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Comparison of Diazinon Toxicity to Embryos of *Xenopus laevis* and *Danio rerio*; Degradation of Diazinon in Water

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Abstract The aim of this study was to determine the toxic effect of diazinon (organophosphate insecticide) to embryos of *Xenopus laevis* and *Danio rerio*. The 96-h LC_{50} values showed higher toxicity of diazinon for *X. leavis* in standard solution (9.84 mg/L) compared to the pond water (12.64 mg/L). Teratogenic index for diazinon was 1.3 and 1.6, respectively. The 96-h LC_{50} diazinon values demonstrated similar sensitivity of embryos *D. rerio* (8.21–9.34 mg/L) and *X. laevis* in standard test solutions. Our results reflect that direct application of diazinon into the water can be associated with significant risks to aquatic organisms.

Keywords FETAX · Diazinon 60 EC · Embryonic test · Zebrafish

Pesticides migrate easily into surface water—either directly due to incorrect application and disposal of unused residues, or indirectly by runoff from treated crops. Although majority of these chemicals are generally less persistent and bioaccumulative than organochlorine pesticides, most of them are acutely toxic to a wide variety of non-target species, including aquatic organisms. Cholinesterase-inhibiting pesticides (most organophosphates and carbamates) are supposed to be more strongly associated with amphibian and native fish species population declines than any other class of pesticides (Davidson 2004). Although some OPs are undergoing increasing scrutiny and restrictions, they are the most widely used agrochemicals for the control of insect pests and constitute 50% of all insecticides applied in the world (Slotkin et al. 2006).

Diazinon (*O*,*O*-diethyl-*O*-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate), is a non-systemic organophosphate insecticide extensively used for pest control e.g. against a variety of sucking and leaf-eating insects in home gardens and farm-land, and in veterinary treatments. Diazinon 60 EC, a water-based diazinon preparation used in the presented study, can be used in the Czech Republic directly into the water in well-grounded cases as a biocide in fishery operations (Machova et al. 2007). Given the environmental fate characteristics of diazinon, relative high concentrations in water can be expected immediately after release. Diazinon is mobile and moderately persistent in the environment. Due to its chemical properties and its widespread use, diazinon is frequently found in wastewater treatment plant effluent and urban and agricultural runoff. Toxicity of OPs results from acetylcholinesterase (AChE) inhibition by their oxon metabolites (Hamm et al. 2001). In fish, diazinon is converted to diazoxon (*O*,*O*-diethyl-*O*-2-isopropyl-4-methyl-6-pyrimidylphosphate) by the cytochrome P450 monooxygenase (Fujii and Asaka 1982). Furthermore, diazoxon has been detected as the oxidation product of diazinon in water and soil (McLaughlin et al. 1993).

Our aim was to study the toxicity of diazinon to early life stages of the South African clawed frog (*X. laevis*) and

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compare these results with toxicity to zebrafish embryos (*D. rerio*). During this 96-h assay, we also monitored the concentration of diazinon and diazoxon in standard FETAX test solution (SS) and test pond water (PW).

Materials and Methods

The commercial preparation Diazinon 60 EC (Nippon Kayaku Co., Japan) (diazinon 600.0 g/L) was used in the bioassays. Standard FETAX solution was composed of (all in mg/L) 625.0 NaCl, 96.0 $NaHCO_3$, 30.0 KCl, 15.0 $CaCl_2$, 60.0 $CaSO_4{\cdot}H_2O$ and 70.0 $MgSO_4$ dissolved in distilled water. Basic physical and chemical indices of the pond water were: pH 7.85, $ANC_{4.5}$ (alkalinity) 3.15 mmol/L, COD_{Mn} (chemical oxygen demand) 5.94 mg/L, BOD_5 (biochemical oxygen demand) 2.22 mg/L, ($NH_4^+ + NH_3$) 0.01 mg/L, $N0_3^-$ 15.6 mg/L, NO_2^- 0.005 mg/L, PO_4^{3-} 0.01 mg/L, Cl^- 20.13 mg/L. The microbiological analysis revealed the total bacterial count of 5.2×10^6/mL (mostly *Aeromonas* spp., G-negative non-fermenting rods, and *Bacillus* spp.).

The South African clawed frog (*X. laevis*) embryos were obtained from an adult pair injected with human chorionic gonadotropin (HCG; Pregnyl 1500, N.V. Organon, Oss, Netherlands) into the dorsal lymph sac (females 300 IU; males 150 IU). Embryo-larval tests were conducted using the standard guide for the frog embryo teratogenesis assay-*Xenopus* (FETAX) (ASTM 1998). Normally developed embryos in mid-blastula (stage 8) to early gastrula (stage 11) (Nieuwkoop and Faber 1994) were selected for testing. Groups of 25 embryos were randomly placed in the plastic Petri dishes (60 mm) with 10.0 mL of the SS or PW and incubated in Zanussi ZT 155 BR at 24 ± 1°C. Stock solutions of particular diazinon concentrations were prepared by dissolving the preparation in SS and PW. They were used both for bath renewal in the FETAX bioassay and determination of diazinon and diazoxon content. The control groups exposed to SS or PW were replicated four times; diazinon treated groups (0.09; 0.9; 12.0, and 18.0 mg/L) were duplicated**.** Subsequently a series of three tests with both SS and PW was performed using embryos produced by different parental pairs. All diazinon concentrations (stock solutions) were prepared at the start of the particular experiments, stored at 5°C and warmed to 24°C before the exchange of the test solutions. The test medium was changed daily and dead embryos were removed. At the end of the experiment (96 h), surviving embryos were euthanized with carbon dioxide and fixed in 3.0% formaldehyde. An assessment of morphological abnormalities was conducted under a dissecting microscope according to the Atlas of Abnormalities (Bantle et al. 1998). The head–tail length of fixed embryos was determined.

The stock solutions were sampled for analyses of diazinon and diazoxon during the assay at 0, 48 and 96 h. Liquid-phase micro-extraction into toluene was used for the extraction of diazinon and diazoxon from the aqueous samples. All analyses were performed using a gas chromatograph 6890 (Hewlett-Packard, USA) equipped with a mass-selective detector 5973 (Hewlett-Packard, USA) and an auto-sampler (HP6890 Series). Limit of detection (LOD) was estimated at 0.016 μg/L for diazinon and diazoxon.

Embryonic toxicity tests on zebrafish embryos (*D. rerio*) were performed according to OECD No. 212 procedure (OECD 1998). The eggs were obtained from two groups of parental fish. A series of six tests was conducted; there were five concentrations of diazinon dissolved in dechlorinated tap water (10; 12; 14; 16; 18 mg/L) and one control in each test. Twenty fertilized eggs were placed per one Petri dish within 8 h after fertilization. The same way as in the FETAX procedure, the semistatic method with the bath replacement at 24 h intervals was used. Eggs were incubated at 25.5 ± 1°C. The number of dead embryos in individual concentrations was recorded every 24 h; the tests were terminated after 96 h. The mortality rate of the control embryos did not exceed 20%.

Results of both bioassays were analysed using the statistical package Unistat® v. 5.1 (Unistat Ltd., GB). Data were subjected to a one-way ANOVA and subsequently to a Tukey-HSD test for multiple comparisons in order to assess the statistical significance of differences among all possible pairs of groups. Differences were considered significant at $p < 0.05$. 96-h LC_{50} values with 95% confidence intervals were calculated using probit analysis (EKOTOX v. 5.2, INGEO Ltd., CZ).

Results and Discussion

Controls for FETAX tests (both in the standard solution and pond water) met the criteria for the test acceptance (mortality and malformation rates) established in ASTM E1439-98. The results of FETAX tests are presented in Table 1. The 96-h LC_{50} values showed higher toxicity of diazinon in SS (9.84 mg/L in SS; 12.64 mg/L in PW). The highest tested concentration of diazinon in both solutions (18 mg/L) resulted in 100% mortality. Diazinon of 12.0 mg/L caused 54% mortality in SS and no increase in mortality in PW. 96-h EC_{50} values of diazinon were comparable between SS and PW (5.36 and 6.79 mg/L, respectively). Severe edema, abnormal gut development, and simple axial abnormality were the most frequent malformations observed in the diazinon of 12.0 mg/L in both types of solutions. Teratogenic index (96-h LC_{50}/96-h EC_{50}) for diazinon was 1.3 in SS and 1.6 in PW. The

Table 1 Effects of diazinon on *Xenopus laevis* using FETAX[b]

Type of test solution	Concentration of diazinon (mg/L)	Mortality (%)	Malformed (%)	Growth (% of control)
SS	Control	1.5	2	–
	0.09	4	0.2	99.2
	0.9	0	6	99.1
	12.0	54	77	93.5[a]
	18.0	100	–	–
PW	Control	0.5	4	–
	0.09	2.5	0.4	98.2
	0.9	1.5	0.5	97.6
	12.0	0.8	86.5	92.8[a]
	18.0	100	–	–

[a] Significant difference between test groups and SS control or PW control, respectively (Tukey-HSD *t* test, $p < 0.01$)

[b] Average values from three series, after 96 h

differences in the mean head–tail length between either SS or PW control and diazinon treated embryos were statistically significant only for diazinon of 12.0 mg/L in both SS and PW.

The LC_{50} of diazinon for embryonic life stage of zebrafish expressed as the 96-h LC_{50} was calculated to be in range of 8.21–9.34 mg/L. The 96-h LC_{50} values demonstrated similar sensitivity of embryonic stages of *D. rerio* and *X. laevis* to diazinon.

pH values of both solutions used for the bioassays were within the same range (7.92–8.15 and 7.85–8.05 for SS and PW, respectively). Diazinon concentrations throughout the bioassays exceeded 80% of the nominal concentration in all test groups (Table 2). The most obvious decrease in diazinon was detected after 48 h in the concentration of 0.9 mg/L in both types of test solutions**.** In contrast, diazoxon increased during the first 48 h and then its concentration either stabilized or slightly decreased until the end of the test in solutions of diazinon with the starting concentrations of 0.09 and 12.0 mg/L (Table 2). In solutions with the starting concentration of diazinon of 0.9 mg/L, there was an initial decline in concentration of diazoxon after first 48 h followed by an increase in the PW only. With exception of this dilution, the concentration curves of diazoxon did not differ between SS and PW.

Toxicity of pesticides is strongly affected by natural conditions. For this reason, a plentitude of studies on organophosphates pesticides has been carried out in natural aquatic environments. Our study has showed that decrease of diazinon concentration during 96 h did not differ between the pond water and the standard solution FETAX. This finding does not correspond to the expectation of faster pesticide degradation in natural aquatic environments due to contribution of microorganisms (Singh and Walker 2006).

Our results document higher sensitivity of *D. rerio* embryos to diazinon (96-h LC_{50} = 8.21–9.34 mg/L) when compared to the early life stages of other fish species. Hamm et al. (2001) examined toxicity of diazinon and diazoxon to early life stages of medaka (*Oryzias latipes)* and reported diazinon 96-h LC_{50} of 31 mg/L. Viant et al. (2006) found diazinon 96-h LC_{50} in eyed eggs and alevins of chinook salmon (*Oncorhynchus tshawytscha*) 545 and 29.5 mg/L, respectively. For adult *D. rerio* 96-h LC_{50} was estimated at 8 mg/L (Keizer et al. 1991), which indicates the same range of sensitivity as that of embryos of this species. Keizer et al. (1991) did not detect diazoxon in the body of *D. rerio*, however, diazinon metabolite 2-isopropyl-6-methyl-4-pyrimidinol was revealed in the test zebrafish. Results of experiments with diazinon in zebrafish is indicative of the accumulation of diazinon as a parent compound in fish and low tendency to formation of toxic metabolites such as diazoxon (Keizer et al. 1993). No studies on the possibilities of a rise in toxic diazoxon in water have been carried out in context of fish experiments.

The findings of our study suggest that the higher mortality rate in *X. laevis* in SS was caused by the higher concentrations of diazoxon in this solution after 48 h. Our results are in accordance with the observation of Sparling and Fellers (2007). They estimated 96-h LC_{50} for diazinon in larval *Rana boylii* at 7.49 mg/L and found the toxicity of diazoxon approximately ten fold higher than that of the parent compound. In comparison with other frog species,

Table 2 Concentrations of diazinon and diazoxon in test solutions

Type of test solution	Diazinon start. conc. (mg/L)	48 h (% of start. conc.)	96 h	Diazoxon start. conc. (μg/L)	48 h	96 h
SS	0.09	93	93	0.036	0.089	0.079
PW	0.09	98	98	<LOD	0.067	0.068
SS	0.90	82.4	80.5	0.273	0.233	0.167
PW	0.90	86.4	85.2	0.227	0.137	0.225
SS	12.0	90.9	84.4	1.723	2.128	1.530
PW	12.0	95.9	81.0	0.145	0.808	0.227

diazinon is the most toxic to *Rana clamitans*, causing a significant reduction in the tadpole length at the exposure concentration of 5.0 μg/L (Harris et al. 1998).

Although toxicity of diazinon is affected by many factors such as biotransformation in the organism, water temperature, presence of other pollutants and other unidentified environmental variables, our results reflect that before its degradation diazinon can be associated with significant risks to aquatic organisms.

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