

Impact of Atrazine on Chlorpyrifos Toxicity in Four Aquatic Vertebrates

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Abstract. Atrazine has been shown previously to potentiate chlorpyrifos toxicity in selected invertebrates. This study examined interactions of atrazine and chlorpyrifos in four aquatic vertebrates. Organisms were exposed to binary mixtures of atrazine and chlorpyrifos during toxicity bioassays. Inhibition of cholinesterase (ChE) enzyme activity and chlorpyrifos uptake kinetics were also examined with and without atrazine exposure. Atrazine alone did not affect organisms at concentrations up to 5000 µg/L; however, the presence of atrazine at 1000 µg/L did result in a significant increase in the acute toxicity of chlorpyrifos in *Xenopus laevis*. Mixed results were encountered with *Pimephales promelas*; some bioassays showed greater than additive toxicity, while others showed an additive response. No effect of atrazine on chlorpyrifos toxicity was observed for *Lepomis macrochirus* and *Rana clamitans*. Atrazine did not affect ChE activity or chlorpyrifos uptake rates, indicating that these toxicodynamic and toxicokinetic parameters may not be related to the mechanism of atrazine potentiation of chlorpyrifos toxicity. Based on the results of this study, it does not appear that a mixture toxicity of atrazine and chlorpyrifos at environmentally relevant concentrations presents a risk to the vertebrate organisms examined in this study.

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-S-triazine) is a commonly used triazine herbicide that inhibits electron transport mechanisms of photosystem II in target plants (Woolhouse 1981; Beste 1983). This herbicide is widely used in the United States and commonly applied to corn, sorghum, sugarcane, orchards, turf grass, and other field crops. The estimated amount of atrazine applied in the United States in 2001 was between 33.6 and 36.3 million kg of active ingredient (a.i.), making it the second most heavily applied herbicide in the country behind glyphosate (Kiely *et al.* 2004). Atrazine is often detected in ground and surface water as a result of agricultural runoff. For example, the U.S. Geological Survey (USGS 1998) reported atrazine concentrations of 0.01 µg/L or greater in over 80% of large streams in mixed land use areas (agricultural and urban). Atrazine has also been

detected in over 70% of smaller streams and shallow ground water aquifers (USGS 1998). Atrazine has a hydrolysis half-life of 30 d and relatively high water solubility (32 mg/L), which aids in its infiltration into ground water (Orme and Kegley 2004). Atrazine concentrations of 20 µg/L have been commonly detected in surface water runoff, while concentrations as high as 700 µg/L have been reported (Perry 1990; Solomon *et al.* 1996; Selim 2003). These concentrations are much higher than the U.S. Environmental Protection Agency Maximum Contamination Level for drinking water (i.e., 3 µg/L) (Thurman *et al.* 1992).

Organophosphate insecticides (OPs) are commonly used in agriculture to control soil arthropods, with applications often coinciding with periods of atrazine use. In 2001, OPs accounted for 70% of all insecticide applications in the United States, totaling 33.1 million kg of active ingredient applied (Kiely *et al.* 2004). Chlorpyrifos (5.0–7.3 million kg a.i.) was second only to malathion (10.4–14.5 million kg a.i.) in OP application in the United States in 2001 (Kiely *et al.* 2004). Organophosphate insecticides affect target species by inhibiting cholinesterase (ChE) activity and affecting neuromuscular function. Chlorpyrifos has been shown to be more toxic after biotransformation within the organism via oxidative desulfuration resulting in the oxon-analog (Klaassen 2001).

Pesticides often co-occur in the environment with many chemicals applied at the same time in agricultural and urban settings. Some chemical combinations have been shown to yield additive results, while others have shown less than additive or greater than additive toxicity. Pape-Lindstrom and Lydy (1997) reported less than additive to greater than additive toxicity when mixtures of atrazine and different OPs were tested using the midge *Chironomus tentans*. Atrazine in binary mixtures with chlorpyrifos, methyl-parathion, trichlorofon, and malathion all yielded greater than additive toxicity. A ternary mixture of atrazine, methyl-parathion, and methoxychlor also yielded greater than additive results. However, in binary mixtures of atrazine and methoxychlor, less than additive toxicity was observed (Pape-Lindstrom and Lydy 1997). Atrazine was also shown to cause greater than additive toxicity in *C. tentans* and *Hyaella azteca* in binary mixtures with the OPs chlorpyrifos, methyl-parathion, and diazinon (Belden and Lydy 2000; Anderson and Lydy 2002). Recently, greater than additive toxicity was noted in *C. tentans* between atrazine in binary mixtures with chlorpyrifos and diazinon (Schuler *et al.* 2005).

To date, most studies on triazine herbicide and OP insecticide interactions have focused on invertebrate species (Pape-Lindstrom and Lydy 1997; Belden and Lydy 2000; Anderson and Lydy 2002). Vertebrates are also found in the same aquatic and semi-aquatic environments affected by agricultural runoff, often filling important niches as both consumers and acting as a food source for other organisms. Also of concern are amphibian species, which have experienced population declines over the past 10 to 15 years (Wake 1991; Blaustein *et al.* 1994). Often the application times of insecticides and herbicides (spring to early summer) coincide with the anuran breeding and developmental periods. These egg and larval stages are thought to be the most sensitive in the amphibian life cycle (Harris *et al.* 1998; Linder *et al.* 2003).

The primary objective of this study was to examine the impact of atrazine on chlorpyrifos toxicity in early life stages of four aquatic vertebrate species including, fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), green frog (*Rana clamitans*), and African clawed frog (*Xenopus laevis*). An additional objective was to examine the variation in ChE activity and chlorpyrifos uptake rate when organisms were exposed to chlorpyrifos in combination with atrazine. Since these vertebrate organisms inhabit the same environments as the invertebrate species previously examined, we expected to observe similar patterns such as the influence of atrazine on chlorpyrifos toxicity, inhibition of ChE activity, and chlorpyrifos uptake rates.

Materials and Methods

Chemicals

Chlorpyrifos and atrazine were purchased from Chem Service (Westchester, PA). Purity of the compounds was verified by the manufacturer to be 99.5% and 98.0% pure for chlorpyrifos and atrazine, respectively. Radiolabelled ^{14}C -chlorpyrifos was purchased from Sigma Chemical Co. (St. Louis, MO) and had a specific activity of 47.7 mCi/mmol. The compound was purified to 94.5% purity using thin layer chromatography (using a solvent system of hexane:acetone, 90:10 v/v) followed by liquid scintillation counting. Analytical grade acetone was used to solubilize the neat pesticides and as the carrier solvent. Atrazine is a triazine herbicide that is water soluble to 32 mg/L, has a log K_{ow} of 2.7, and a hydrolysis half-life of 30 d. Chlorpyrifos, an organophosphate insecticide, is water soluble to 1.39 mg/L, has a log K_{ow} of 5.0, and a hydrolysis half-life of 58 d (Orme and Kegley 2004).

Organisms

All organisms were maintained at Southern Illinois University (SIUC) (Carbondale, IL) under institutional animal care use committee protocol # 04-017. *Pimephales promelas* were cultured at SIUC following standard procedures (US EPA 1994). Culture water was filtered with activated carbon and aged at least 24 h prior to use. Eggs were removed from breeding tanks, incubated in a water bath, and treated with methylene blue (3 $\mu\text{g/L}$) to prevent fungal growth. The prophylactic addition of methylene blue was not a treatment of a known fungal infection. After hatching, larval fish were contained as a cohort in 5-L plastic tanks with aeration and were fed freshly hatched brine shrimp (*Artemia salina*) nauplii twice daily until they were used in a

bioassay or were approximately 100 d old. Older fish were fed Zeigler's fin-fish starter food (Gardners, PA) twice daily. Hatchery raised *L. macrochirus* fry (Logan Hollow Fish Hatchery, Murphysboro, IL) were removed from ponds by seining, graded for size, and transported to SIUC. Once acclimated to laboratory conditions, *L. macrochirus* care was identical to juvenile *P. promelas*. Fish were held for at least one week in the laboratory prior to use in bioassays.

Xenopus laevis were cultured at SIUC by artificially induced breeding using injections of human chorionic gonadotropin (HCG) (Etheridge and Richter 1978). Two different breeding pairs were used for each spawning session. After the final injection, the organisms were placed in a common tank and remained undisturbed until amplexus was completed (approximately 24 h). Eggs were allowed to mix and the cohort as a whole was used in the toxicity bioassay. After eggs were produced, adult frogs were removed and viable embryos were collected. Embryos were housed in an aerated aquarium and allowed to hatch. Larvae reached stage 35 at approximately 48 h post-hatch and were free swimming with a developed adhesive gland (Nieuwkoop and Faber 1994). Larvae reached stage 45 at approximately 96 h post-hatch and had developed a mouth, heart, nose, and intestine (Nieuwkoop and Faber 1994). *Rana clamitans* egg clutches were collected from ponds in the Shawnee National Forest (McClure, IL). Six egg masses were transported to the laboratory in pond water with protection from sunlight and excessive heat and egg masses were acclimated to laboratory conditions. After hatching, organisms were housed in one aerated aquarium to ensure thorough mixing of all clutches and fed blanched spinach daily during holding and testing periods.

Toxicity Bioassays

Experimental design for bioassays consisted of a 3×5 factorial design for each experiment with atrazine at 0, 100, and 1000 $\mu\text{g/L}$ and five concentrations of chlorpyrifos over a 96-h exposure period. A sub-set of organisms for each species was also exposed to 5000 $\mu\text{g/L}$ atrazine to determine whether a high concentration of atrazine affected these species. Chlorpyrifos concentrations targeted the EC10, EC25, EC50, EC75, and EC95 for each species based on preliminary dose-response experiments and the time-weighted measured concentrations for each of these effective levels are provided in Table 1. Temporally, the design consisted of a 48-h pre-exposure to atrazine followed by an atrazine and chlorpyrifos mixture or atrazine alone treatment for the remaining 48-h period. This pulsed-dose method was employed to simulate an environmental exposure with repeated pesticide applications and run-off events. In addition, an atrazine pre-exposure allowed time for stimulation of the P450 enzyme system prior to chlorpyrifos exposure. This helped to avoid toxic effects of chlorpyrifos prior to stimulation of the P450 enzymes by atrazine, which may have weakened or even masked the ability to observe the interaction. During each experiment, dead organisms and uneaten food were removed from test chambers daily and water renewals were performed at 48 and 72 h of exposure, during which >90% of test water was replaced with clean, dosed water. For this study, a behavioral endpoint was used exclusively. The behavioral response variable consisted of avoidance of a probe, while simultaneously maintaining equilibrium. Probing was repeated twice, and an organism was considered affected if it did not respond positively both times. This endpoint was chosen over mortality after preliminary experiments indicated that sublethal behavioral impacts would likely limit survival in natural settings (i.e., organisms often swam erratically or maintained a visible heartbeat without any other movement and were clearly affected by the OP). At the end of each bioassay, 48-h chlorpyrifos EC50s were estimated and surviving organisms were euthanized with MS-222. Negative controls (water only), solvent controls, and atrazine controls (1,000 $\mu\text{g/L}$) were included in each test. Solvent controls consisted of clean water with

Table 1. Time-weighted chlorpyrifos concentrations over 24-h exposure periods for each chlorpyrifos × atrazine mixture bioassay

Targeted EC Level	<i>P. promelas</i> <24h (Test1)	<i>P. promelas</i> <24h (Test2)	<i>P. promelas</i> (80 d)	<i>X. laevis</i> (Stage 35)	<i>X. laevis</i> (Stage 45)	<i>R. clamitans</i>	<i>L. macrochirus</i>
EC10	42.0 (±1.7)	45.3 (±6.3)	70.9 (±3.2)	57.1 (±4.8)	42.4 (±1.6)	60.5 (±14.3)	0.04 (±0.01)
EC25	73.0 (±2.3)	73.9 (±9.2)	102.1 (±5.6)	96.7 (±3.6)	75.6 (±12.8)	160.3 (±26.4)	0.89 (±0.03)
EC50	106.1 (±2.0)	118.1 (±1.6)	166.5 (±23.5)	202.6 (±13.0)	139.1 (±17.4)	302.9 (±22.2)	1.77 (±0.07)
EC75	149.6 (±11.3)	163.1 (±12.6)	222.1 (±21.9)	344.4 (±29.5)	278.0 (±8.3)	455.9 (±2.6)	3.30 (±0.16)
EC95	223.0 (±16.0)	229.7 (±5.6)	343.9 (±37.6)	473.9 (±101.3)	612.7 (±173.8)	587.9 (±0.71)	6.58 (±0.32)

Values are means (± standard deviation) from time-weighted chlorpyrifos concentrations from the control, 100-, and 1000- µg/L atrazine treatments (i.e., $n = 3$) within each targeted EC level.

the addition of acetone at the highest volume used in the bioassay, never exceeding 300 µL and typically around 100 µL.

Bioassays using *Pimephales promelas* (<24 h), *Xenopus laevis* tadpoles (stage 35 and stage 45; Nieuwkoop and Faber 1994), and *Rana clamitans* tadpoles (approximately 8 d post hatch) were conducted in 0.5- or 1-L glass beakers with reconstituted moderately hard water (RMWH) in environmental chambers at 25°C and a 16:8 light:dark photoperiod (Precision Scientific model 818, Chicago, IL). Three replicate beakers of 10 organisms were examined for each treatment. Dissolved oxygen, temperature, pH, and conductivity were recorded at the beginning and end of the 96-h testing period to check for consistency of water quality parameters among test chambers.

Experimental chambers for 80-d-old *P. promelas* consisted of 9.5-L glass aquaria. Bioassays were conducted in a temperature controlled room with a 16:8 light:dark photoperiod. Ten *P. promelas* were exposed in each tank with three replicate tanks for each treatment combination. In this system, 7 L of carbon-filtered tap water was added to each aquarium. Due to the water demands of these large-scale tests, RMWH was replaced with carbon-filtered tap water. Replicate tests were only compared within like water sources, never between water sources. Carbon-filtered tap water was aged prior to use for >24 h to allow equilibration to room temperature. During water renewals, 5 L of test water was replaced with clean water and the appropriate chambers dosed with the proper amount of atrazine and chlorpyrifos from initial dosing stocks. The same testing conditions were used for juvenile *L. macrochirus* (mean mass ± SD = 0.35 ± 0.12 g per fish) except experiments were conducted in 5-L glass jars with 3.5 L of carbon-filtered tap water with five organisms per jar, there were three replicate jars per treatment combination, and renewals consisted of 3 L of clean dosed water.

Cholinesterase Activity

Preliminary experiments indicated that fish surviving toxicity bioassays using the behavioral endpoint had greatly inhibited (~90%) ChE activity. This was primarily due to chlorpyrifos exposure periods of 48 h coupled with concentrations capable of eliciting a substantial behavioral impact or even lethal response. A second set of bioassays was conducted using lower chlorpyrifos concentrations, and was designed to examine variation in ChE activity in response to atrazine-chlorpyrifos interactions. In these bioassays, *P. promelas* (<24 h) and *X. laevis* (stage 35) were exposed to atrazine, chlorpyrifos, or a mixture of the two chemicals in a 2 × 4 factorial design. Two concentrations of atrazine (0 and 1000 µg/L) and four concentrations of chlorpyrifos (0, 1.03, 8.87, and 47.14 µg/L for *P. promelas* and 0, 0.60, 4.25, and 33.31 µg/L for *X. laevis*) were used in these experiments. Based on toxicity data, the highest chlorpyrifos concentrations used (33.3 and 47.1 µg/L) were close to the EC1 for each species. Organisms were exposed in five replicate beakers per treatment with 10 animals in each beaker and pre-exposed to atrazine for 48 h. Following a 90% water renewal, organisms were exposed simultaneously to atrazine and chlorpyrifos for an additional 12 h. Chlorpyrifos exposure time was reduced as in previous tests from 48 h to 12 h to ensure that ChE activity could be measured before effective or lethal body residue levels were reached (Belden and Lydy 2001). At the end of the testing period, all organisms were rinsed with deionized water and frozen at -80°C.

P. promelas and *X. laevis* were assayed for ChE activity using methods modified from Ellman *et al.* (1961) for use with 96-well microplates. All individuals from an experimental unit were split into sub-sample replicates for analysis. Activity values for individual fish were averaged to derive a mean for each experimental unit. Organisms were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 0.1% (v/v) Triton X-100 using a Teflon homogenizer. Whole body homogenates were centrifuged at 1378g for 20 min at

4°C (Eppendorf 5702R, Hamburg), and the supernatants were assayed for ChE activity using acetylthiocholine iodide (AThCh) and 5',5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Denton *et al.* 2003). In addition, tetraisopropylpyrophosphoramidate (Iso-OMPA) was used to differentiate acetyl and butyryl ChE. The AThCh, DTNB, and Iso-OMPA concentrations used were either previously reported in the literature (Denton *et al.* 2003) or derived from optimization experiments. The AThCh concentrations for *P. promelas* and *X. laevis* were 2×10^{-3} and 6×10^{-3} M, respectively, and Iso-OMPA concentrations were 4.4×10^{-5} and 3.3×10^{-4} M, respectively. For both species, a DTNB concentration of 3.2×10^{-4} M was used. The final reaction volume was 250 μ l and included 30 μ l of the sample supernatant. Absorbance was measured with a spectrophotometer (Thermo Lab-systems, Vantaa) at a wavelength of 405 nm at 23°C, and readings were taken 45 times over a 6-min reaction period. Total ChE values were expressed as μ mol of AThCh hydrolyzed/min/mg protein. All reagents and buffers were purchased from Sigma-Aldrich (St. Louis, MO).

A detergent-compatible protein assay based on the Lowry method was used to measure total protein levels in samples (Bio-Rad Laboratories, Hercules, CA). Protein concentrations were measured colorimetrically at $\lambda = 750$ nm using bovine serum albumin as a standard.

Uptake Experiment

An uptake study was conducted on *P. promelas* to examine the effects of atrazine on the rate of chlorpyrifos uptake. This toxicokinetic study followed methods modified from Schuler *et al.* (2003). Briefly, *P. promelas* (<24 h old) were placed in 250-ml beakers containing 200 ml of RMHW. Organisms were pre-exposed, in groups of 15, for 48 h to either RMHW or RMHW spiked with 1000 μ g/L atrazine. After this period, organisms were transferred to fresh RMHW spiked with 1.68 μ g/L 14 C-labelled chlorpyrifos and either 0 or 1000 μ g/L atrazine. Sampling times were 15, 30, 60, 90, and 120 min from the initial transfer to chlorpyrifos-spiked water. At each time point, two replicate beakers for each treatment were sampled, organisms (as a batch) were rinsed thoroughly with deionized water, blotted dry, and wet weight measured. Organisms were weighed to the nearest 0.1 mg on a Mettler model B154-S analytical balance (Columbus, OH). After weighing, organisms were placed in liquid scintillation vials containing 10 ml of ScintiSafe Plus 50% scintillation cocktail (Fisher, Fair Lawn, NJ). Samples were sonicated for 30 s (Tekmar High-Intensity Ultrasonic Processor, Tekmar Corp., Solon, OH) and allowed to equilibrate for 24 h prior to being radioassayed to allow for complete extraction of radio-labeled chlorpyrifos from organisms tissues. The surface of each vial was cleaned and samples were radioassayed by liquid scintillation counting (LSC). The LSC was performed using a Tri-Carb 2900 TR liquid scintillation counter (Packard Bioscience Company, Meridian, CT) using the external standards method to ensure background radiation was removed from samples.

The uptake data for chlorpyrifos in *P. promelas* were analyzed using an iterative least squares procedure, which fit the data using a first-order one compartment model in the software package Scientist[®] (Micromath, Salt Lake City, UT) (Lydy *et al.* 2000, Schuler *et al.* 2003). The following equation was employed:

$$\frac{dC_a}{dt} = (k_u C_w) - (k_e C_a)$$

where C_a = concentration of total compound in the animal (μ g/g wet wt), C_w = concentration of water (μ g/L), k_u = uptake clearance coefficient (ml compound/g organism wet wt \times min), k_e = elimination rate constant (min^{-1}), and t = time (min). This

model assumes that the concentration of the compound in the water stays constant, which it did during the 120-min period.

Chemical Analyses

All dosing stock solutions were analyzed prior to use to confirm chemical concentrations. Chlorpyrifos stocks were analyzed using an Agilent Technologies 6890N gas chromatograph (Palo Alto, CA) set in pulsed split mode with a nitrogen-phosphorus detector (GC-NPD) at 260°C. The Agilent Technologies HP5 capillary column was a 30 m \times 0.320 mm with a 0.25- μ m film thickness. The oven program started at 160°C, ramped to 190°C at 10°C/min, was maintained for 3 min, then continued to ramp at 10°C/min to 270°C, and was maintained an additional 3 min. Combined helium flow was 10 ml/min with make-up flow at 7.8 ml/min and column flow at 2.2 ml/min. Test chamber water was also analyzed to ensure proper chemical concentrations throughout the testing period. The second replicate of each treatment was tested exclusively throughout the entire test. The testing regime included all controls and totaled 18 samples per time point. These samples included three control samples and five samples from each atrazine concentration (0, 100, and 1000 μ g/L). Samples were taken at 1 h after initial chlorpyrifos dosing and at the 24-h time point prior to water renewal. A water:hexane extraction (1:1 v/v) was used to analyze test chamber chemical concentrations. In this extraction, 5 ml of test water was added to 5 ml of hexane in a 25-ml scintillation vial. This mixture was shaken briskly by hand for 5 min and then allowed to settle for 10 min. A 1.5-ml aliquot of the hexane layer was then transferred to a GC vial for analysis. For *L. macrochirus*, 100 ml of test water was extracted with hexane. The hexane wash was evaporated under nitrogen until it reached a final volume of 1.0 ml, which ensured a 100-fold enrichment. The remaining hexane was then analyzed by GC-NPD following the methods of Belden *et al.* (2000). Analyzed chemical peaks were compared to calibration curves for each compound. The instrument was re-calibrated with four chlorpyrifos standards within each set of samples for quality control. Qualitative identity was established using a retention time window of 0.5% (Belden *et al.* 2000).

Atrazine stocks and exposure water were analyzed using an Agilent Technologies 1100 high-performance liquid chromatograph (HPLC). Signal detection was at 254 nm (VWD 1A) with a peak width of >0.025 min. Water and acetonitrile (ACN) were used as the carrier solvents with flow set at 1.2 ml/min. A solvent gradient was established starting with 5% ACN and shifting to 99% ACN over 8.0 min. The ACN was held at 99% for 0.25 min before returning to 5% over 1.0 min for a total runtime of 9.25 min. The instrument was re-calibrated with four atrazine standards within each set of samples for quality control.

Statistical Analyses

Initial EC50 estimates based on measured water concentrations were calculated using Trimmed Spearman-Kärber analysis (EPA version 1.5, Duluth, MN). From these estimates, synergistic ratios (SR) were calculated to describe the magnitude of effects encountered in the study and allow simple comparison of EC50s. The SRs were calculated by dividing the chlorpyrifos EC50 for a non-atrazine treatment by the chlorpyrifos EC50 from an atrazine treatment. Analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare the percent effects of chlorpyrifos toxicity among atrazine treatments. Two-factor ANOVA was used to compare the behavioral response endpoint and ChE activities among treatment combinations (SAS Institute, Cary, NC). A two-sample *t*-test was used to compare control and solvent control ChE activity. Because there were no sig-

nificant differences in ChE activity between controls and solvent controls for all cases, control values were pooled. Statistical differences between uptake rates of chlorpyrifos with and without atrazine were analyzed using an approximate z -test (Schuler *et al.* 2003).

Results

Water Chemistry

Water chemistry was similar between the two sources and likely was not a modifier of toxicity nor did it influence stability of atrazine and chlorpyrifos. For RMHW, pH was 7.99 ± 0.02 and conductivity was $307 \pm 3.39 \mu\text{S/cm}$; for carbon-filtered tap water pH was 8.07 ± 0.02 and conductivity was $193 \pm 1.19 \mu\text{S/cm}$.

Chemical Analyses

Atrazine concentrations were reported as nominal, since concentrations were within 95% of nominal and did not significantly drop throughout the duration of any bioassay. Actual chlorpyrifos water concentrations were more variable, so measured concentrations were reported for this compound (Table 1). For *P. promelas*, chlorpyrifos concentrations averaged 92.7, 79.8, and 80.9% of the initial concentration for three respective tests. *X. laevis* chlorpyrifos concentrations were 67.3 and 66.1% of the initial concentrations and *R. clamitans* concentrations were 101.3% of initial concentrations. More specifically, in most bioassays chlorpyrifos concentrations remained fairly consistent throughout each 24-h static renewal period. The time-weighted measured water concentrations from each test were used for all chlorpyrifos EC50 estimates and other statistical calculations (Table 1).

Toxicity Bioassays

Preliminary bioassays with atrazine exposure at 5000 $\mu\text{g/L}$ for 96 h did not result in acute responses (i.e., mortality or behavioral response to a stimulus) for any of the organisms tested. For all bioassays, there was no difference between control and solvent controls and they were pooled into one group for statistical analysis. For larval *P. promelas* (<24 h old), the addition of atrazine resulted in a significant increase in chlorpyrifos toxicity based on mean percent effects calculated with ANOVA in one bioassay (test 2: $F_{2,30} = 8.22$, $p = 0.001$) (Tables 2 and 3). Although the second experiment did not show a significant affect of atrazine when analyzed with ANOVA, synergistic ratios calculated from EC50s suggest atrazine may have been responsible for a subtle difference in chlorpyrifos toxicity (Table 2). In one toxicity test with 80-d-old *P. promelas*, experimental units that received both atrazine and chlorpyrifos had a lower EC50 than those that were dosed with chlorpyrifos alone ($F_{2,30} = 4.09$, $p = 0.027$) (Tables 2 and 3). This test had a synergistic ratio less than one, a trend that was opposite of what was predicted based on the greater than additive toxicity noted in other *P. promelas* bioassay results.

Table 2. Summary of chlorpyrifos EC50 values ($\mu\text{g/L}$) with 95% confidence intervals (in parenthesis) for each atrazine concentration ($\mu\text{g/L}$) calculated using Trimmed Spearman-Kärber (TSK)

Species	Age	Atrazine	EC50	SR
<i>P. promelas</i>	<24 h-Test 1	0	131.2 (117.6–146.4)	
		100	119.7 (103.4–138.6)	1.10
		1000	99.98(88.83–112.5)	1.31
	<24 h-Test 2	0	148.6 (134.4–164.4)	
		100	139.7 (120.0–162.7)	1.06
		1000	126.7 (109.6–146.5)	1.17
	~80 d	0	133.9 (115.4–155.4)	
		100	156.5 (137.1–178.6)	0.86
		1000	155.5 (140.2–172.4)	0.86
<i>X. laevis</i>	Stage 35	0	115.1 (94.26–140.5)	
		100	80.05 (59.65–107.41)	1.44
		1000	61.98 (45.80–83.87)	1.86
	Stage 45	0	155.6 (130.6–185.5)	
		100	164.1 (137.4–196.0)	0.95
		1000	86.92 (72.79–103.8)	1.79
<i>R. clamitans</i>	~8d	0	235.9 (211.6–263.1)	
		100	218.0 (192.8–246.6)	1.08
		1000	235.2 (206.9–267.4)	1.00
<i>L. macrochirus</i>	0.35 g	0	1.78 (1.46–2.17)	
		1000	1.95 (1.61–2.38)	0.91

Synergistic ratios were calculated by dividing the chlorpyrifos EC50 of the non-atrazine treatment by the chlorpyrifos EC50 from an atrazine treatment.

For both *X. laevis* bioassays, a significant effect of atrazine on chlorpyrifos toxicity was observed (test 1: $F_{2,30} = 13.24$, $p < 0.001$, test 2: $F_{2,30} = 4.12$, $p = 0.026$) and this observation was also supported by the calculated synergistic ratios (Tables 2 and 3). For both *R. clamitans* and *L. macrochirus*, no effect of atrazine on chlorpyrifos toxicity was observed (*R. clamitans*: $F_{2,30} = 0.56$, $p = 0.575$, *L. macrochirus*: $F_{1,20} = 1.23$, $p = 0.280$) and this was supported by synergistic ratios around 1 (Tables 2 and 3).

Cholinesterase Activity

In both *P. promelas* and *X. laevis*, greater than 95% of total ChE enzyme activity was attributed to acetylcholinesterase; therefore, total ChE is reported throughout. A similar percentage was reported for *X. laevis* whole body homogenates (Gindi and Knowland 1979; Richards and Kendall 2002).

For experiments in which ChE activity was the response variable, there was a significant dose-dependent effect of chlorpyrifos on ChE enzyme activity for both *P. promelas* ($F_{2,20} = 85.69$, $p < 0.001$) and *X. laevis* ($F_{2,22} = 60.88$, $p < 0.001$) (Fig. 1). However, there was no effect of atrazine (*P. promelas*: $F_{1,20} = 0.03$, $p = 0.863$; *X. laevis*: $F_{1,22} = 0.32$, $p = 0.574$) or interaction effect of chlorpyrifos and atrazine (*P. promelas*: $F_{2,20} = 0.52$, $p = 0.602$; *X. laevis*: $F_{2,22} = 1.01$, $p = 0.379$) on ChE activity for either species. At 1.0, 8.9, and 47.3 $\mu\text{g/L}$ of chlorpyrifos, ChE inhibition was 21.4, 60.5, and 77.9%, respectively, for *P. promelas* relative to control organisms. For *X. laevis*, chlorpyrifos concentrations of 0.6, 4.6, and 33.3 $\mu\text{g/L}$ resulted in 15.2, 43.7, and 69.6% ChE enzyme inhibition, respectively.

Table 3. Summary of acute toxicity tests to *Pimephales promelas*, *Xenopus laevis*, *Rana clamitans*, and *Lepomis macrochirus*

Species	Age	Atrazine Concentration ($\mu\text{g/L}$)		
		0	100	1000
<i>P. promelas</i>	<24 h–Test 1	42.85 \pm 9.4 ^A	44.67 \pm 8.8 ^A	52.00 \pm 10.0 ^A
	< 24 h–Test 2	32.92 \pm 9.5 ^A	43.33 \pm 9.0 ^B	46.46 \pm 9.2 ^B
	~80 d	58.26 \pm 8.9 ^A	48.43 \pm 9.7 ^B	50.86 \pm 10.8 ^{AB}
<i>X. laevis</i>	Stage 35	68.41 \pm 9.1 ^A	77.56 \pm 7.3 ^{AB}	82.73 \pm 6.1 ^B
	Stage 45	47.33 \pm 10.8 ^A	46.55 \pm 10.2 ^A	66.00 \pm 9.5 ^B
<i>R. clamitans</i>	~8 d	56.67 \pm 12.1 ^A	59.29 \pm 11.9 ^A	54.69 \pm 11.4 ^A
<i>L. macrochirus</i>	0.35 g	53.33 \pm 11.8 ^A	—	50.67 \pm 11.9 ^A

Values represent the percent effect caused by an atrazine treatment, considering the percent effects from all five chlorpyrifos concentrations for a given mixture toxicity test. Values are shown with mean and standard error of the mean. Treatments with the same letter indicate no significant difference among atrazine concentrations (ANOVA, $p < 0.05$).

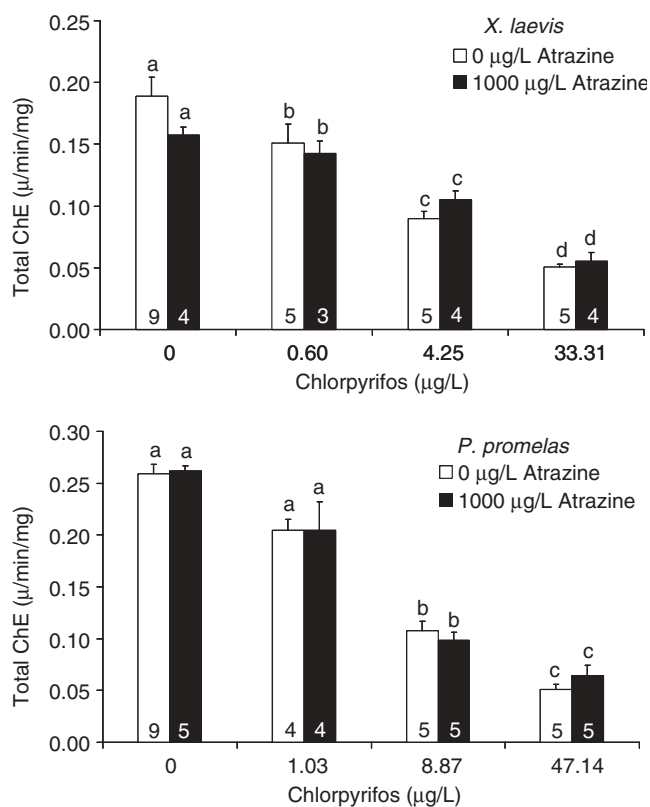


Fig. 1. Total cholinesterase (ChE) enzyme activity for *Pimephales promelas* and *Xenopus laevis*. Bars that do not share a common letter within a species are significantly different ($p < 0.05$). Within chlorpyrifos concentrations, total ChE activity did not differ between groups with and without atrazine. Error bars represent standard error and sample sizes are indicated at the base of each bar

Uptake Experiment

A significant difference in chlorpyrifos uptake rates in *P. promelas* was noted with and without the addition of atrazine ($F_{1,19} = 5.28$, $p = 0.033$). The uptake clearance coefficient (k_u) for the 0 $\mu\text{g/L}$ atrazine treatment was 6.33 melamin and the k_u for the 1,000 $\mu\text{g/L}$ atrazine treatment was 5.86 melamin. This suggests that without atrazine in the system, uptake of chlorpyrifos was accelerated compared to when

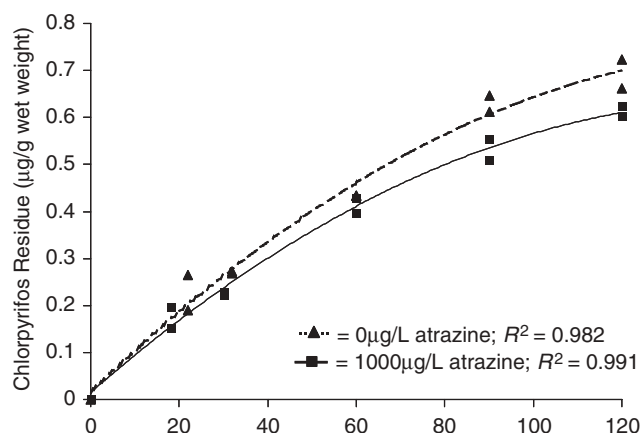


Fig. 2. Uptake rates of chlorpyrifos by *P. promelas* with and without simultaneous atrazine exposure. Uptake rates were found to be significantly different by approximate z test ($p < 0.001$)

atrazine was present in the system. An approximate z -test indicated differences between the two uptake rates ($p < 0.001$) (Fig. 2). *P. promelas* chlorpyrifos body residues at the final time point (120 min) did not differ between the atrazine ($0.61 \pm 0.01 \mu\text{g/g}$) and non-atrazine treatments ($0.69 \pm 0.02 \mu\text{g/g}$) ($p = 0.149$). The uptake experiment was only conducted for *P. promelas*.

Discussion

Toxicity Bioassays

In this study, an approximately two-fold increase in chlorpyrifos toxicity was observed on *Xenopus laevis* tadpoles as a result of the addition of atrazine. This is the first report of potentiation of chlorpyrifos toxicity by atrazine in a vertebrate species. Previous studies have shown the addition of atrazine to cause a two- to seven-fold increase in chlorpyrifos toxicity to selected aquatic invertebrates (Pape-Lindstrom and Lydy 1997; Jin-Clark *et al.* 2002; Anderson and Lydy 2002; Lydy and Linck 2003). Mixed results were found in toxicity bioassays with *Pimephales promelas*. No effect of atrazine on chlorpyrifos toxicity was observed for *Lepomis macrochirus* or

Rana clamitans. Organisms examined here appeared to be different in their susceptibility to organophosphate insecticide toxicity and tolerance with the addition of atrazine (i.e., *X. laevis* was the only organism that showed a clear effect of atrazine potentiation in this study).

This interspecific variation may be explained by variation in the physiological systems of each species related to biotransformation and metabolism of xenobiotics (Rocha-e-Silva *et al.* 2004). Specifically for chlorpyrifos, toxicity is directly related to biotransformation to a more toxic metabolite by the cytochrome P450 monooxygenase enzymes (Goksøyr and Förlin 1992) via phase I biotransformation in the cytochrome P450 pathway (Andersson and Förlin 1992). These enzymes vary among species, but serve the same general functions of detoxification and metabolism of xenobiotics (Klaassen 2001). For example, relative to the species examined in the current study, *X. laevis* and *L. macrochirus* hepatic microsomal mixed-function oxidases were similar between the species; however, interspecific variation was observed in the content and activity of cytochrome P450, cytochrome *c* reductase, and various epoxidases and hydroxylases between the species (Doherty and Khan 1981). Furthermore, fish (*Oreochromis niloticus* and *Brycon cephalus*) and amphibians (*Rana catesbeiana* and *Bufo marinus*) were reported to vary in specific content of cytochrome P450, cytochrome *b*₅, ethoxyresorufin *O*-deethylase (EROD), cytochrome *c* reductase, and several cytosolic anti-oxidant enzymes (Rocha-e-Silva *et al.* 2004). All of these typically function to detoxify and excrete xenobiotic compounds from an organism and variation in basal levels among species could be related to the variation in toxicity and sensitivity to the atrazine-chlorpyrifos mixture observed in the current study.

In terms of atrazine potentiation of chlorpyrifos, similar variation in the degree of potentiation (as indicated by synergistic ratios) among species has also been observed among invertebrate taxa (Pape-Lindstrom and Lydy 1997; Belden and Lydy 2000; Jin-Clark *et al.* 2002; Anderson and Lydy 2002; Lydy and Linck 2003; Schuler *et al.* 2005; Trimble and Lydy 2006). These results, and the results from the current study on aquatic vertebrates, suggest that potentiation of chlorpyrifos toxicity by atrazine may not occur uniformly throughout aquatic communities, but rather may be limited to certain species sensitive to this type of chemical mixture. Identifying the mechanism and source of this sensitivity should be an objective for future study.

Cholinesterase Activity

Since chlorpyrifos is a potent ChE inhibitor, quantification of ChE inhibition was used as an indicator of exposure. Previous research on the midge *C. tentans* indicated that atrazine alone does not significantly alter ChE activity. However, when organisms were exposed to a binary mixture of atrazine and chlorpyrifos, ChE levels were significantly lower than organisms exposed to chlorpyrifos alone (Belden and Lydy 2001). In the current study, very similar experiments were performed for vertebrate species yielding different results. Both *P. promelas* and *X. laevis* showed a dose-dependent ChE inhibition response to increasing chlorpyrifos concentrations, but ChE inhibition was not enhanced with the addition of atrazine.

For some OPs such as chlorpyrifos, phase I biotransformation yields oxon-analog metabolites that are more potent ChE inhibitors (Klaassen 2001). Previous studies have suggested the co-occurrence of atrazine leads to induction of the cytochrome P450 system, causing increased biotransformation and, ultimately, increased chlorpyrifos toxicity. If phase I detoxification processes were indeed stimulated by the presence of atrazine, an increase in oxon-analog formation would be expected, yielding increased chlorpyrifos toxicity and thus increased ChE inhibition as shown for the midge *C. tentans* (Belden and Lydy 2000). Toxicity data supported this hypothesis; however, ChE inhibition in the current study did not differ between organisms exposed to chlorpyrifos alone and those exposed simultaneously to chlorpyrifos and atrazine. This suggests that the increase in chlorpyrifos toxicity with the addition of atrazine observed in the bioassays was not a result of increased ChE inhibition and that increased biotransformation rates of chlorpyrifos through some phase I processes in *X. laevis* and *P. promelas* may not be the mechanism for the interaction effect.

Other phase I biotransformation processes such as hydrolysis via carboxylesterases can produce OP breakdown products that are less toxic, and have higher water solubility; therefore, they are more easily excreted by an organism (Klaassen 2001). Perhaps the occurrence of additional phase I biotransformation processes, such as hydrolysis, as well as additional stress responses due to multiple chemicals are responsible for the interaction. This should be examined in future studies by measuring residue concentrations of parent, oxon, and other metabolites in organisms after exposure.

Uptake Experiment

Belden and Lydy (2000) reported increased chlorpyrifos uptake in the presence of atrazine on the midge *C. tentans* with a corresponding increase in chlorpyrifos toxicity. Based on these findings, it was hypothesized that the addition of atrazine may cause an increase in chlorpyrifos uptake in vertebrate species and contribute to the understanding of the observed potentiation and elevated synergistic ratios. The chlorpyrifos uptake rate was slightly reduced with atrazine in the system, but the magnitude of this change was quite small. While uptake rates were statistically different with or without the addition of atrazine in *P. promelas*, a difference of only 7.4% in uptake slope (k_u) between the two groups does not appear to be biologically significant. Furthermore, body residues at the 120-min endpoint of the study were similar. Therefore, changes in chlorpyrifos uptake were not thought to be responsible for the potentiation of chlorpyrifos toxicity observed for the bioassay experiments.

Conclusions

Understanding the monooxygenase system for each species examined would undoubtedly contribute to identifying the mechanisms responsible for atrazine potentiation of OP toxicity in vertebrates. In earlier work, microsomal cytochrome P450 activity was induced in *C. tentans* and this activity was

isolated to a 45-kDa protein (Miota *et al.* 2000). A similar P450 enzyme has been isolated in *X. laevis* with a molecular weight of 52 kDa (Saito *et al.* 1997). Proteins in the range of 45 to 60 kDa are heme-thiolate membrane-associated proteins, a large family of P450 enzymes thought to play an important role in biotransformation (Miota *et al.* 2000). Similar-sized heme-thiolate proteins have also been identified for fish species (Oleksiak *et al.* 2000). When exposed to atrazine levels of 1 to 10 mg/L, *C. tentans* larvae showed a significant elevation of *O*-demethylase activity, a cytochrome P450-dependent enzyme (Londoño *et al.* 2004). In general, the cytochrome P450 system in *P. promelas* and other aquatic vertebrates is not well understood and significant induction of P450s by atrazine has not been noted. Examining P450 induction by atrazine for each species tested here would be useful for understanding the mixed results among species. If specific proteins could be isolated and induced in the presence of different xenobiotics, the pathways of biotransformation could also be examined more thoroughly.

Binary combinations of atrazine and chlorpyrifos did not result in variation of ChE inhibition and chlorpyrifos uptake rate compared to the chlorpyrifos-only treatment for the organisms examined in this study. High concentrations (5000 µg/L) of atrazine alone also did not result in toxicity to the organisms; however, an interaction effect of atrazine and chlorpyrifos was observed for a behavioral endpoint in *X. laevis*. Physiological processes other than those related to mixed function oxidases described above may be related to the observed potentiation of chlorpyrifos. One possibility is that general health and stress levels of exposed organisms may be an important factor for this interaction. Potentially, atrazine may have elicited a stress or related response in tested organisms that influenced tolerance to chlorpyrifos exposure without detection by the behavioral effect and enzyme biomarker responses. Previous studies have shown that atrazine can influence a number of physiological and immunological processes. For example, in vertebrates, atrazine has been shown to reduce the number of eosinophils in wood frogs (*Rana sylvatica*) during a trematode infection (Kiesecker 2002); to increase the proportion of successfully encysting trematodes in wood frogs (Kiesecker 2002); to promote mitochondrial malformations and reduce glycogen in grey mullet (*Liza ramada*) (Biagianti-Risbourg and Bastide 1995); and to cause hermaphroditism and demasculinization in *X. laevis* (Hayes *et al.* 2002). In aquatic invertebrates, atrazine has been shown to up- and down-regulate numerous genes in the Pacific oyster, *Crassostrea gigas*, that were linked to metabolic functions such as energy production, immune response, and transcription (Tanguy *et al.* 2005) and affect behavioral and movement patterns as well as kidney cell lysis in two freshwater mollusks (*Physa acuta* and *Ancylus fluvialtilis*) (Rosès *et al.* 1999). Other potential indicators of stress by atrazine exposure for use in future studies might include heat shock proteins, adrenal cortical function, cortisol levels, MHC class II protein expression indicating immunosuppression, or glutathione S-transferase levels indicating oxidative stress. Many of these endpoints have been previously used to quantify general stress responses to other contaminants (Oruç and Üner 2002; Pruett *et al.* 2003; Harvey and Everett 2003; Mukhopadhyay *et al.* 2003; Lower *et al.* 2005). Further study on possible stress responses to atrazine exposure is needed to help

explain the mechanism of the interaction of atrazine and chlorpyrifos reported here.

Although greater than additive effects of atrazine were observed at the highest exposure level, it is unlikely these concentrations would be encountered in an agricultural run-off scenario. In conclusion, it does not appear that mixture toxicity of atrazine and chlorpyrifos at environmentally relevant concentrations pose a risk to the vertebrate organisms examined in this study. However, this study focused on acute toxicity. Thus, chronic or indirect effects at the ecosystem level should be considered for future work on these compounds at environmental relevant concentrations. Also, while three of these vertebrates are representative of species native to areas where simultaneous atrazine and chlorpyrifos exposure typically occur, they may not represent the most sensitive species. Also, since an organism's physiology may change throughout the course of development, sensitivity to atrazine and chlorpyrifos toxicity may change as well. Since juvenile organisms were used exclusively in this study, it should not be assumed that older individuals would elicit the same responses to atrazine, chlorpyrifos, or a mixture of the two compounds.

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