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Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: *Pelophylax ridibundus*, *Pseudepidalea viridis*, and *Xenopus laevis*

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ABSTRACT

The assessments of pesticide toxicity on nontarget organisms have largely been focused on the determination of median lethal concentration (LC_{50}) values using single/laboratory species. Although useful, these studies cannot describe the biochemical mechanisms of toxicity and also cannot explain the effects of pesticides on natural species. In this study, the toxic effects of glyphosate and methidathion were evaluated comparatively on early developmental stages of 3 anurans—2 natural (*Pelophylax ridibundus*, *Pseudepidalea viridis*) and 1 laboratory species (*Xenopus laevis*). The 96-h LC_{50} values for methidathion and glyphosate were determined as 25.7–19.6 mg active ingredient (AI)/L for *P. viridis*, 27.4–22.7 mg AI/L for *P. ridibundus*, and 15.3–5.05 mg AI/L for *X. laevis* tadpoles. Furthermore, as early signs of intoxication, glutathione *S*-transferase (GST), acetylcholinesterase (AChE), carboxylesterase (CaE), glutathione reductase, lactate dehydrogenase, and aspartate aminotrasferase were assayed in 4-day-old tadpoles after 96-h pesticide exposure. The GST induction after 3.2 mg AI/L methidathion exposure was determined to be 173%, 83%, and 38% of control, and the AChE inhibition for the same dose was determined to be 86%, 96%, and 30% of control for *P. ridibundus*, *P. viridis*, and *X. laevis*, respectively. Unlike the application of methidathion, all enzyme activities showed statistically significant increases on glyphosate exposure compared to controls. However, these increases in enzyme activities were not shown to be parallel with the increase of concentration. The levels of increases of GST and AChE were determined to be 111% and 31% for *P. ridibundus*, 13% and 51% for *P. viridis*, and 15% and 36% for *X. laevis* after 3.2 mg AI/L glyphosate exposure, respectively. The findings of the study suggest that the most sensitive species to pesticide exposure is *X. laevis*. The selected biomarker enzymes AChE, CaE, and GST are useful in understanding the toxic mechanisms of these pesticides in anuran tadpoles as early warning indicators.

1. Introduction

Amphibians are a diverse class of vertebrates, with approximately 7113 species distributed worldwide; 6274 amphibians belong to anurans (AmphibiaWeb, 2013). The World Conservation Union currently lists 30.5% of the world's amphibian species as threatened, and this number will likely continue to increase if current conditions creating these losses remain the same (IUCN, 2013). Therefore, declining amphibian populations are a major concern of many scientists worldwide in last 20 years (Perkins et al., 2000). Much of the interest on declines in amphibians is currently focused on the role of pesticides and endocrine disrupters (Johansson et al., 2006; Falfushinska et al., 2008). Amphibians are exposed to pesticides by many routes, but perhaps the most common route is agricultural runoff. Agricultural practices affect natural habitats in several ways, such as through land conservation, increased fragmentation, and agrochemical contamination (Ezemonye and Tongo, 2010a). Biomonitoring studies on natural frog populations have shown correlations between population declines and proximity to agricultural lands. Especially, postmetamorphic amphibians that inhabit the littoral region of wetland ecosystems may be exposed to pesticides (Edge et al., 2011).

Among various pesticides, one of the most common herbicides-glyphosate and one commonly used insecticide-methidathion, which both are organophosphate (OP) compounds, were tested in this study. Methidathion is a nonsystemic insecticide, registered for the control of a wide range of agricultural mite and insect pests in terrestrial food crops (EPA, Washburn, 2003). OP insecticides impair the nervous system of target organisms by inhibiting the activity of acetylcholinesterase (AChE). This enzyme normally facilitates breakdown of the neurotransmitter acetylcholine in muscle and nerve synapses and thus ends transmission of a neural impulse (Zimmerman and Soreq, 2006; Tripathi and Srivastava, 2008).

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The isopropylamine (ipa) salt of glyphosate (N-phosphonomethyl glycine), formulated with a surfactant (Roundup, Monsanto), is currently used as a herbicide to control weeds in dry-land situations in many countries (Perkins et al., 2000) and is widely detected in European ecosystems due to extensive use and deposition (Quaghebeur et al., 2004). Glyphosate inhibits the enzyme enolpyruvylshikimate phosphate synthase in plants, which is an essential enzyme in the biosynthesis of phenylalanine, tyrosine, and tryptophan. This pathway is not present in animals (Marrs, 2004). Glyphosate does not inhibit cholinesterases, has low mammalian toxicity, and is nontoxic to bees, birds, and most aquatic organisms, according to the World Health Organization/Food and Agriculture Organization (Lajmanovich et al., 2011). Previous studies showed that the toxicity of Roundup herbicides to aquatic organisms is largely caused by the surfactant in the mixture (Edginton et al., 2004; Moore et al., 2012).

The living members of the amphibian order Anura constitute the majority of the class, with 6274 known species (AmphibiaWeb, 2013). Therefore, the laboratory toxicity tests and ecotoxicologic impact studies about amphibians have been conducted exceedingly on anurans, such as the genera *Rana* (*Pelophylax*), *Bufo* (*Pseudepidalea*), and *Xenopus* (Widder, 2001). *Pseudepidalea viridis* and *Pelophylax ridibundus* eggs are frequently found in vegetated areas, wetlands, agricultural areas, and urban regions. These species are distributed in mainland Europe, Asia, Northern Africa, and Turkey. On the other hand, *X. laevis* is found naturally in ponds and rivers of sub-Saharan Africa and is also a common laboratory animal that was chosen for the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). FETAX has been used for assessing ecological and human health hazards of different kinds of xenobiotics (Perkins et al., 2000; Burýsková et al., 2006).

Biomarkers might be used to detect the impacts of pesticides in different species of amphibians, and they can give a good indication of the potential sublethal toxicity and inform about the possible mechanisms of action; they have also been used as effective tools in assessing environmental risk (Burýsková et al., 2006; Lajmanovich et al., 2010). Among the variety of available biomarkers, the activity of detoxification enzymes [e.g., glutathione-*S*-transferase (GST), carboxylesterase (CaE)], oxidative stress enzymes (glutathione reductase), and metabolic enzymes [e.g., acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and aspartate aminotransferases (AST)] were assayed as biomarkers in predicting the effects of pesticide exposure.

The main objectives of this study were: (1) determine the toxic effects of 2 OP pesticides in amphibian species in early life stages, (2) investigate the interspecies variation in biomarker enzymes of different amphibian species that are exposed to sublethal OP pesticides, and (3) determine whether or not these biomarkers could be used to assess the toxic effects of glyphosate and methidathion on *X. laevis* tadpoles at short periods of exposure.

2. Materials and methods

2.1. Chemicals and reagents

The technical formulations of glyphosate (Roundup) and methidathion (Supracide) that were considered in this study were purchased from local agrochemical stores. The active ingredient of Roundup is the isopropylamine salt of glyphosate in commercial solutions at 441 g/L. The remaining substance of the Roundup formulation consists of the active surfactant polyoxyethyleneamine. The active ingredient of methidathion in a commercial solution of Supracide is 426 g/L.

Reagents for enzymatic reactions—1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), *p*-nitrophenyl acetate (PNPA), acetylthiocholine iodide (ACTI), reduced glutathione (GSH), bovine serum albumin (BSA), and Bradford reagent—were obtained from Sigma (MO, USA). Oxidized glutathione (GSSG) and reduced β-nicotinamide adenosine-diphosphate (NADPH) were purchased from MP Biomedicals (USA). The AST and LDH diagnostic kits were obtained from Biolabo (France). Human chorionic gonadotropin (hCG; Pregnyl, 5000 IU) was provided by Organon (Istanbul, Turkey).

2.2. Test organisms

The FETAX solution was used in all breeding tanks for all test species, identified by the American Standards for Testing and Materials guideline (ASTM, 2003). Test media of controls and all treatment groups were prepared using FETAX solution. It was composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4{\cdot}2H_2O$, and 75 mg $MgSO_4$/L distilled water. The mean values of pH, conductivity, and dissolved oxygen in the used FETAX solution were determined to be 7.5 ± 0.03, 1.51 ± 0.05 mS/cm, and 8.2 ± 0.25 mg/L, respectively.

X. laevis tadpoles were obtained from adult frog pairs that were maintained in a frog colony in our laboratory. The breeding of *X. laevis* adults and the collection of embryos were performed as described in ASTM-E1439-98 (ASTM, 2003). For this aim, males and females were injected with 500 and 600 IU hCG into the dorsal lymph sac, respectively. All injections were done at midnight; deposition of eggs occurred in the early morning. Fertilized *X. laevis* eggs were selected under a stereomicroscope, and normal developing embryos were kept in well-aerated FETAX solution until embryos reached the tadpole stage 46 (Nieuwkoop and Faber, 1956). However, *P. viridis* and *P. ridibundus* eggs were collected during April and May 2011, respectively, from a creek near the Campus of Inonu University (38°20′03 N/38°27′16 E, Turkey). All the eggs were collected in one night after spawning, so they likely had minimal exposure to environmental contaminants and hatched in well aerated aquaria within FETAX solution at 23 °C and a 12:12-h light–dark cycle. Four-day-old tadpoles that were obtained from eggs were used as test organisms in this study. All tadpoles used for the assays were in the same developmental period. According to Nieuwkoop and Faber (1956) stage 46 tadpoles of *X. laevis* were equal to stage 24 of *P. ridibundus* and *P. viridis* described by Gosner (1960).

The tadpoles of control groups of each species were maintained in FETAX solution. All dilutions of pesticides were prepared daily in FETAX solution. Tadpoles were exposed to pesticide solutions at 23 °C (±1 °C) with a 12:12-h light:dark photoperiod in static test conditions.

2.3. Bioassays

For the toxicity tests, groups containing 20 tadpoles were randomly placed into covered polycarbonate dishes with 50 mL of different concentrations of methidathion (6.4–51.2 mg AI/L for *P. viridis*, 4.92–51.2 mg AI/L for *P. ridibundus* and 1.6–32.3 mg AI/L for *X. laevis*) and glyphosate (18.8–51.2 mg AI/L for *P. viridis* and *P. ridibundus*, 12.8–56.2 for *X. laevis*) solutions. All groups were tested with 4 replicate dishes, containing 20 tadpoles each, using a total of 80 tadpoles. Test solutions were changed every 24 h during the 4-day test periods. The dead tadpoles were removed, and the incidences were recorded. At the end of the experiment, median lethal concentrations (LC_{50}) were determined for 24-, 48-, 72-, and 96-h exposure periods.

For the enzymatic assays, groups containing 20 tadpoles were randomly placed into covered polycarbonate dishes with 50 mL of different sublethal concentrations (below LC_{50} values) of methidathion (0.1–12.8 mg AI/L) and glyphosate (0.1–25.6 mg AI/L) solutions with increasing concentrations by a factor 2x. All groups