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NeemAzal T/S – toxicity to early-life stages of common carp (*Cyprinus carpio* L.)

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ABSTRACT: In the European Union, the use of the insecticide NeemAzal T/S (standardised variant 1% of the active ingredient azadirachtin) is authorised in organic agriculture. The objective of this study was to determine the toxic effects of NeemAzal T/S at concentrations of 3, 10, 30, and 60 mg/l on the morphometric and condition characteristics, mortality, hatching, and histopathology of early-life stages of common carp (*Cyprinus carpio* L.) as a non-target aquatic organism, as well as related effects of NeemAzal T/S on selected indices of oxidative stress in the same organism. The embryo-larval toxicity test was performed according to OECD Guidelines 210 (Fish, Early-life Stage Toxicity Test). NeemAzal T/S exposure induced slow hatching on the first day and increased cumulative mortality in groups exposed to the insecticide. No effect on morphometric or condition characteristics was observed after 31 days of exposure. Histopathological changes of the gills were found at the highest concentration of 60 mg/l of NeemAzal T/S. Exposure at 30 mg/l was associated with significantly ($P < 0.01$) increased glutathione peroxidase and glutathione S-transferase ($P < 0.05$) activities compared to the control group. The content of oxidised lipids was significantly higher ($P < 0.05$) in the 3, 10, and 30 mg/l experimental groups than in the controls. NeemAzal T/S exerted a significant negative influence on histopathological parameters in the embryo and larvae of common carp, as well as on the indices of oxidative stress in the same organism.

Keywords: Neemazal T/S; embryo-larval toxicity test; azadirachtin; oxidative stress; histopathology; insecticide

List of abbreviations

$ANC_{4.5}$ = acid neutralising capacity, **CAT** = catalase, **COD_{Mn}** = chemical oxygen demand, **FCF** = fulton's condition factor, **GPx** = glutathione peroxidase, **GR** = glutathione reductase, **GST** = glutathione S-transferase, **HE** = haematoxylin and eosin, **I** = inhibition of specific growth, **LC_{50}** = median lethal concentration, **OECD** = Organization for Economic Cooperation and Development, **SGR** = specific growth rate, **TBARS** = thiobarbituric acid reactive substances, **TL** = total length, **W** = weight

The use of synthetic pesticides and their entry into the environment has had a destructive influence on the aquatic ecosystem. In addition, synthetic pesticide usage has resulted in the development of resistant pests. In view of the environmental problems caused by the use of synthetic chemicals, there is a growing need for alternative methods of pest control that would minimise this damage. The

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use of eco-friendly botanical and soft-pesticides is considered to be of considerable importance (Wan et al. 1996; Kumar et al. 2012). Botanical pesticides are based on the phyto-chemicals that have evolved in plants for defence against phytophagous insects. There is growing evidence that many plant-derived chemicals are now being used for the control of insect pests (Dubey et al. 2010).

Azadirachtin was isolated from the seeds of kernels from the neem tree (*Azadirachta indica*) by Butterworth and Morgan (1968). Azadirachtin, a mixture of limonoids with azadirachtin A as the major component, has been demonstrated to act as an antifeedant, and its effects on insect growth and reproduction are now well understood and documented. The mode of action of azadirachtin lies in: (i) effects on chemoreceptors resulting in antifeedancy, (ii) effects on ecdysteroid and juvenile hormone titres through a blockage of morphogenetic peptide hormone release, and (iii) direct effects on most other tissues studied resulting in an overall loss of fitness in the insect (Mordue and Blackwell 1993; Girish and Shankara 2008). NeemAzal T/S is approved as an insecticide in organic agriculture in the European Union. In the Czech Republic, a standardised variant of NeemAzal T/S with 1% of the active ingredient, azadirachtin, is authorised for use in pest control in potatoes (against Colorado potato beetle) and pear and apple trees (against aphids). The maximal hectare dose for selected crops is 3 l and the recommended interval between applications is from seven to 14 days.

Fish and other aquatic organisms are affected by pesticides that pollute surface water through agricultural runoff and are considered a significant bio-indicator of aquatic pollution (Bhat et al. 2012). The reproductive ability and early life stages of fish, such as eggs and larvae, are particularly sensitive to chemical pollutants present in the aquatic environment. Young growing stages of fish are most sensitive to these pesticides, which can be disastrous for the future of the entire population (Hutchington et al. 1998; Kumar et al. 2012).

Our study was conducted in order to evaluate the effects of NeemAzal T/S and its ecological impacts on the early-life stages of common carp (*Cyprinus carpio* L.) as a non-target aquatic organism. The toxic effects of NeemAzal T/S on the morphometric and condition characteristics, mortality, hatching, and histopathology of early-life stages of common carp *(Cyprinus carpio* L.*)* were assessed, as well as related effects on selected indices of oxidative stress in the same organism.

MATERIAL AND METHODS

Experimental animals. Fertilised eggs of common carp (*Cyprinus carpio* L.) were provided by a commercial fish farm (Rybnikarstvi Pohorelice PLC, Czech Republic). The eggs were produced according to guidelines for the production of eggs by Kocour et al. (2005).

Water parameters. The basic physical and chemical parameters of the dilution water used in the embryo-larval toxicity test were as follows: acid neutralising capacity ($ANC_{4.5}$), 3.5–3.7 mmol/l; chemical oxygen demand (COD_{Mn}), 1.4–1.9 mg/l; total ammonia < 0.04 mg/l; nitrates, 12.1–13.6 mg/l; nitrites < 0.01 mg/l; Cl^- 17.5–18.5 mg/l; and Σ Ca + Mg, 3.06 mmol/l. Temperature, pH, and oxygen saturation were measured daily. The water temperature ranged from 21 to 23 °C; pH was between 7.7 and 8.4, and dissolved oxygen did not fall below 60%.

Experimental protocol. The trial was carried out using the modified test design of OECD guidelines 210 (Fish, Early-life Stage Toxicity Test) (OECD 2013). At 24 h post-fertilisation, 100 fertilised eggs were randomly transferred into each of fifteen 900 ml crystallisation dishes containing one of four ascending concentrations of NeemAzal T/S solution (3, 10, 30 and 60 mg/l), and into NeemAzal T/S-free tap water as a control. The lowest concentration was selected according to expected environmental level of azadirachtin, which could be found in treated locations after direct overspray at the maximum allowable application rate (Pest Management Regulatory Agency 2012). Each treatment level was replicated three times (a total of 300 fertilised eggs for each concentration and control). A semi-static trial with solution replacement twice daily was used. Replacement was conducted with care to avoid interfering with the development of embryos and larvae. Observations of hatching, survival, and behaviour were made twice daily and dead embryos and larvae were collected. Larvae were fed *ad libitum* twice daily on fresh *Artemia salina* nauplii.

Samples in the control and each of the tested groups were collected on Days 6 and 13 (30 specimens per group), Days 20 and 27 (15 specimens per group), and Day 31 (45 specimens per group) and were fixed in 4% formaldehyde. Samples of

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embryos and larvae were sampled to monitor total length (TL) (stereomicroscopically to 0.01 mm using a micrometer), weight (W) (to 0.1 mg), developmental stages, Fulton's condition factor (FCF), specific growth rate (SGR), and the inhibition of specific growth (I) and morphological anomalies. Sample fish were assigned to developmental stages according to Penaz et al. (1983), who described the determination of developmental stages of common carp, including nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages.

Trial test plan. The first day post-fertilisation was designated as the beginning of the embryo-larval toxicity test; on Day 3, the eggs began hatching; on Day 5, hatching was completed; on Day 6, the hatchlings began to be fed *ad libitum* twice daily on fresh *Artemia salina nauplii*; on Day 31, the test was finished (all larvae in the control groups had entered the first juvenile stage).

Morphometric and condition characteristics. FCF was calculated at each sampling time according to the formula:

$$FCF = (W \times 10^5)/TL^3$$

The SGR for fish was calculated for each group beginning on Day 6 (the first sampling time) and on Day 31 (the completion of the test) according to the formula:

$$SGR = 100 \times ((\ln W_2 - \ln W_1)/(T_2 - T_1))$$

where:

W_1 = weight (mg) at time T_1 (Day 6)

W_2 = weight (mg) at time T_2 (day 31)

T_1 = first sampling time

T_2 = end of the test

I in each experimental group was calculated as follows:

$$I\ (\%) = 100 \times [(SGR_{control} - SGR_{group})/SGR_{control}]$$

where:

I = the inhibition of specific growth in a selected experimental group after 25 days of exposure

Histopathology. At the end of the test (Day 31), thirty fish in all groups were fixed in buffered 4% formaldehyde, dehydrated, embedded in paraffin wax, cut into cross sections of 4 µm thickness, and stained with haematoxylin and eosin (HE). Histological changes in skin, gills, kidney, and liver samples were examined using light microscopy.

Evaluation of antioxidant defence enzymes and lipid peroxidation. At the end of the test, a further 30 specimens from each test and control group were killed, weighed, homogenised with phosphate buffer (pH 7.2), and stored at –85 °C until analyses. The catalytic concentrations of glutathione S-transferase (GST), glutathione reductase (GR), catalase (CAT), and glutathione peroxidase (GPx) were measured spectrophotometrically (Habig et al. 1974; Carlberg and Mannervik 1975; Aebi 1984; Flohe and Gunzler 1984). The protein concentration was determined using a Bicinchoninic Acid Protein Essay Kit (Sigma-Aldrich, St. Louis, USA) with bovine serum albumin as standard (Smith et al. 1985). To assess lipid peroxidation in the samples, malondialdehyde was measured by lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) method by Lushchak et al. (2005).

Statistical analysis. Statistical analysis was conducted using Statistica 10 for Windows (StatSoft, Prague, Czech Republic). Data on morphometric and condition characteristics and oxidative stress parameters did not show a normal distribution and were therefore subjected to Kruskal-Wallis ANOVA and Dunn's test. Differences in cumulative mortality and hatching were tested using a contingency table. All figures were also generated using Statistica 10. Statistical significance was accepted at the level $P < 0.05$.

RESULTS

Hatching

Eggs began hatching on Day 3 and hatching was completed on Day 5. On the first day of hatching, significant delays ($P < 0.05$) were observed in groups exposed to 3 mg/l, 30 mg/l and 60 mg/l concentrations compared to the control group (Table 1).

Cumulative mortality

Cumulative mortality was lowest in the control group (17%) and in the group exposed to 3 mg/l (16%). Increased mortality was detected in experimental groups exposed to 30 (22%) and 60 mg/l (27%) of NeemAzal T/S. The highest ($P < 0.05$) cumulative mortality (32%) was observed in the group exposed to 10 mg/l compared to the control (Figure 1).

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Table 1. Effects of NeemAzal T/S on egg hatching

NeemAzal T/S (mg/l)	3rd day (%)	4th day (%)	5th day (%)
0 (control)	6.83	67.83	100
3	0.67*	59.39	100
10	1.67	71.67	100
30	0.33*	55.33	100
60	1.00*	70.00	100

*$P < 0.05$

Morphometric and condition characteristics

There was no statistically significant effect after six and 31 days of exposure to NeemAzal T/S on morphometric and condition characteristics (Table 2).

Early ontogeny

No significant changes in early ontogeny between control and tested concentrations were recorded.

Morphological anomalies

A low frequency of morphological anomalies (tail defects, lordosis, and kyphosis) was observed in the 3, 10, 30 and 60 mg/l concentrations on Day 6. Interestingly, a high number of pigmentation changes had appeared by Day 6 in all NeemAzal T/S-exposed groups compared with the control. In later samplings, no further changes in pigmentation were reported. Embryo stages of fish appeared to be the most sensitive in this respect.

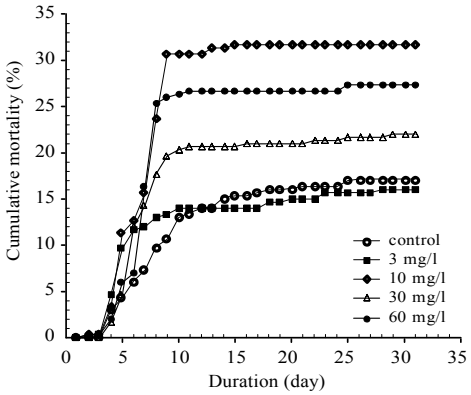

Figure 1. Cumulative mortality of common carp after 31 days of exposure to NeemAzal T/S

Histopathology

Histopathological examination revealed slight pathological changes at the 30 mg/l concentration of NeemAzal T/S and significant changes at the highest concentration of 60 mg/l. The following morphological changes were found in the gills: (a) oedema of gill lamellae; (b) focal separation of gill lamellar surface; (c) aggregates of mononuclear cells (Figure 2B) compared with sample tissue sections from the negative control group (Figure 2A). No histopathological changes were observed in other organs (kidney, liver) and skin.

Oxidative stress indices

Glutathione peroxidase exhibited highly significant activity ($P < 0.01$) in the group exposed to a concentration of 30 mg/l compared to the control

Table 2. Morphometric condition characteristics after 6 and 31 days of the embryo-larval test on common carp

Group	Control	Concentration of NeemAzal T/S (mg/l)			
		3	10	30	60
W_6 (mg)	2.25	2.35	2.30	2.40	2.40
W_{31} (mg)	130.20	121.00	136.70	131.50	147.60
TL_6 (mm)	6.78	6.68	6.69	6.72	6.91
TL_{31} (mm)	21.21	20.40	21.34	21.29	22.33
FCF_{31}	1.36	1.40	1.36	1.35	1.37
SGR	16.29	15.61	15.98	16.30	16.25
I (%)	–	4.15	1.87	–0.09	0.23

W_6, W_{31} = median fish weights on days 6 and 31; TL_6, TL_{31} = median total body lengths on days 6 and 31; FCF_{31} = median Fulton's condition factor on day 31; SGR = specific growth rate after 25 days of exposure; I = inhibition of specific growth after 25 days of exposure

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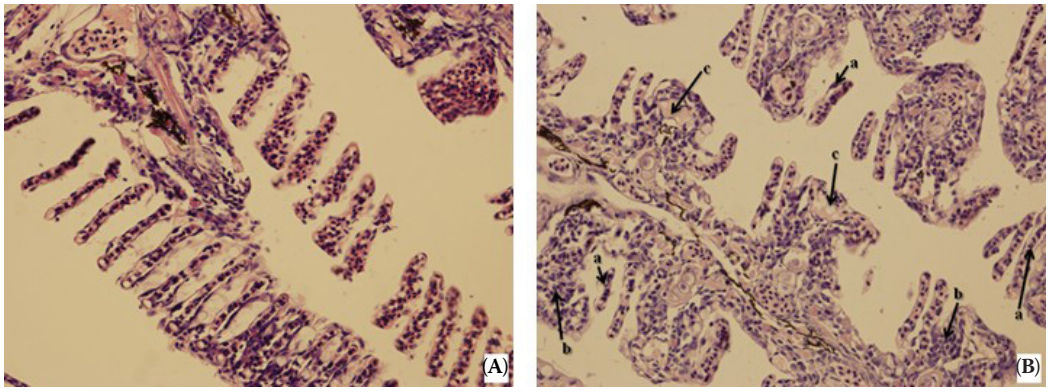

Figure 2. **A** – gill of common carp larvae from the control group after 31 days. **B** – pathological changes in the gill of common carp larvae exposed to NeemAzal T/S at a concentration of 60 mg/l after 31 days (HE, × 400). a = oedema of gill lamellae, b = focal separation of gill lamellar surface, c = aggregates of mononuclear cells

group (Figure 3A). Glutathione reductase exhibited highly significant activities ($P < 0.01$) in the groups exposed to 30 and 60 mg/l compared to the control group (Figure 3B). Glutathione S-transferase exhibited increased activity in all experimental groups exposed to NeemAzal T/S compared to the control group, but a significant difference ($P < 0.05$) from the control group was found only at a concentration of 30 mg/l (Figure 3C). No significant effects on catalase activity were found in any NeemAzal T/S-exposed groups (Figure 3D). TBARS contents were significantly increased at concentrations of 3 and 10 mg/l ($P < 0.01$) and also at 30 mg/l ($P < 0.05$) compared to the control group (Figure 4).

DISCUSSION

The present study revealed no significant negative effect on the morphometric and condition charac-

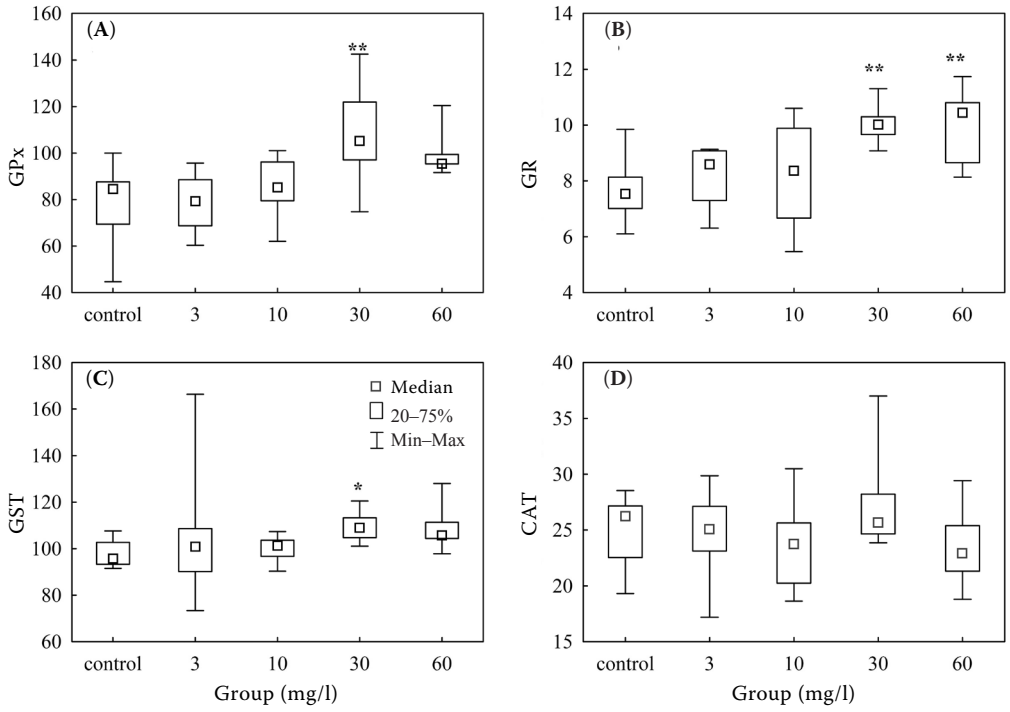

Figure 3. Activities of antioxidant enzymes in common carp larvae after 31 days of exposure to NeemAzal T/S. **A** – GPx (nmol/min/mg protein); **B** – GR (nmol/min/mg protein); **C** – GST (nmol/min/mg protein); **D** – CAT (μmol/min/mg protein); *$P < 0.05$, **$P < 0.01$

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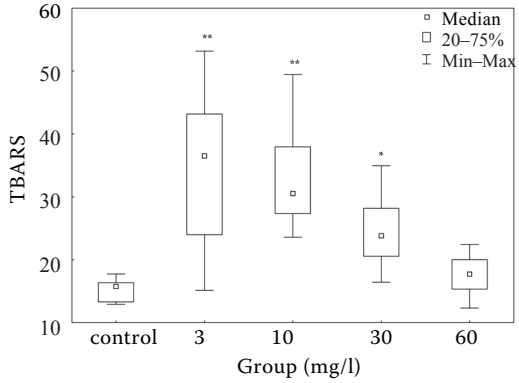

Figure 4. TBARS content (nmol/g wet tissue) in common carp larvae after 31 days of exposure to NeemAzal T/S; $^{*}P < 0.05$, $^{**}P < 0.01$

teristics or early developmental stages of common carp after 31 days of exposure to NeemAzal T/S at the tested concentrations.

However, NeemAzal T/S exposure was observed to have an effect on the hatching of embryos on the first day. The process of hatching was retarded on the first day in all experimental groups compared to the control group. In a study by Singh and Ansari (2010), the toxic effects of two neem-based pesticides on the reproductive ability of zebrafish were investigated. Females and males of zebrafish were exposed for one month to 96 h LC_{50} values of 0.27 µg/l for Nimbecidine (0.03% of azadirachtin as active ingredient; neem oil-based) and 0.08 µg/l for Ultineem (3% of azadirachtin as active ingredient; from the leaves of the neem tree). Then, the zebrafish were returned to pesticide-free water and allowed to breed to observe fecundity and hatchability. Both pesticides were found to cause alterations in the reproductive ability of zebrafish at very low concentrations. The results showed a significant decrease in hatchability: up to 21.69% in Nimbecidine and 26.99% in Ultineem treated fish.

Mondal et al. (2007) studied the acute toxicity of two neem-based biopesticides, Nimbecidine and Neem Gold, both separately and in combination with fingerlings of *Lepidocephalichthys guntea*. The 96 h LC_{50} values for Nimbecidine (neem oil based, 0.03% of azadirachtin as active ingredient) and Neem gold (neem seed kernel extract, 0.15% of azadirachtin A as active ingredient) were 0.0135 mg/l and 0.0525 mg/l, respectively, and 0.0396 mg/l for the combination of the two. The authors concluded that these biopesticides were highly toxic to fingerlings of loach, *L. guntea* and that if their rampant use continued, it could cause high mortality and thus reduce the gene pool of the fish. On the other hand, Stalin et al. (2008) compared the non-target toxicity of azadirachtin (0.1% stock solution), a natural pesticide of plant origin, with a synthetic pyrethroid, deltamethrin (0.1% stock solution), on *Poecilia reticulata* and found that the pesticide containing azadirachtin (with a 96 h LC_{50} of 0.011 mg/l) was less toxic to fish compared to deltamethrin (with a 96 h LC_{50} of 0.0019 mg/l). The 96 h LC_{50} values for adult zebrafish were 2.37 µg/l for Nimbecide and 0.83 µg/l for Ultineem (Singh and Ansari 2010). Ansari and Sharma (2009) observed a negative effect of Achook (0.03% of azadirachtin) on zebrafish. Achook was reported to be safe for non-target animals, but the authors found it to be toxic to zebrafish at low concentrations. The toxicities of neem-based pesticides with different concentrations of azadirachtin are different for various fish species. Mousa et al. (2008) assessed the effects of a water leaf extract of the neem plant (500 g of dried leaves *A. indica* per litre of water, for 24 h at room temperature) on the survival and health status of Nile tilapia (*Oreochromis niloticus*) and African cat fish (*Clarias gariepinus*). The 24 h LC_{100} values for neem leaf extract were found to be 4 g/l and 11 g/l for juvenile *O. niloticus* and *C. gariepinus*, respectively, while the 96 h LC_{50} values were 1.8 g/l and 4 g/l. Adult catfish *Heteropneustes fossils* were exposed to different concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225, 250 mg/l) of azadirachtin (Ozoneem Aza containing azadirachtin A (23.78%) and azadirachtin B (3.59%)) and the following LC_{50} values at various sampling times were reported: 173.06 mg/l at 24 h, 80.69 mg/l at 48 h, 58.57 mg/l at 72 h, and 52.35 mg/l at 96 h (Kumar et al. 2011). The 96 h LC_{50} value for *Labeo rohita* exposed to Neem-on (0.15% of azadirachtin as active ingredient, neem seed kernel) was found to be 42.66 mg/l (Bhat et al. 2012).

In our study, histopathological examination revealed significant changes in gills at the highest concentration of 60 mg/l of NeemAzal T/S. Winkaler et al. (2007) described frequent histological alterations in the gills (lamellar aneurism, rupture of the lamellar epithelium) and kidney (reduction of Bowman's space, cytoplasmic vacuolation, granular degeneration, and the narrowing of tubular lumen) of fish exposed to neem extracts of 5 g/l and 7.5 g/l (neem aqueous extracts prepared from 25 g of dried leaves per litre of water).

The production of reactive oxygen species and the induction of oxidative stress are important responses in aquatic organisms exposed to negative environ-

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mental conditions such as pollution. Many chemical pollutants such as pesticides induce oxidative stress in fish (Lesser 2006; Winkaler et al. 2007; Blahova et al. 2013; Chromcova et al. 2013). In organisms, this leads to the formation of oxygen free radicals and other reactive oxygen species that cause oxidative damage to membrane lipids, DNA, and proteins, and induce changes in antioxidant enzyme activities; they can even cause cell death. One defence mechanism is the formation and maintenance of antioxidant enzymes. Such enzymes are biomarkers of oxidative stress and include glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase and products of lipid peroxidation, TBARS (Van der Oost et al. 2003; Di Giulio and Meyer 2008; Stepanova et al. 2013).

In the present study, the larvae of common carp exposed to NeemAzal T/S showed significantly increased activities with respect to almost all antioxidant enzymes (GPx, GR, GST) as well as increased TBARS content compared to the control group. In a study by Winkaler et al. (2007), juvenile *Prochilodus lineatus* were exposed for 24 h to neem extract concentrations of 2.5, 5, and 7.5 g/l (neem aqueous extract prepared from 25 g of dried leaves per litre of water) and alterations in biochemical, physiological and histopathological parameters were observed, compared to the control. The 24 h LC_{50} of neem leaf extract for juvenile *P. lineatus* was estimated to be 4.8 g/l. The authors documented enhanced hepatic GST activity following exposure to 5.0 g/l of neem extract, which should be related to the metabolism of organic compounds in the extract. On the other hand, the lack of increased GST activity at 7.5 g/l neem may reflect impairment of the detoxifying capacity of the fish at levels above the LC_{50}. In a study by Mutawie and Hegazi (2011), *Oreochromis niloticus* was exposed to 1/10 LC_{50} (0.03 mg/l) of Nimbecidine (neem oil based, 0.03% of azadirachtin as active ingredient) for four weeks. The authors observed a significant resulting reduction in the activity of catalase and total protein in the muscles of *O. niloticus*. Also, in our study with NeemAzal T/S, it was confirmed that at the highest used concentration of 60 mg/l a weak inhibition of GPx and GST activity and a marked inhibition of TBARS were documented compared to the effects of 30 mg/l. Winkaler et al. (2007) reported a significant reduction in hepatic CAT activity, which is likely to affect the capacity of liver cells to defend themselves and respond to contaminant-induced oxidative stress. In other studies, the authors described this phenomenon as damage to organisms due to the weakening of their antioxidant capacity (Wiegand et al. 2000; Blahova et al. 2013).

In conclusion, this study indicates that the range of neem-base products, i.e. those containing different percentages of the active ingredient, azadirachtin, are not harmless to non-target aquatic organisms, i.e. fish and their developmental stages. Therefore, it is especially important that the application of such pesticides complies with measures to prevent their penetration into the aquatic environment.

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