Comparative Assessment of *In Vitro* and *In Vivo* Toxicity of Azinphos Methyl and Its Commercial Formulation

Abbas Güngördü, Miraç Uçkun

Department of Biology, Laboratory of Environmental Toxicology, Faculty of Arts and Science, Inonu University, Malatya 44280, Turkey

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ABSTRACT: The toxic effects of Gusathion (GUS), which is a commercial organophosphate (OP) pesticide, and also its active ingredient, azinphos methyl (AzM), are evaluated comparatively with *in vitro* and *in vivo* studies. Initially, the 96-h LC_{50} values of AzM and GUS were estimated for two different life stages of *Xenopus laevis*, embryos, and tadpoles. The actual AzM concentrations in exposure media were monitored by high-performance liquid chromatography. Also, the sub-lethal effects of these compounds to tadpoles were determined 24 h later at exposure concentrations of 0.1 and 1 mg/L using selected biomarker enzymes such as acetylcholinesterase (AChE), carboxylesterase (CaE), glutathione *S*-transferase (GST), glutathione reductase, lactate dehydrogenase, and aspartate aminotrasferase. Differences in AChE inhibition capacities of AzM and GUS were evaluated under *in vitro* conditions between frogs and fish in the second part of this study. The AChE activities in a pure electrical eel AChE solution and in brain homogenates of adult *Cyprinus carpio, Pelophylax ridibundus*, and *X. laevis* were assayed after *in vitro* exposure to 0.05, 0.5, 5, and 50 mg/L concentrations of AzM and GUS. According to *in vivo* studies AChE, CaE and GST are important biomarkers of the effect of OP exposure while CaE may be more effective in short-term, low-concentration exposures. The results of *in vitro* studies showed that amphibian brain AChEs were relatively more resistant to OP exposure than fish AChEs. The resistance may be the cause of the lower toxicity/lethality of OP compounds to amphibians than to fish. Environ Toxicol 30: 1091–1101, 2015.

Keywords: azinphos methyl; Gusathion; biomarker; fish; frog

INTRODUCTION

A pesticide is defined as a chemical or chemical mixture used to prevent, eliminate, remove, or reduce a pest. The majority of pesticides are not designed only for specific target species. Therefore they have potential risks of adverse effects on a diverse spectrum of organisms due to similarities in biochemical and physiological mechanisms both in the target and non-target organisms. Mammals, birds, fish, and frogs are considered to be non-target organisms for organophosphate (OP) insecticides, however, they have been affected by OP exposure along with target species, with irreversible acetylcholinesterase (AChE) inhibition (Ozmen et al., 1998; Küster, 2005; Lazarevic-Pasti et al., 2011; Rosenbaum et al., 2012; Foudoulakis et al., 2013). On the other hand, the sensitivity of non-target organisms to pesticide exposure may differ among species, due to differences in their detoxification routes and mechanisms of toxic action. These differences in the responses of various organisms and different life stages of one organism to pesticide exposure make it difficult to draw generalizations about the effects of pesticides on biota and ecosystems from data obtained from the life stage of only one bioindicator organism. This situation highlights the importance of comparative studies aimed

Correspondence to: Dr. A. Güngördü;
e-mail: abbas.gungordu@inonu.edu.tr

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at the assessment of the responses and susceptibility of different organisms. In addition, the elucidation of the mechanisms regulating the toxicity of pollutants on non-target organisms deserves more attention.

OPs have been important agents for pest management, industry, government, and military applications and are still widely used as alternatives to organochlorine compounds for pest control in the world today due their high potency/toxicity and relatively non-persistent characteristics (Chambers and Levi, 1992; Ozmen et al., 1999; Arufe et al., 2007). The OP insecticides that bind to the active site of the enzyme AChE can provoke toxicity by irreversibly inhibiting AChE both in target and non-target organisms (Tryfonos et al., 2009; Ferrari et al., 2011). AChE inhibition causes synaptic accumulation of acetylcholine, resulting in the excessive stimulation of postsynaptic cholinergic receptors (Sturm et al., 2007). Thus, continuous transmission of nerve signals causes tetany, paralysis, and often death (Fulton and Key, 2001).

Azinphos methyl (*O*,*O*-diethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl) methyl] ester (phosphorodithioate, type OP; AzM) was first registered in the United States in 1959 for use as a wide-spectrum insecticide to control fruits, vegetables, grains and forage crops from a variety of pests. AzM has been used as the active ingredient in several commercial OP pesticide formulations such as Guthion, Gusathion (GUS), Gusathion-M, Crysthyron, Cotnion, Cotnion-methyl, Metriltrizotion, Carfene, Bay 9027, Bay 17147, and R-1852 (Lewis et al., 2004). More than 900 tons of AzM were used annually as the active ingredient in pesticides in the United States according to the United States Environmental Protection Agency (US EPA, 2006). AzM has been banned in the European Union as a plant protection product based on Commission regulation 1376/2007 (EC, 2007). The distribution or sale of AzM products was prohibited after September 30, 2012, in the United States, but the EPA amended this regulation to permit the use of existing stocks of AzM products (US EPA, 2012). AzM was also recently banned in Turkey (Official Gazette of the Republic of Turkey, 2013). On the other hand, the use of AzM has been still continued in Australia and partly in New Zealand (APVMA, 2014).

The effects of AzM on amphibian development have been studied using physiological, morphological, and behavioral markers and may be summarized as reduced size, notochord bending, abnormal pigmentation, defective gut and gills, swimming in circles, body shortening, and impaired growth (Venturino et al., 2003). A determination of exposure to these pesticides (AzM and other OP insecticides) and also the evaluation of the physiological effects on exposed animals in aquatic environments were assessed using high anti-cholinesterase and carboxylesterase inhibitory actions as specific biochemical markers (Ozmen et al., 1999; Thompson, 1999; Beauvais et al., 2000; Fulton and Key, 2001; Pretti and Cognetti-Varriale, 2001; Wogram et al., 2001). Besides its anti-cholinesterase action, AzM, as well as other OPs, can affect detoxification and antioxidant enzymes such as glutathione S-transferase (GST) and glutathione reductase (GR) and also metabolic enzymes such as aspartate aminotrasferase (AST) and lactate dehydrogenase (LDH) (Agrahari et al., 2007; Ferrari et al., 2007, 2009; Anguiano et al., 2012). Antioxidant and other metabolic enzymes have been extensively evaluated in toxicological and ecotoxicological studies performed with diverse aquatic organisms such as fish species, amphibian embryos, and other invertebrates (Cunha et al., 2007; Ferrari et al., 2007, 2009; Attademo et al., 2011; Kristoff et al., 2011).

In general, commercial pesticides are mixtures of a certain amount of active ingredients together with additional compounds (US EPA, 2006). Although commercial formulations are useful determining the environmental toxicology of pesticide exposure in laboratory conditions, they may not always provide reliable results about the mechanisms of the toxic effects of the active ingredients due to the presence of additional compounds in the formulation. In addition, studying only the effects of analytically pure active ingredients for the assessment of impacts of the pesticide may also provide incomplete results due for similar reasons. Therefore, the toxic effects of AzM and its commercial formulation, GUS, were assessed comparatively in different embryonic stages of *X. laevis* in this study. Also, the differences in the responses of fish and frog brain AChEs after AzM and GUS exposure were evaluated under *in vitro* conditions.

ABBREVIATIONS

AchE	acetylcholinesterase
ACTI	Acetylthiocholine iodide
AST	aspartate aminotrasferase
BSA	bovine serum albumin
CaE	carboxylesterase
CDNB	1-chloro-2,4-dinitrobenzene
CYP	cytochrome P450
DTNB	5,5′-dithio-bis(2-nitrobenzoic acid)
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione *S*-transferase
GUS	Gusathion
HPLC	high-performance liquid chromatographic
LC	lethal concentrations
LDH	lactate dehydrogenase
MAC	maximum acceptable concentration
NADPH	β-nicotinamide adenosine-diphosphate reduced
OP	organophosphate
PNPA	*p*-nitrophenyl acetate

MATERIALS AND METHODS

Chemicals and Reagents

A test chemical, GUS M WP 25, was purchased from a local agro-chemical store. The percentage of AzM as an active

ingredient in GUS was declared by the manufacturer to be 25%. AzM was purchased from Sigma-Aldrich (98.5%). Acetylthiocholine iodide (ACTI), *p*-nitrophenyl acetate (PNPA), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), Bradford reagent, and bovine serum albumin (BSA) were purchased from Sigma (MO, United States). β-nicotinamide adenosine-diphosphate reduced (NADPH) and oxidized glutathione (GSSG) were obtained from MP biomedicals (United States). Human chorionic gonadotropin (hCG; Pregnyl, 5000 IU) was provided by Organon (Istanbul, Turkey).

In vivo Tests

Test Organisms

Xenopus laevis tadpoles were obtained from adult frogs of both sexes maintained in our laboratory. The breeding of *X. laevis* adults and the collection of embryos were performed as described in the American Society for Testing and Materials guidelines (ASTM, 2003). Males and females were injected with 500 and 600 IU of hCG into the dorsal lymph sac, respectively. All injections were done at midnight; deposition of eggs occurred in the early morning. Fertilized eggs were selected and eighth stage embryos were used for FETAX assay. Furthermore, normal developing embryos, which were not used for the FETAX assay, were kept in a well-aerated FETAX solution until the embryos reached the tadpole stage (stage 46) (Nieuwkoop and Faber, 1956). These tadpoles were used for the tadpole toxicity assays.

The FETAX solution used in all breeding tanks, controls, and treatments conformed to ASTM-E1439-98 (ASTM, 2003). It was composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4.2H_2O$, and 75 mg $MgSO_4$ per liter of distilled water. The values of pH and conductivity in the FETAX solution were determined to be 7.24 and 1.56 mS/cm, respectively. The embryos and tadpoles of control groups were maintained in a FETAX solution. The dilutions of AzM and GUS were prepared daily in the FETAX solution. Tadpoles were exposed to the pesticide dosing solutions at 23°C (±1°C) with a 12:12-h light:dark photoperiod under static test conditions.

Bioassays

Twelve-well cell culture plates were used both for the FETAX and tadpole toxicity assays. Groups containing five embryos (stage 8) or tadpoles (stage 46) were randomly placed into each well and different AzM and GUS exposure concentrations were applied. The total dosing volume was 3 mL. Each exposure concentration was repeated using six replicates; the total number of exposed tadpoles for each concentration was 30. Test solutions were changed every 24 h during the 4-day testing period. Dead embryos/tadpoles were removed and mortality was recorded. At the end of the experiment, the 96-h median lethal concentrations (LC_{50}) were determined.

For the enzymatic assays, groups containing 20 tadpoles at stage 46 were randomly placed into covered polycarbonate dishes with 50 mL of 0.1 and 1 mg/L concentrations of AzM and GUS solutions and each concentration was tested with four replicates with 80 tadpoles in total. Tadpoles were exposed to pesticide solutions for a 24-h test period. Tadpoles from each dish were collected and placed into a chilled microfuge vial after the exposure period. All vials were stored at −80°C in a deep freeze until the enzymatic assays.

High-Performance Liquid Chromatographic Analyses

For the purposes of determining the actual concentration of AzM in the exposure media, the concentration of AzM was monitored by a high-performance liquid chromatographic (HPLC) method using an Agilent 1100 system equipped with a diode array detector and an auto-sampler. A reversed-phase C18 (5-μm, 4.6-mm internal diameter, and 250-mm length) column was used in the experiments. Twenty microliters of the sample was injected. The column was eluted with a mixture of mobile phase: 40% water (containing 0.1% phosphoric acid) and 60% acetonitrile (v/v) with a flow rate of 1.4 mL/min. AzM was determined at 282 nm and was quantified by comparing with a standard curve of known concentrations.

Biochemical Analyses

Tadpoles were thawed, weighed, and homogenized on ice in a homogenization buffer (0.1 M K-phosphate buffer (pH 7.4) with 0.15 M KCl, 1 mM EDTA, 1 mM DTT). The homogenate was centrifuged at 16,000*g* for 20 min at 4°C and the supernatant was transferred into a clean microfuge tube. Enzyme activity in post-mitochondrial fractions was determined immediately after the centrifugation procedures, without freezing. All enzyme activities were determined spectrophotometrically according to the previously described methodology using a microplate reader system (VersaMax, Molecular Devices Corporation, United States) at 25°C. Samples were assayed in triplicate. Specific activities were expressed as nmol/min/mg protein.

The AChE activity of homogenates was measured in the sub-mitochondrial fraction, following the Ellman et al. (1961) method modified for microplate readers, as described by Ozmen et al. (2006). Ten microliters of supernatant were added to flat-bottom microplate wells. The final concentrations of ACTI and DTNB, prepared in a Trizma buffer (0.1 M, pH 8.0), were 0.7 mM and 0.14 mM, respectively. Changes in absorbance were monitored at 412 nm for 1 min.

The CaE activity was assayed with a modification of the procedure developed by Santhoshkumar and Shivanandappa (1999) for a microplate reader. A reaction mixture that

contained 5 μL of sample and 0.1 mM 250 μL of Trizma buffer (pH 7.4) was incubated for 3 min at 25°C. The reaction was initiated by the addition of 5 μL of the PNPA (final concentration, 0.5 mM) into the reaction solution. The liberated *p*-nitrophenol was followed at 405 nm for 2 min. Enzyme activities were calculated from the extinction coefficient of *p*-nitrophenol (1830 M^{-1} cm^{-1}).

GST activity was determined according to Habig et al. (1974) with some modifications. The reaction mixture contained 210 μL of 0.1 M K-phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB, and a 10-μL sample. Change in absorbance was monitored at 344 nm and the activity was calculated using an extinction coefficient of 9600 M^{-1} cm^{-1}.

The GR activity was measured with the method described by Stephensen et al. (2000) with some modifications. The reaction solution contained 0.075 mM DTNB, 1.2 mM NADPH, and 20 μL of sample in a total volume of 190 μL. The addition of 20 μL of 3.25 mM GSSG initiated the reaction. All the reagents were dissolved in 0.1 M K-phosphate buffer (pH 7.5) containing 1 mM EDTA. The reduction of DTNB was monitored at 405 nm and the enzyme activity was calculated using an extinction coefficient of 14 151 M^{-1} cm^{-1}.

The LDH and AST assays were conducted with a microplate reader at the appropriate wavelengths using the respective commercial test kits provided by the manufacturer (Biolabo, France). For LDH, 5 μL of supernatant was used, while for AST, 10 μL of supernatant was dispensed into each well and mixed with 200 μL of test reaction solution according to the methods described in the manufacturers' manuals.

The total protein concentration in the supernatant was measured using the Bradford method with BSA as a standard (0–1.4 mg BSA/mL) (Bradford, 1976). All protein measurements were carried out using a microplate reader. The following solutions were added to each microplate well: 5 μL of diluted supernatant (1:4) and 250 μL of the Bradford reagent. Absorbance was read at $\lambda = 595$ nm and the protein concentration was calculated from the calibration curve constructed from standard BSA solutions (0–1.4 mg BSA mL^{-1}). The calculated protein values were used to calculate the specific activity values of each tested enzyme.

In vitro Tests

Preparation of Enzymatic Samples

Brain samples of three adult individuals of each species, *C. carpio* (mirror carp), *X. laevis*, and *P. ridibundus* (marsh frog) were used. *X. laevis* were obtained from the frog colony in our laboratory. *P. ridibundus* were collected from a creek near the Campus of Inonu University (38° 20' 03 N, 38° 27'16 E, Turkey). The carp were caught from Karakaya Dam Lake by local fisherman. The protocol (Research Protocol No. 2013/A-44, May 22, 2013) for using animals in the *in vitro* experiments was reviewed and approved by the Inonu University Research Animals Ethics Committee.

Specimens were sacrificed after tricain anesthesia and the brain tissue of each animal was immediately dissected, weighed, and homogenized on ice in a 35:1000 (w/v) trizma buffer (pH 7.4) with a teflon-glass homogenizer (model RZR-2021, Heidolph, Germany) according to the method described by Ozmen et al. (1999). Homogenates were centrifuged at 16,000*g* for 20 min at 4°C. After centrifugation, the supernatants were collected into clean microfuge tubes. The supernatants were used as the AChE enzyme source in the *in vitro* assays.

Also, AChE (type VI-S) of electric eels (*Electrophorus electricus*), which typically exists as large multi-subunit complexes, was used to determine the *in vitro* anti-AChE effects of the tested Ops Ozmen et al. (1999). Before *in vitro* AChE inhibition with commercial electric fish, 500 units of enzyme were dissolved in 1 mL of Trizma buffer (0.05 M, pH = 7.4).

In Vitro AChE Inhibition Tests

For the determining AChE inhibition levels, 100 μL of the supernatant of brain homogenates of exposed animals or the commercial electric eel AChE solution (2.5 units/mL) were added to the wells of 96-well microplate, than 100 μL of 100, 10, 1, and 0.1 mg/L solutions of AzM and GUS were added to the supernatants. The nominal final concentrations of the tested compounds in the incubation media were 50, 5, 0.5, and 0.05 mg/L. The AChE activity in the assay mixture, incubated at 25°C, was measured at 0, 5, 15, 30, 45, 60, 90, and 120 min.

The total protein concentration in the supernatant after the centrifugation of the brain homogenates was measured using the Bradford method (1976), as discussed above. The difference in this case was the use of 5 μL of diluted supernatant (1:10). In this study, the levels of protein in the submitochondrial samples were determined to be 0.99, 1.61, and 0.86 mg/L for *C. carpio, P. ridibundus*, and *X. laevis*, respectively. The amounts of protein were used to calculate the specific activities for the AChE enzyme.

Statistical Analysis

The LC_{50} values were calculated by Finney's Probit Analysis using EPA Computer Program (Version 15.0). Statistical analyses of biochemical markers were performed using SPSS Version 15.0 software (SPSS, United States). One-way ANOVA (Dunnett's test) was used to evaluate the significant differences between AzM and GUS exposure and control groups. Significance was designated at $p < 0.05$and $p < 0.001$ for biomarker analysis.

RESULTS

The 96-h LC_{50} values of AzM were determined to be 31.6 and 30.1 mg/L for the eighth stage embryos and 46th stage

TABLE I. The 96-h LC_{50} values of AzM and GUS on embryos (stage 8) and tadpoles (stage 46) of *X. laevis*

	Measured Concentration			
	AzM (mg/L)		GUS (mg/L AzM)	
Nominal Concentration (mg/L)	Before exposure	After 24 h exposure	Before exposure	After 24 h exposure
Control	0	0	0	0
10.8	16.0	3.5	26.9	6.9
14.01	19.6	10.8	33.9	12.6
18.2	23.2	26.0	43.0	22.0
23.7	27.1	27.2	54.4	37.9
30.8	32.6	40.5	63.0	50.1
40	35.4	50.9	74.2	53.1
LC50 for stage 8	31.6 (29.9–33.3)[a]		18.4 (17.4–19.4)[a]	
LC50 for stage 46	30.1 (28.5–31.8)[a]	31.6 (30.5–32.5)[b]	12.4 (11.4–13.2)[a]	30.4 (28.3–32.2)[b]

[a]These values were calculated according to nominal concentrations.
[b]These values were calculated according to measured AzM concentrations in exposure solution before exposure by HPLC.
Values in parentheses represent 95% confidence limits.

tadpoles, respectively, according to the nominal exposure concentration (Table I). On the other hand, 96-h LC_{50} values of GUS were 18.4 and 12.4 mg AI/L for the eighth stage embryos and 46th stage tadpoles, respectively, according to the nominal exposure concentration. Furthermore, the LC_{50} values of AzM and GUS that were calculated according to measurement concentrations for stage 46 tadpoles were 31.6 mg/L and 30.4 mg AI/L, respectively.

According to the measured values, AzM levels in the two exposure chambers decreased after 24 h (Table I). AzM concentrations in all GUS applications decreased after 24 h. The lowest AzM concentrations (10.8 mg/L) decreased both in the AzM and GUS applications at rates of 78% and 74%, respectively (Table I). Figure 1 shows that the transformation/degradation of azinphos methyl in both the AzM and GUS applications seem to lead to the formation of the same metabolites.

The sub-lethal effects of pure and commercial AzM were evaluated with changes of biomarker enzymes. The stage 46 tadpoles of *X. laevis* were exposed to 0.1 and 1 mg/L or mg AI/L concentrations of both AzM and GUS for 24 h. The esterases, AChE, and CaE were inhibited after both exposure concentrations while GST activities increased after 1 mg/L AzM and GUS exposure ($p < 0.05$, Table II). However, no statistically significant differences were observed for the other selected biomarker enzyme activities (GR, AST, and LDH) for the control and AzM exposed groups ($p > 0.05$).

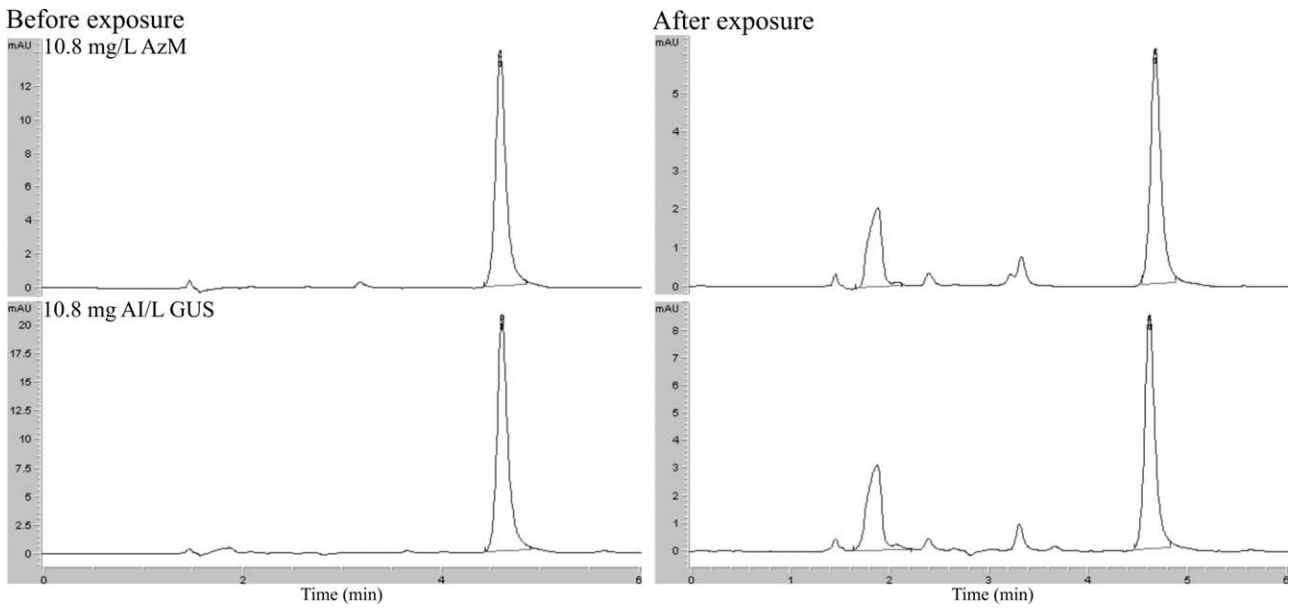

Fig. 1. The HPLC chromatograms of 10.8 mg/L AzM and GUS before exposure and 24 h after exposure.

TABLE II. The enzyme activities in stage 46 tadpoles after 24 h AzM and GUS exposure

Concentration (mg/L)		n	AChE	CaE	GST	GR	LDH	AST
Control		4	126.7 ± 8.5	181.4 ± 7.5	214.1 ± 3.7	4.59 ± 0.13	445.1 ± 34.5	198.6 ± 5.9
AzM	0.1	4	134.0 ± 4.6	120.4 ± 7.1[b]	230.6 ± 4.7	4.62 ± 0.08	443.9 ± 14.7	206.9 ± 7.8
	1	4	69.2 ± 5.2[b]	115.9 ± 7.5[b]	284.3 ± 9.8[b]	5.00 ± 0.24	482.8 ± 24.0	208.5 ± 13.7
GUS	0.1	4	112.0 ± 7.1	117.2 ± 4.8[b]	236.8 ± 10.8	4.76 ± 0.24	463.6 ± 9.6	202.6 ± 10.7
GUS	1	4	69.9 ± 2.6[a]	99.5 ± 5.4[b]	258.0 ± 6.1[a]	4.95 ± 0.18	444.1 ± 7.4	197.2 ± 5.7

Enzyme activities were expressed as nmol/min/mg protein ± standard error.
n: each concentration was tested with 4 replicates with 80 tadpoles in total.
[a] $p < 0.05$ was showed statistical significance compared with control.
[b] $p < 0.01$ was showed statistical significance compared with control.

The potential of AChE inhibition due to AzM and GUS exposure was tested in brain homogenates of *C. carpio*, *X. laevis*, and *P. ridibundus* and also in pure commercial samples of electric eel AChE under *in vitro* conditions (Tables III–VI). The AChE inhibition tests showed that AzM and GUS caused higher inhibition in fish enzyme samples than in frog enzyme samples (Fig. 2). The rate of inhibition in fish enzymes were dose and time dependent. 0.05 mg/L AzM or GUS exposure caused 34–27%, 24–13%, 0–0%, and 2–1% AChE inhibition after a 120 min exposure period for pure electric eel, *C. carpio, P. ridibundus*, and *X. laevis* brain enzyme samples, respectively. AzM and GUS caused 100–100%, 92–94%, 13–29%, and 22–24% AChE inhibition in pure electric eel, *C. carpio*, *P. ridibundus*, and *X. laevis* enzyme samples, respectively, due to 50 mg/L exposure in *in vitro* tests.

DISCUSSION

In this study, the 96-h LC_{50} values for AzM and GUS that were calculated according to nominal exposure concentrations were 31.6–30.1 mg/L (8th–46th stages) and 18.4–12.4 mg AI/L (8th–46th stages), respectively. These results suggest that GUS was more toxic than pure AzM due to its mixture properties as an active ingredient in commercial pesticides, whose increased toxicity may be attributed to this mixture. However, the 96-h LC_{50} values of AzM and GUS for stage 46 tadpoles which calculated according to measured exposure concentrations (31.6 and 30.4 mg/L, respectively) were very similar to each other. These results show that the real concentration of azinphos metyl is higher than the concentration of the active ingredient stated on the label of the commercial solution (GUS). Furthermore, the calculated LC_{50} values from this study and also from previous studies show that amphibian species are less sensitive to the toxicity of the tested chemicals than fish. According to the literature, the 96-h LC_{50} values for the embryonic stages (10–11) of *X. laevis* are between 6.1 and 11.9 mg/L (Schuytema et al., 1994). The 24-h LC_{50} values of GUS were determined to be 21.98 mg AI/L for 46th stage tadpoles of *X. laevis* in our previous study (Güngördü et al., 2013). Also, the 96-h LC_{50} values of AzM were determined to be 10.44 and 15.6 mg/L for *Rhinella arenarum* embryos and larvae, respectively (Ferrari et al., 2004, 2009). On the other hand,

TABLE III. Time-dependent inhibition of commercial electric eel AChE exposed to test chemicals with *in vitro* tests

	AChE Activity (μmol/L)															
	0th min		5th min		15th min		30th min		45th min		60th min		90th min		120th min	
Concentration	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)
Control	62.4		56.0		56.1		55.8		50.4		49.8		43.2		32.9	
AzM (mg/L)																
0.05	67.7	0	57.0	0	50.7	10	48.5	13	42.6	15	36.7	26	30.9	29	21.9	34
0.5	67.0	0	51.6	8	39.2	30	29.2	48	21.4	58	15.7	68	9.0	79	5.6	83
5	62.5	0	21.7	61	10.3	82	3.8	93	1.6	97	1.1	98	0	100	0	100
50	44.4	29	3.3	94	3.0	95	1.2	98	0	100	0	100	0	100	0	100
GUS (mg AI/L)																
0.05	69.2	0	61.6	0	54.7	3	53.6	4	48.1	5	44.6	10	39.3	9	24.0	27
0.5	67.0	0	52.0	7	41.4	26	33.4	40	25.2	50	18.9	62	12.5	71	4.5	86
5	63.8	0	26.5	53	13.1	77	4.3	92	1.1	98	0	100	0	100	0	100
50	47.9	23	9.7	83	5.1	91	4.5	92	4.3	92	2.2	96	0.8	98	0	100

Act.: Unit activity, Inh. (%): Inhibition percent according to control activity.

TABLE IV. Time-dependent inhibition of *C. carpio* brain AChE exposed to AzM and GUS with *in vitro* tests

	AChE Activity (nmol/min/mg protein)															
	0th min		5th min		15th min		30th min		45th min		60th min		90th min		120th min	
Concentration	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)
Control	84.4		69.1		71.6		67.4		62.6		64.5		62.4		62.3	
AzM (mg/L)																
0.05	81.9	3	72.6	0	69.9	2	64.4	4	62.0	1	57.0	12	51.9	17	47.6	24
0.5	82.1	3	66.9	3	52.9	26	40.0	41	33.4	47	25.9	60	21.0	66	14.7	76
5	74.9	11	38.5	44	16.7	77	9.4	86	9.5	85	8.6	87	9.4	85	3.3	95
50	60.3	29	12.6	82	8.6	88	5.5	92	7.7	88	7.5	88	8.9	86	4.7	92
GUS (mg AI/L)																
0.05	84.1	0	71.9	0	72.0	0	70.4	0	61.1	2	57.6	11	54.8	12	54.2	13
0.5	79.1	6	65.7	5	61.2	15	51.8	23	41.4	34	36.9	43	29.5	53	25.1	60
5	73.8	13	45.5	34	21.5	70	15.1	78	12.1	81	10.0	85	6.0	90	5.7	91
50	58.2	31	21.5	69	6.0	92	6.6	90	8.8	86	5.3	92	8.4	87	4.0	94

Act.: Specific activity, Inh. (%): Inhibition percent according to control activity. ($n = 3$).

TABLE V. Time-dependent inhibition of *P. ridibundus* brain AChE exposed to AzM and GUS with *in vitro* tests

	AChE Activity (nmol/min/mg protein)															
	0th min		5th min		15th min		30th min		45th min		60th min		90th min		120th min	
Concentration	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)
Control	41.4		44.2		44.5		45.4		44.5		43.3		43.8		44.0	
AzM (mg/L)																
0.05	42.8	0	44.2	0	43.7	2	44.6	2	44.6	0	44.4	0	45.0	0	45.5	0
0.5	42.8	0	44.5	0	42.8	4	45.2	1	44.7	0	42.6	2	45.0	0	43.7	1
5	42.1	0	43.5	2	42.7	4	45.4	0	43.6	2	42.9	1	43.4	1	43.1	2
50	42.1	0	43.5	2	41.9	6	44.1	3	41.0	8	41.4	4	40.5	7	38.1	13
GUS (mg AI/L)																
0.05	43.6	0	44.5	0	44.7	0	45.0	1	43.8	1	42.7	1	43.1	1	44.2	0
0.5	40.1	3	44.0	0	44.0	1	42.4	7	43.5	2	42.2	2	42.4	3	44.1	0
5	39.0	6	42.3	4	41.4	7	41.8	8	41.0	8	41.6	4	42.6	3	42.1	4
50	40.3	3	42.7	3	40.2	9	40.6	11	39.1	12	37.7	13	36.8	16	31.3	29

Act.: Specific activity, Inh. (%): Inhibition percent according to control activity. ($n = 3$).

TABLE VI. Time-dependent inhibition of *X. laevis* brain AChE exposed to AzM and GUS with *in vitro* tests

	AChE Activity (nmol/min/mg protein)															
	0th min		5th min		15th min		30th min		45th min		60th min		90th min		120th min	
Concentration	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)	Act.	Inh. (%)
Control	55.6		54.5		51.8		49.8		50.5		45.3		44.6		45.0	
AzM (mg/L)																
0.05	50.8	9	52.0	5	51.0	1	51.7	0	51.5	0	47.1	0	45.7	0	44.2	2
0.5	51.0	8	48.8	11	50.0	3	47.2	5	48.8	3	47.1	0	46.6	0	42.9	5
5	47.7	14	48.0	12	49.6	4	47.0	6	49.1	3	44.4	2	45.9	0	42.9	5
50	48.6	12	48.3	11	46.2	11	46.2	7	44.0	13	41.8	8	40.7	9	35.0	22
GUS (mg AI/L)																
0.05	57.6	0	55.5	0	59.4	0	54.4	0	54.1	0	48.6	0	48.4	0	44.3	1
0.5	58.1	0	55.0	0	57.0	0	50.0	0	47.8	5	44.9	1	43.6	2	44.1	2
5	56.0	0	51.8	5	54.0	0	47.6	5	45.9	9	40.9	10	41.6	7	45.0	0
50	54.3	2	49.0	10	49.9	4	42.9	14	39.8	21	36.9	19	34.8	22	34.3	24

Act.: Specific activity, Inh. (%): Inhibition percent according to control activity ($n = 3$).

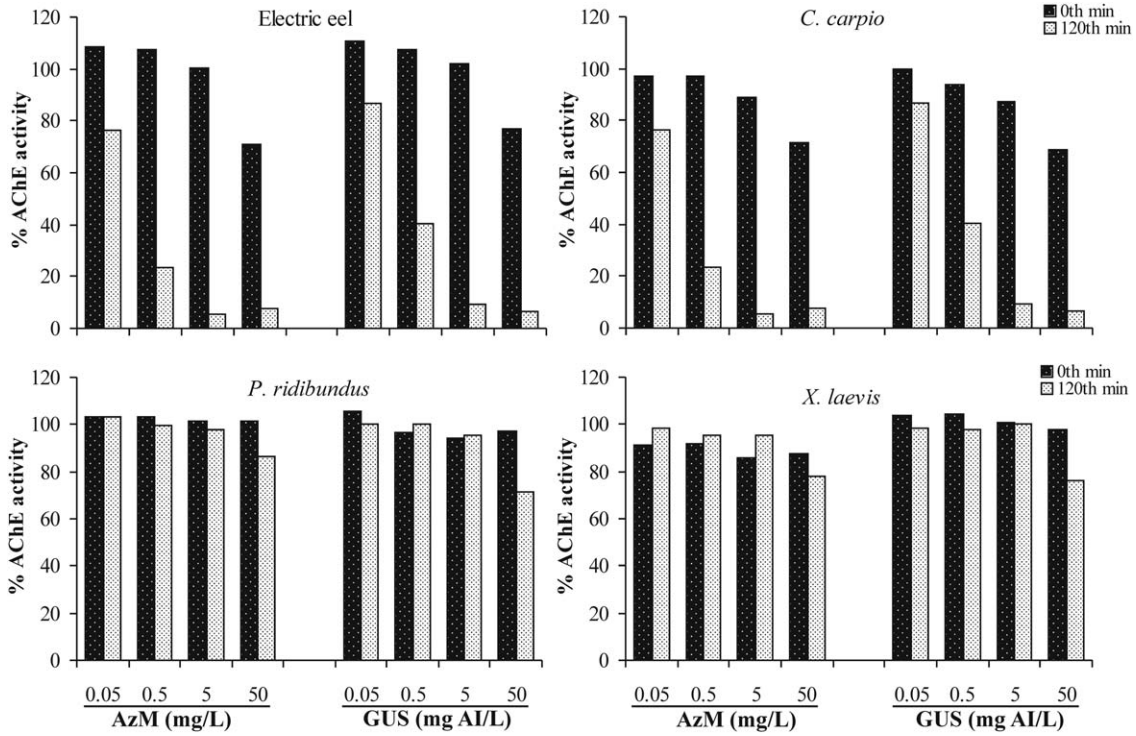

Fig. 2. % AChE activities of tested organisms at initial time of exposure (0th min) and after the 120 min of exposure with AzM and GUS under *in vitro* conditions.

the 96-h LC_{50} values of AzM for fish were determined to be 0.09 mg/L for *Oreochromis niloticus* (Oruç and Uner, 2000) and 0.007 mg/L for *Oncorhynchus mykiss* (Ferrari et al., 2009). The 72-h LC_{50} value was determined to be 0.0046 mg/L for *Sparus aurata* (Arufe et al., 2007).

The determined AzM concentrations in exposure media before and 24 h after exposure showed that low concentrations of AzM were transformed more than the higher concentrations. The degradation ratios in 10.8 mg/L AzM applications were more than 70% after a 24 h exposure period. Similar to our laboratory results, a mesocosm study aimed to determine persistence, distribution, and mass balance of AzM in the exposure pond and the half-life and long-term persistence (95% loss) of AzM within the water column was determined to be 1–2 days and 5–10 days, respectively (Knuth and Heinis, 2000). The maximum AzM concentration in the water phase was determined 1 h post-treatment and all concentration levels were below the detection level within 15 days. Furthermore, the half-life under environmental conditions was determined by modeling to be 0.9–5.5 days for German spring and summer conditions (APVMA, 2006).

In this study, the changes of several biochemical markers were determined 24 h after exposure to 0.1 and 1 mg/L AzM applications, for determining sub-lethal effects. The selected sub-lethal concentrations were approximately 5% and 5‰ of the 24 h LC_{50} reading [21.98 mg/L, according to Güngördü et al. (2013)] for *X. laevis* tadpoles and were still higher than acceptable concentrations in freshwater ecosystems; however, AzM concentrations may actually reach these values in environmental conditions. For example, reported concentrations of AzM in surface waters are in the range of 0.06 μg/L to 0.42 mg/L (Cacciatore et al., 2013). On the other hand, the maximum acceptable concentration (MAC) and health value for drinking water for AzM are 0.02 mg/L and 0.003 mg/L, respectively, according to the Guidelines for Canadian Drinking Water Quality and the Australian Drinking Water Guidelines, respectively (Canada FPT, 2003; NHMRC/NRMMC, 2004). However, according to "National Institute for Public Health and the Environment" of The Netherlands, the maximum permissible concentration in freshwater based on ecotoxicological data, the MAC for freshwater ecosystems and the serious risk concentration for freshwater ecosystems were 0.002 μg/L, 0.014 μg/L and 0.0048 mg/L, respectively (Moermond et al., 2008).

The induction of the phase II enzyme, GST, was determined only after 1 mg/L of AzM and GUS exposure; 0.1 mg/L did not cause to any GST induction ($p < 0.05$). GST induction was also shown in *B. arenarum* and *X. laevis* tadpoles after 6 mg/L (3 mg/L did not cause induction) and 5.5 mg/L AzM exposure, respectively, in previous studies (Ferrari et al., 2011; Güngördü et al., 2013). AzM has a thiophosphoryl bond (P═S) instead of a phosphoryl bond (P═O) and possesses minimal or no anti-cholinesterase activity and requires metabolic activation to their oxon analogs to inhibit ChE and CaE activities (Cacciatore et al.,

2013). The bioactivation of AzM to highly toxic oxons forms and metabolic detoxification pathways including phase I enzymes such as cytochrome P450s (CYPs) and esterases and other phase II enzymes (Cacciatore et al., 2013). Incubation of AzM with various subcellular fractions confirmed that the metabolism of AzM in the liver was mediated by GST and CYPs (APVMA, 2006). The metabolism of AzM by GST resulted in the formation of desmethyl isoazinphos-methyl and glutathionyl methylbenzazimide (Lewis et al., 2004).

The most commonly used biomarker for OP exposure is AChE inhibition. Dose-related AChE inhibitions were demonstrated in different OP pesticide exposures. On the other hand, OPs caused the inhibition of esterase enzymes other than AChE, such as CaE and BChE (Arufe et al., 2007; Wogram et al., 2001). CaE is involved directly in metabolism and subsequent detoxification of OP insecticides such as malathion (Wheelock et al., 2008) and also indirectly acts as an alternative phosphorylation site for OPs, thus irreversibly binding these enzymes to OPs and preventing the binding of OPs to AChE, thereby causing AChE inhibition (Denton et al., 2003; Küster, 2005; Ferrari et al., 2011). AChE and CaE inhibition after OP pesticides, AzM, diazinon, methidathion, and parathion exposure was shown in fish and frog species (Arufe et al., 2010; Leite et al., 2010; Tridico et al., 2010; Rosenbaum et al., 2012; Güngördü, 2013; Güngördü et al., 2013).

However, previous studies showed that AChE was more sensitive to AzM exposure. For example, exposure to 21.98 (the LC_{50} value after 24 h), 10.99 (the $LC_{50}/2$ value after 24 h), and 5.5 (the $LC_{50}/4$ value after 24 h) mg/L of AzM caused an 83%, 88%, and 90% inhibition of AChE, respectively, but the CaE inhibition ratios were determined to be 69%, 71.5%, and 71.9% with the specified doses. Although both enzymes were inhibited, the CaE inhibition exhibited a weak dose-response relationship (Ferrari et al., 2007, 2011; Denton et al., 2003). This situation is emphasized by the presence of some CaE isoforms with capacities to hydrolyze *p*-nitrophenyl but which are not inhibited by OP compounds. However, exposures to low concentration of AzM (0.1 mg/L) did not cause AChE inhibition while both *in vivo* AzM exposures (0.1 and 1 mg/L) caused CaE inhibition in the range of 31% to 45% in this study. Therefore, CaE may actually be a better biomarker to identify low-level OP exposures in amphibians. This sensitivity of CaE to OP pesticides and methyl paraoxon was also demonstrated in zebra fish in a previous study (Küster, 2005).

On the other hand, we also showed important differences among fish (electrical eels and the common carp) and frog (*P. ridibundus* and *X. laevis*) brain AChE activities in terms of sensitivity to *in vitro* AzM exposure in this work. Species-related differences in the inhibition capacity of brain AChE by OP compounds that were closely related to the susceptibility of the species to OP poisoning were shown in previous studies. For example, AChE inhibition related to the toxicity of mipafox, paraoxon, somon, tabun, sarin, dimethylphosphorylfluoride, and diethylphosphorylfluoride were higher in chickens and in rats than in frogs (Andersen et al., 1977; Chattopadhyay et al., 1986). Wang and Murphy (1982) also showed that chicken brain AChE was 100-fold more sensitive to inhibition by methyl paraoxon than frog brain AChE. Similarly, amphibian species such as *B. arenarum* tadpoles were less sensitive to AChE inhibition produced by AzM than rainbow trout (Ferrari et al., 2004). The susceptibility or resistance to acute intoxication of anti-ChE compounds have been attributed, in large part, to differences in the kinetics of inhibition of AChE (Qadri et al., 1984). The resistance of frogs could be explained on the basis that frog brain AChE has a lower affinity and a slower rate of phosphorylation. Furthermore, Ferrari et al. (2004) suggest that the level of susceptibility may be mainly related to the different availability of alternative targets to be phosphorylated by OPs. Also, Shapira et al. (1998) demonstrated that the insensitivity of *Xenopus* tadpoles to anti-cholinesterases was not due to the inefficient penetration of chemicals to those cells, which were alive in the tissues, since enzyme inhibition was linear and dose-dependent in all subcellular compartments of live *Xenopus* embryos. Rather, the relatively low toxicity of paraoxons toward *Xenopus* tadpoles could be attributed to the resilience of their AChE protein toward these inhibitors.

Our investigation provides further data indicating that *in vivo* exposure of *X. laevis* to sub-lethal concentrations of AzM and also its commercial form, GUS, caused serious brain intoxication and a modification of detoxification pathways and metabolic processes. AChE is a most important biomarker of the effect of OP exposure while CaE may be more effective in short-term, low-concentration exposures. The results of *in vitro* studies showed that amphibians were relatively more resistant to AzM poisoning than fish species and these differences may be due to the different AzM affinities for AChEs. Finally, we propose that toxicity data/results obtained from one species or only one life stage of a species may be insufficient to evaluate the ecological effects of pesticides due to differences in responses.

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