early-life stage toxicity test was used. Liver, intestine, kidneys, and gills of surviving larvae were examined, and the activity of the detoxifying and antioxidative enzymes glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST), as well as lipid peroxidation (TBARS) was determined. Eggs of common carp 24 h post-fertilisation were exposed for 35 days to Cyperkill 25 EC at concentrations of 7.2, 36, 72, 144, and 360 µg/l containing the active ingredient cypermethrin at concentrations of 1.8, 9, 18, 36, and 90 µg/l, respectively. All larvae exposed to concentrations higher than 144 µg/l showed signs of damage after five days and died in the next two days; at concentrations of 72 and 36 µg/l total mortality was observed several days after hatching. Larvae exposed to 7.2 µg/l survived to the end of the test but showed significantly lower growth ($P < 0.01$) and retarded ontogenetic development compared to controls. Examination of these larvae did not reveal histological changes. Activity of GST, GR, and GPx in the exposed group was significantly lower ($P < 0.01$), while CAT and TBARS did not show significant differences from controls. Exposure to Cyperkill 25 EC affected hatching and survival at tested concentrations above 7.2 µg/l. Alterations in oxidative stress parameters and retarded growth and ontogenetic development were evident at 7.2 µg/l.

**Keywords**: embryo-larva toxicity test; Cyperkill 25 EC; oxidative stress; pyrethroid; mortality; ontogenesis

Pyrethroids are synthetic analogues of the natural pyrethrins that occur in the ornamental pyrethrum daisy *Chrysanthemum cinerariaefolium* and related species. Synthetic analogues that show less rapid photodegradation have been developed to replace natural pyrethrins as agricultural pesticides. They are non-systemic insecticides that act through disruption of the nervous system leading to hyperactivity, paralysis, and death. The pyrethroids are also toxic to some non-target animals including crustaceans and fish. Median lethal concentrations of the more commonly used pyrethroids are generally less than 10 µg/l in fish, while birds and mammals show lower sensitivity (Bradbury and Coats 1989a). Pyrethroids are among the most commonly used pesticides worldwide, and pose a threat to the natural environment, including aquatic ecosystems (Richterova and Svobodova 2012). Contamination of surface water by pesticides is widespread (Hill 1985; Sibley and Kaushik 1991).

Cyperkill 25 EC contains 250 g/l cypermethrin. It is classified as a type II pyrethroid, which is more

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), and by the GAJU (No. 018/2014/Z).

doi: 10.17221/8417-VETMED

effective than type I (Narahashi 1986; Soderlund et al. 2002). Cypermethrin blocks sodium channels of nerve filaments by lengthening their depolarisation phase as well as affecting gamma-aminobutyric acid receptors and chloride and calcium channels in nerve filaments (Bradbury and Coats 1989b; Hayes 1994; Burr and Ray 2004). Cypermethrin contains chlorine atoms in a vinyl side chain of the compound. The presence of halogens contributes to greater insecticidal activity and higher stability, as well as providing better residual activity against insects. Further, halogen presence leads to a higher potential for negative effects on the environment (Brown at al. 1973; Bradbury and Coats 1989a). Cypermethrin is used to control pests including moths in cotton, fruits, and vegetable crops (Crawford et al. 1981). It is used in public health and animal husbandry, including in marine fish production to control ectoparasites (Treasurer and Wadsworth 2004).

The aim of this study was to assess the effect of synthetic cypermethrin, an active ingredient in the commonly used pesticide Cyperkill 25 EC, on early life stages of common carp using an embryo-larval toxicity test (EL test) supplemented by determination of oxidative stress parameters.

## MATERIAL AND METHODS

**Experimental substance**. Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate] was tested in the form of Cyperkill 25 EC pesticide (Agriphar S.A., Belgium) containing 250 g/l active substance. Emulsifiable concentrate (EC) formulations of pyrethroids are usually two to nine times as toxic as the technical-grade pyrethroids, most likely due to synergistic interactions (Smith and Stratton 1986). Alpha-cypermethrin as an active ingredient of this emulsifiable concentrate formulation contains two isomers. The cis/trans ratio is 40 : 60.

**Experimental animals**. Fertilised eggs of common carp (*C. carpio* L.) were obtained from the Breeding Station of the Department of Fish Genetics and Breeding of the Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Czech Republic. Eggs were produced according to standard methods of artificial reproduction (Kocour et al. 2005). All experimental procedures involving animals were in accordance with European Community guidelines. The Law on Protection of Animals against Cruelty Act No 246/1992 Coll. adopted by The Czech National Council as amended was followed throughout the whole experiment.

### Experimental design

**Early-life stage toxicity test**. This test followed the OECD 210 Guideline for Testing of Chemicals (OECD 2013), modified in that only fertilised eggs were selected for testing. One-hundred eggs at 24 h post-fertilisation were placed in crystallisation dishes containing 1 l of dechlorinated tap water (control) or 1 l aqueous Cyperkill 25 EC solution at 7.2, 36, 72, 144, or 360 µg/l representing cypermethrin concentrations of 1.8, 9, 18, 36, and 90 µg/l in solution, respectively. The beginning of the test was considered to be 24 h post-fertilisation and was designated Day 1. The test was conducted in two replicates. Water conditions were pH 7.98, $NH_4^+$, N < 0.02 mg/l; $NO_2^-$, N 0.006 mg/l; $NO_3^-$, N 1.55 mg/l, $PO_4^{3-}$, P 0.09 mg/l; $COD_{Mn}$ 0.6 mg/l. During the test, water temperature ranged from 21 to 23 °C, pH values from 7.5 to 8.5, and oxygen saturation did not drop below 60%. Water in all dishes was continuously gently aerated, and was renewed once daily. Unhatched eggs and dead embryos and larvae were removed and recorded. Hatching was essentially complete on Day 4. Beginning on Day 8, larvae were fed with freshly hatched brine shrimp *Artemia salina* nauplii *ad libitum*. Samples of embryos/larvae for observation of length and weight growth, ontogenetic development, and malformations were taken from the control group and from the 7.2 µg/l concentration on Days 5, 12, 19, 26, 33, and 35. During these days, ten embryos/larvae were taken from the control group and from the experimental group. Sampled embryos/larvae were fixed in 4% formalin. The experiment was terminated on Day 35, when the majority of control fish reached the juvenile stage. Ontogeny stages comprised nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages (Penaz et al. 1983). At the end of the test, samples from the control and the 7.2 µg/l concentration were taken for histological examination, fixed in 10% formalin solution and processed using conventional paraffin techniques.

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Tissue sections were stained with haematoxylin and eosin. Samples of liver, intestine, kidneys, and gills were examined at magnification 100–1000× using light microscopy.

**Cumulative mortality and biometric data**. Cumulative mortality was recorded daily. The fixed samples of embryos/larvae were weighed to the nearest 0.1 mg using a WAS 220/C/2 analytical balance (RADWAG Balances & Scales, Poland). Total length was measured to the nearest 0.01 mm using a binocular loupe and a scale. Fulton's condition factor (FCF) was calculated at the end of the trial for nineteen fish from the control and the lowest concentration of cypermethrin using the formula

$$FCF = W \times TL^{-3} \times 100$$

where:

W = weight in g

TL = total length in cm

The software program Statistica, v. 10.0 for Windows (StatSoft, Prague, Czech Republic) was used to compare length and weight differences among the test groups.

**Determination of oxidative stress**. Samples of larvae/juveniles for investigation of oxidative stress parameters were taken on Day 35 and placed immediately into liquid nitrogen for transport to a screening laboratory. Whole bodies were homogenised in a 50mM potassium phosphate buffer with 1mM EDTA (pH 7.4) and centrifuged at 11 200 g for 20 min at 4 °C. The supernatant was pipetted into individual Eppendorf tubes and held at –85 °C until analysis. Supernatant was used for determination of GR (glutathione reductase), GPx (glutathione peroxidase), CAT (catalase), and GST (glutathione-S-transferase) activity and protein concentration. Non-centrifuged homogenate (stored at –85 °C) was used to estimate lipid peroxidation. Protein concentration was quantified with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard (Smith et al. 1985). Total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm (Habig et al. 1974). Specific activity was expressed as the nmol of the formed product per min/per mg of protein. The catalytic concentration of GR was determined by measuring NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidation at 340 nm (Carlberg and Mannervik 1975). The catalytic concentration of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm (Flohe and Gunzler 1984). Specific activity of GR and GPx was expressed as nmol of NADPH consumption per min/per mg of protein. The CAT activity was determined by measuring $H_2O_2$ breakdown at 240 nm and expressed as μmol of decomposed $H_2O_2$ per min/per mg of protein (Aebi 1984). Lipid peroxidation was determined using the TBARS method at 535 nm (Lushchak et al. 2005), with concentration expressed as nmol/g wet weight of tissue. All parameters were measured spectrophotometrically using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific). Obtained parameters of oxidative stress were checked for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, a one-way ANOVA was employed to determine whether there were significant differences in measured variables among experimental groups. When a difference was detected ($P < 0.05$), Dunnett's multiple-range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal-Wallis) was used. Obtained data of oxidative stress were controlled by the Shapiro-Wilk test for assessing normality of data, which determined that the data were normally distributed. Test of homogeneity of variances (Levene test) and an analysis of variance (ANOVA) were conducted, followed by a multiple comparison (Tukey-HSD test). Differences were considered to be significant when $P < 0.05$.

**Determination of the active ingredient**. The GC/ECD (gas chromatography/electron capture detector) method was used to control the level of active substance throughout the test (Kocourek and Hajslova 1989). Cypermethrin in water samples was determined after extraction using isooctane by gas chromatography with electron capture detection in the screening laboratory. Chromatography was performed on a column designated HP-5MS (60 m × 0.32 mm, film 0.25 μm). The carrier gas was helium with a flow rate of 25 ml/min a splitless injection volume of 2 μl, and a temperature of 250 °C was used. The temperature column program was 100 °C/2 min, increased to 230 °C at 14 °C/min, increased to 285 °C at 4 °C/min, and subsequently held for 40 min. This method confirmed the presence of cypermethrin at > 80% throughout the course of 24 h.

doi: 10.17221/8417-VETMED

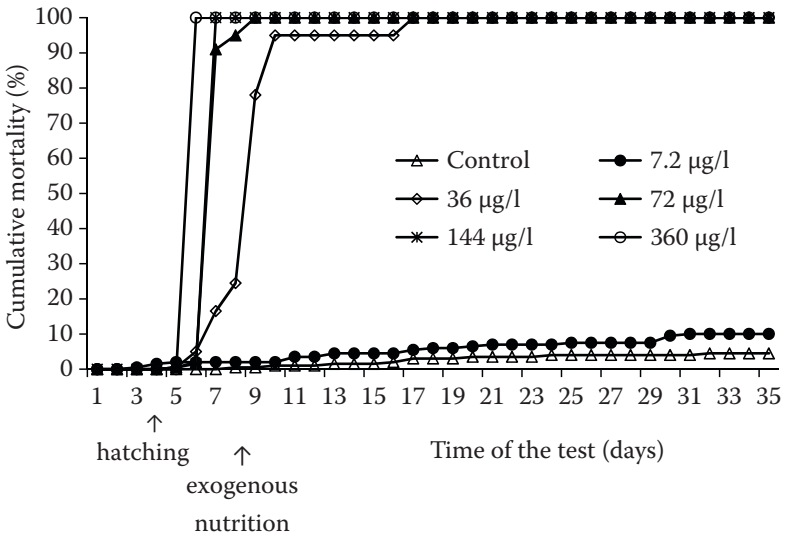


Figure 1. Cumulative mortality (%) of common carp embryos and larvae in EL toxicity test using Cyperkill 25 EC

## RESULTS

### Cumulative mortality

Mortality increased over the course of the trial (Figure 1). No significant differences in mortality were observed among test groups until Day 5. Total mortality was observed at 360 µg/l on Day 5 and at 144 µg/l on Day 6. Delayed and reduced hatching, along with post-hatch mortality, was observed at 72 µg/l. Only 5% of embryos from this group survived to initiation of exogenous nutrition, and total mortality was seen after several days. On Day 1 of feeding, 24% and 2% mortality was observed at 36 and 7.2 µg/l, respectively, with 0.5% mortality in the control. Total mortality at 36 µg/l was seen on Day 17. At the conclusion of the trial, 90% of larvae in the 7.2 µg/l group and 95.5% in the control group were viable.

### Growth

Samples from all concentrations were taken only on Day 5, since insufficient numbers of larvae survived beyond Day 11 at higher concentrations. Only the control and the 7.2 µg/l group were compared at the completion of the trial. Survival at 36 µg/l concentration was not sufficient to include individuals in growth comparisons. Significant differences in weight and length growth were observed ($P < 0.01$) (Figures 2 and 3).

On Day 35, FCF was calculated as an index of thriving in fish from the control group and the 7.2 µg/l group. Values of 19 fish from each group were averaged (± SD). The mean FCF of control fish was 1.269 ± 0.3714 and mean FCF of the 7.2 µg/l concentration was significantly lower at 1.157 ± 0.0807 ($P < 0.05$).

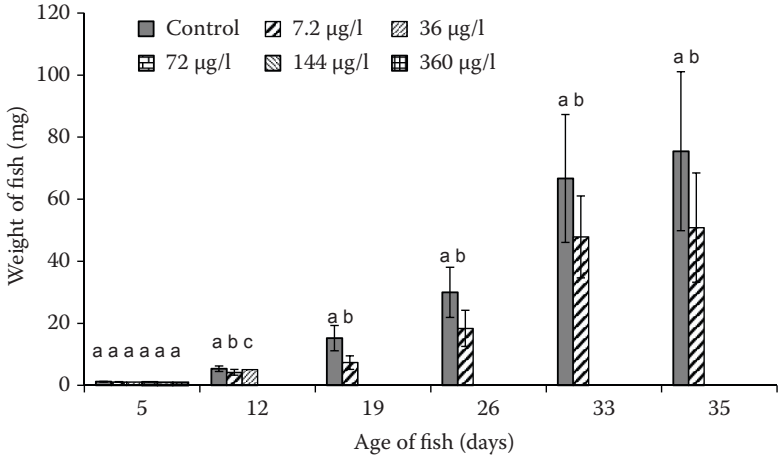


Figure 2. Effect of Cyperkill 25 EC on weight in mg (mean ± SD) of common carp larvae and juveniles in EL test. Significant differences ($P < 0.01$) between groups at each sampling time are indicated by different letters (a, b)

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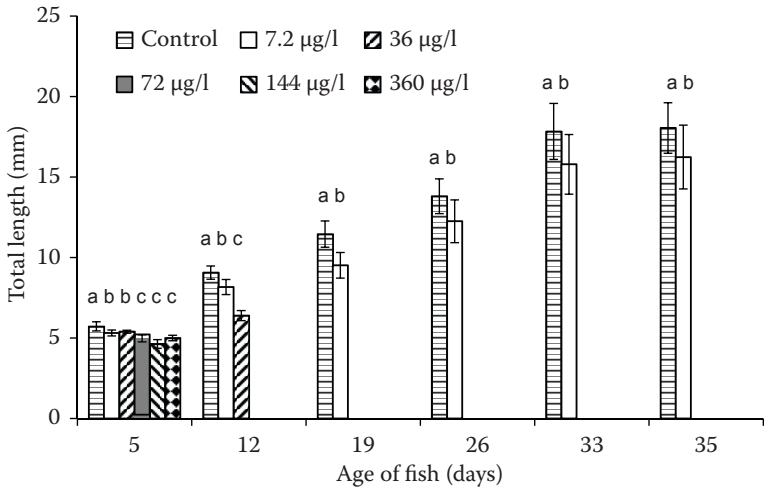


Figure 3. Effect of Cyperkill 25 EC on total length in mm (mean ± SD) of common carp larvae and juveniles. Significant differences ($P < 0.01$) between individual groups at each sampling time are indicated by different letters (a, b)

## Early ontogeny

Ontogenetic developmental stages were investigated concurrently with growth indices (Table 1). Ten larvae on Day 5, 12, 19, 26, 33, and nineteen larvae on Day 35 from 7.2 µg/l and control groups were examined. From Day 19, developmental stages of controls and the 7.2 µg/l group showed visually observable differences. No fish from the 7.2 µg/l concentration had reached the juvenile stage by the end of the test with some remaining two stages behind. Morphological anomalies such as curvature of the spine, changes in yolk sac, and shortening of body were rare in both groups and could be considered chance occurrences. Deeper pigmentation was observed in 68% of the fish from the 7.2 µg/l concentration on Day 35. Larvae from the 36 µg/l that died later showed similar colour changes before death and no presence of food in the digestive tract.

## Histology

Light microscopy did not reveal significant differences between 7.2 µg/l and controls in examined tissues.

Table 1. Ontogeny of common carp from control group and in response to exposure to Cyperkill 25 EC at 7.2 µg/l

| Day | Developmental stages | |
|---|---|---|
| | control | 7.2 µg/l |
| 5 | E8–E9 | E8–E9 |
| 12 | L3–L4 | L3–L4 |
| 19 | L4–L5 | L3–L4 |
| 26 | L5–L6 | L5 |
| 33 | L6–J1 | L5–L6 |
| 35 | L6–J1 | L5–L6 |

## Oxidative stress

Activity of GR, GPx, and GST of controls and the 7.2 µg/l concentration showed significant differences ($P < 0.01$) on Day 35 (Figures 4–6). The mean level of TBARS was 12.1 ± 2.58 and 10.7 ± 3.55 nmol/g of wet weight in control and treated fish, respectively, and mean CAT activity was 31.4 ± 5.07 and 24.5 ± 2.71 µmol $H_2O_2$/min/mg of protein, respectively. There were no significant differences in TBARs and CAT activity in control and treated fish.

## DISCUSSION

The tests described here on the effects of Cyperkill 25 EC on common carp revealed low hatching rate, high mortality soon after hatching at concentra-

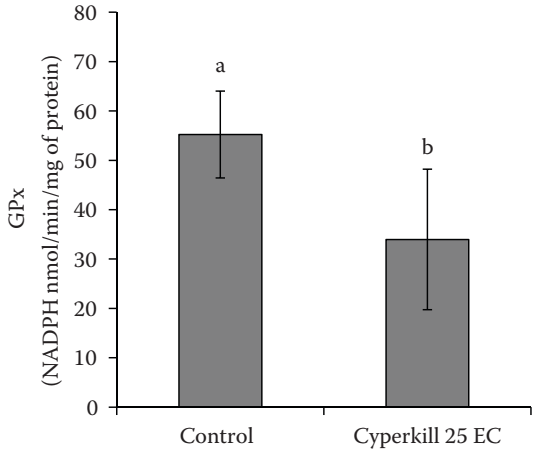


Figure 4. Effect of Cyperkill 25 EC at 7.2 µg/l on GPx activity in common carp on Day 35 of exposure compared to control ($P < 0.01$)

doi: 10.17221/8417-VETMED

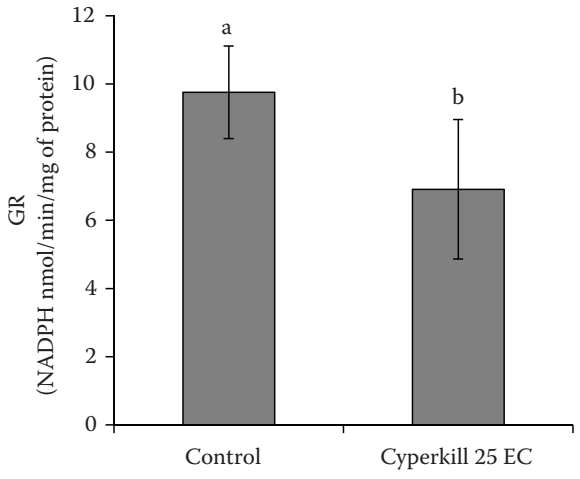


Figure 5. Effect of Cyperkill 25 EC at 7.2 µg/l on GR activity in common carp on Day 35 of exposure compared to control ($P < 0.01$)

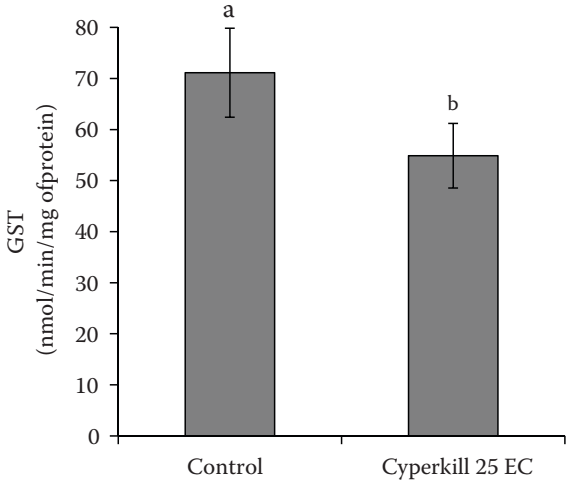


Figure 6. Effect of Cyperkill 25 EC at 7.2 µg/l on GST activity in common carp on Day 35 compared to control ($P < 0.01$)

tions of 36 µg/l (9 µg/l of cypermethrin) and higher, and significantly reduced growth and FCF in surviving larvae at a concentration of 7.2 µg/l (1.8 µg/l of cypermethrin). These larvae showed protracted ontogenetic development and colour changes.

Our results confirm that cypermethrin is highly toxic to common carp. High acute toxicity of cypermethrin to freshwater fish has been reported by many authors. The acute toxicity indicated for roho labeo (*Labeo rohita*) is 4.0 µg/l (Marigoudar et al. 2009). The 96 h LC50 value for cypermethrin in rainbow trout (*Oncorhynchus mykiss)* is 8.2 µg/l and in bluegill sunfish *(Lepomis macrochirus)* it is 1.8 µg/l (Bradbury and Coats 1989b). Similar values were found in other studies: 96 h LC50 for juvenile trout (mean weight 11.71 g and length 88.9 mm), 3.14 µg/l, and 96 h LC0, 96 h LC50, and 96 h LC100 for common carp were 1.82, 2.91, and 4.64 µg/l, respectively (Velisek et al. 2011). The 96 h LC50 for juvenile carp (mean weight 37.63 mg and length 13.85 mm) has been reported to be 1.38 µg/l (Stara et al. 2013a). We observed increased mortality dependent on concentration and duration of exposure. Similarly, acute toxicity tests of deltamethrin and cypermethrin in embryos and larvae of common carp have shown concentration-dependent decreases in hatching success (Koprucu and Aydin 2004; Aydin et al. 2005).

Larvae exposed to cypermethrin at 36 µg/l and higher often remained at the bottom and started to swim only upon stimulation. We did not observe curvature of the body as a result of spasms as was described, nor did we observe the spastic movement and 5–15 s immobility that was reported in zebrafish (*Danio rerio*) embryos exposed to cypermethrin (De Micco et al. 2010).

Treated larvae exhibited dark pigmentation at the end of the trial, as has been observed with exposure to pyrethroids (Ural and Saglam 2005; El-Sayed and Saad 2008; Richterova et al. 2014).

The reduced growth of fish observed in our test is in agreement with findings of reduced growth and feeding in fathead minnow (*Pimephales promelas*) larvae exposed to the pyrethroid esfenvalerate (Werner and Moran 2008). Decreased growth was described for mysid shrimp chronically exposed to cypermethrin at the very low concentration of 0.00078 µg/l (NOEC value) (Werner and Moran 2008).

Calculated FCF indicated reduced growth of exposed larvae compared with the control group. Significantly lower FCF values were observed in surviving larvae exposed to 7.2 µg/l compared to controls. These results were in agreement with reports demonstrating a decline in condition factor in fish exposed to pollutants (Khan 2003).

Although some authors report histopathologies in fish exposed to pyrethroids, we did not observe significant histological changes in liver, intestine, kidneys, or gill tissues of larvae exposed to cypermethrin. Degeneration of hepatocytes has been described after exposure of bifenthrin and cyhalothrin (Velisek et al. 2009a; Velisek et al. 2009b; Richterova et al. 2014), and damage of gill lamellae was de-

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scribed (Velisek et al. 2006; Velisek et al. 2009a). The concentration of 3.14 µg/l cypermethrin in the form of Alimethrine 10 EM was found to be associated with fatty degeneration of liver and severe teleangioectasiae of gills in juvenile rainbow trout (*O. mykiss*) (Velisek et al. 2006). It is possible that our tested concentration (1.8 µg/l of cypermethrin corresponding to 7.2 µg/l of Cyperkill 25 EC) was too low to cause histological damage.

The exposure of organisms to Cyperkill 25 EC was associated with differences in biochemical parameters of oxidative stress at a concentration of 7.2 µg/l. Activity of GR, GPx, and GST were decreased in our test. Many pesticides have been shown to be associated with induction of oxidative stress in aquatic organisms, via the formation of ROS (reactive oxygen species) and alterations in antioxidant or free oxygen radical scavenging enzyme systems (Uner et al. 2006; Slaninova et al. 2009; Lushchak 2011; Stara et al. 2012; Stara et al. 2013b). An increase or inhibition of antioxidant enzyme activity can depend on the intensity and the duration of exposure as well as on the susceptibility of the exposed fish (Oruc and Usta 2007). Manifestation of oxidative stress varies with fish species and organ (Slaninova et al. 2009). Exposure to 3 µg/l of cypermethrin for ten days caused increased activity of SOD (superoxide dismutase), CAT, and MDA (malondialdehyde) levels in the liver of common carp (*C. carpio*) and Nile tilapia (*Oreochromis niloticus*) while the level of GPx activity increased in tilapia but decreased in common carp (Uner et al. 2001). This study also found increases in SOD, GPx, CAT, and MDA in kidneys of common carp. The observed higher antioxidant enzyme activity in kidney suggested that this organ participates in the detoxification of cypermethrin or its metabolites (Uner et al. 2001). Significantly higher activity of SOD and GR and lower activity of CAT were observed in juvenile carp exposed to 1.38 µg/l of zeta-cypermethrin (Stara et al. 2013a). High levels of the antioxidant enzymes SOD and CAT followed exposure to cypermethrin in common carp, while the enhanced lipid peroxidation reported in blood and tissue demonstrated that cypermethrin-induced ROS were not totally scavenged by the antioxidant enzymes (Yonar 2013).

We can conclude that exposure to Cyperkill 25 EC (containing 250 g/l cypermethrin) may pose a risk for fish.

**Acknowledgements**

The authors would like to thank The Lucidus Consultancy (Alan Pike and Kathleen Hills from The Lucidus Consultancy, United Kingdom) for the improvement of this paper and for English correction.

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Received: 2015–01–21
Accepted after corrections: 2015–07–24

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