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IMPACT OF ATRAZINE ON ORGANOPHOSPHATE INSECTICIDE TOXICITY

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Abstract—Acute toxicity of selected organophosphorus insecticides (OPs; chlorpyrifos, methyl parathion, diazinon, and malathion) was determined for individual OPs and binary combinations of the OPs with atrazine to larvae of the midge *Chironomus tentans.* Atrazine individually was not acutely toxic even at high concentrations (10,000 μg/L); however, the presence of atrazine at much lower concentrations (40–200 μg/L) increased the toxicity of chlorpyrifos, methyl parathion, and diazinon. Atrazine did not increase the toxicity of malathion. Possible mechanisms for the synergistic toxicity found between atrazine and chlorpyrifos were investigated, including increased uptake rate and increased biotransformation into a more toxic metabolite. Although the uptake rate was increased by more than 40%, the resulting increase in toxicity would be minimal as compared to the 400% decrease estimated to occur in EC50 values for the same atrazine exposure (200 μg/L). Body residue analysis of midges exposed in vivo to atrazine and chlorpyrifos mixtures for 96 h indicated that a larger amount of metabolites was generated in atrazine treatments as compared to controls. Additionally, in vitro assays of microsomal proteins obtained from treated and control midges indicated that an increase in toxic metabolite (chlorpyrifos-*O*-analog) was generated in atrazine-treated midges. Therefore, the increase in toxicity is thought to be due to an increase in biotransformation rates of the OPs, resulting in more *O*-analog within the organism.

Keywords—Atrazine Organophosphate insecticides Mixtures Synergism Biotransformation

INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropyl amino-*s*-triazine) is a widely used selective herbicide. In 1996, more than 24 million kilograms of atrazine were applied to corn and related silage crops in the agricultural regions of the United States [1]. Atrazine is present in surface water and groundwater throughout the Midwest because of its high usage and relatively high water solubility. Atrazine concentrations found in surface and groundwater are generally not considered to be ecologically harmful [2]. However, recent investigations have demonstrated that the presence of atrazine may cause sublethal effects such as induction of xenobiotic metabolizing systems [3–5] and may influence the toxicity of other xenobiotics [4,6,7].

Organophosphorus insecticides (OPs) are used in the same regions of the country as atrazine for control of a wide variety of insects and other invertebrates. Chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) and methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate) are used extensively in agriculture. In 1996, more than 2.7 million kilograms of chlorpyrifos and 640 thousand kilograms of methyl parathion were applied to corn, related silage crops, and wheat in agricultural regions of the United States [1]. Malathion (diethyl mercaptosuccinate, *O*,*O*-dimethyl phosphorodithioate) and diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methyl-4-pyrimidinyl phosphorothioate) are widely used as general insecticides. Both compounds are used commercially on fruits and vegetables [1] as well as domestically. Although OPs are a broad class of chemicals structurally, they all are acutely toxic to animals through interference with cholinergic nerve transmission. Some OPs can directly cause anticholinesterase effects (phosphate class); however, the OPs used in this study are thought to be toxic only after metabolism by cytochrome P-450–dependent monooxygenases (phosphorothioate and phosphorodithoate classes). This bioactivation step results in the replacement of sulfur with oxygen within the chemical structure of the OP (desulfoxidation), creating a metabolite that is a much stronger inhibitor of acetylcholinesterase. The resulting metabolite is structurally similar to compounds in the phosphate class of OPs [8]. For the purpose of this study, OP desulfoxidation metabolites will be referred to as the *O*-analog of the OP.

Although the pesticides used in this study are usually applied to terrestrial habitats, the high usage of the chemicals coupled with their relatively high water solubility makes it inevitable that mixtures of herbicides and insecticides will contaminate aqueous environments. Historically, several terms have been used to describe the joint action of toxicants. To avoid confusion, interactions will be classified as greater than additive, less than additive, or additive. Terminology original reported in cited studies will appear in parentheses. Mixtures of toxicants with different modes of action are generally accepted to have additive or less than additive toxicity [9–11]. However, a recent study used a toxic unit approach to demonstrate that atrazine in binary combinations with chlorpyrifos, malathion, and methyl parathion (thiophosphate- or dithiophosphate-class OPs) exhibited greater than additive (synergistic) toxicity to the fourth instar of the midge *Chironomus tentans.* However, atrazine in binary combination with mevinophos (phosphate class OP) exhibited less than additive (antagonistic) toxicity [7]. The authors suggested that the mechanism of the joint action noted between atrazine and various OPs may be the result of atrazine increasing the biotransformation of the OPs, thereby converting them into more (or less) toxic metabolites. Other studies have provided support for this hypothesis by demonstrating that atrazine induces xenobiotic metabolizing enzymes in other organisms [3–5]. In addition, Estenik and Collins [12] demonstrated that an organism in the

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same genus as our test organism, *Chironomus riparius*, has an active mixed-function oxidase system, and they reported that piperonyl butoxide (PBO) inhibited this system.

The current study, which comprises a four-tier experimental design, continues the investigation started by Pape-Lindstrom and Lydy [7]. Further investigation was necessary because the previous study utilized a single concentration of atrazine that was well above environmentally relevant concentrations and no evidence of mixed-function oxygenase induction, increased biotransformation, or other mechanism was provided. The first tier investigated the toxic interactions between OPs and atrazine at multiple concentrations to determine if greater than additive toxicity existed at environmentally relevant concentrations of atrazine. The remaining tiers investigated possible mechanisms to help explain the joint action found between atrazine and chlorpyrifos. Chlorpyrifos was chosen for further investigation because of its widespread usage in regions that also use atrazine and the finding, presented within, that the two compounds have a significant joint action (greater than additive) on the test organism. The second tier investigated whether atrazine increased the uptake rate of chlorpyrifos, thereby increasing dose and ultimately toxic action. The third tier investigated whether atrazine increased OP biotransformation by measuring the body residues of midges for both parent compound and metabolites after in vivo exposure to one of the following combinations: OP only, OP + atrazine, OP + PBO, and OP + atrazine + PBO. Finally, the fourth tier further investigated the effect of atrazine on biotransformation rates. In vitro assays were used to compare the mixed-function oxidase system activity of microsomal enzymes, obtained from midges treated with atrazine against untreated midges, using chlorpyrifos as a substrate.

MATERIALS AND METHODS

Organisms

Midge larvae (*C. tentans*) were cultured from egg masses obtained from the Environmental Research Laboratory in Duluth, Minnesota, USA. Animals were cultured in accordance with standard operating procedures for static cultures [13], with slight modifications. Rather than collecting adults to lay eggs in separate containers, thus providing cohorts of instars, animals were maintained in mixed-age brood cultures. Fourth instars were harvested directly from the mixed-age cultures and larvae were considered to be fourth instar if the head capsule width was 0.63 to 0.71 mm wide and the body length was 1.0 cm or longer [14]. *Chironomus tentans* were chosen as the test organism because of its environmental importance, ease of culture in the laboratory, and because it has been used previously in similar studies [7].

Chemicals

Analytical-grade standards of chlorpyrifos, chlorpyrifos-*O*-analog, and trichloropyridinol (TCP) were provided in kind from DowElanco (Indianapolis, IN, USA). Atrazine was obtained from Chemservice (Westchester, PA, USA). Methyl parathion and malathion were provided in kind from Cheminova (Lemvig, Denmark). All analytical-grade standards were certified by the supplier to have a purity of greater than 98%. Radioactive chlorpyrifos was obtained from Sigma (St. Louis, MO, USA) and was determined to have a purity of greater than 97% by thin-layer chromatography (using a solvent system of benzene:hexane:acetone, 50:45:5 v/v/v) followed by liquid scintillation counting. The specific activity for [^{14}C] chlorpyrifos was 26 mCi/mmole.

Tier I—Determining acute toxicity

Fourth instars of *C. tentans* were used in all tests. Ninety-six–hour static toxicity tests were conducted using 10 larvae in each 1-L beaker. Tests were conducted at 20°C in Precision Scientific™ (Grand Rapids, MI, USA) environmental chambers using a photoperiod of 16:8 h light:dark. Solvent and negative controls were conducted with each toxicity test. Because of the cannibalistic nature of *C. tentans*, a layer of fine silica sand substrate, approximately 3 mm deep (40 g), was placed in each beaker to allow the animals to escape from other midge larvae and to provide substrate for the midges to build cases. Silica sand does not affect the toxicokinetics or toxicity of these compounds [15]. Each beaker was filled with 1 L of moderately hard, reconstituted water. The water and sand used in the tests were the same as used within the cultures. Temperature, dissolved oxygen, pH, and conductivity were monitored before and after each test. Test chemicals were delivered to the water using 50 μl of acetone. The endpoint of acute toxicity tests was measured as an effective concentration (ECxx), which represents the concentration where a percentage (xx) of the test population was unable to perform normal swimming motion when pinched with a pair of forceps [7,12,15,16]. Preliminary acute toxicity tests were performed using atrazine and each OP individually. Five concentrations for each toxicant were run in triplicate; thus, 30 midges were run for each concentration. The EC1, EC5, EC15, and EC50 values were determined for each OP using log probit analysis [17]. The effect of atrazine on OP toxicity was then examined using a four by five factorial design. Doses of OP corresponding to the EC1, EC5, EC15, and EC50 were applied in combination with atrazine levels of 0, 10, 40, 100, and 200 μg/L. Test concentrations were chosen to evaluate the impact of environmentally possible levels of atrazine on OP toxicity. Five replicate beakers were used for each atrazine + OP combination. Solvent controls and atrazine-only exposures were run with each toxicity test.

Statistical comparisons were performed using SAS [17]. Comparisons of treatment classes used in tier I acute toxicity tests were conducted from two-way analyses of variance using Tukey's multiple comparisons. Percent effect data were arcsine transformed before analysis. Because atrazine is not acutely toxic to *C. tentans* at water solubility limits (50 times the highest concentration used in this test [7]), no atrazine effect on toxicity would be expected at the levels used in the present experiment. Therefore, a significant F statistic for the atrazine treatment class is demonstration of greater than additive toxicity. This statistical approach was utilized to avoid estimating EC50 values, which are necessary for many other methods for evaluating joint toxicity such as toxic units [7] and additive index approaches [18]. Because atrazine is not acutely toxic, any estimation of an EC50 for atrazine would bias the results. This approach also allows comparisons to be made at multiple levels of the toxicity curves regardless of slope and prevents the need for rigorous preliminary testing that is necessary if log-probit 95% confidence intervals are used to evaluate differences.

Estimation of EC50 values for each OP was also obtained for each atrazine concentration used in the mixture tests using log-probit procedures. Synergistic ratios (SRs) were calculated by dividing the EC50 of the control (no atrazine) by the EC50

value obtained for each atrazine treatment. Synergistic ratio values are used to give an indication of how large of toxicity increase exists among treatments.

To verify that toxicant levels remained stable throughout testing, water from the toxicity tests was collected and pesticide concentrations were determined using solid-phase extraction and analysis by gas chromatography with nitrogen phosphorus detection following previously described procedures [19]. Samples were extracted using solid-phase extraction techniques. Solid-phase extraction cartridges (Supelco C_{18}, Bellefonte, PA, USA) were preconditioned by eluting with 3 ml of hexane:acetone 1:1 v/v, 3 ml of methanol, and 3 ml of reagent-grade water. Samples were subsequently extracted by passing 250 ml of water through the column at 15 psi (10–18 ml/min). The column was allowed to air dry for 5 min before the analytes were extracted from the column using three 3-ml rinses of hexane:acetone 1:1. The extracts were then evaporated to 1 ml under a gentle stream of nitrogen. Gas chromatographic analysis of the extracts was performed on a Hewlett Packard 6890 gas chromatograph (Palo Alto, CA, USA), equipped with nitrogen phosphorus detector (220°C, split-less, 0.75-min purge time). The J&W Analytical (Folsom, CA, USA) capillary column was a DB-608, 30 m × 0.320 mm with a 0.50-μm film thickness. The oven program started at 100°C for 1 min, increased 6°C/min to 280°C, and was then held for 4 min. Carrier and make-up gas was helium at 7 and 15 ml/min, respectively. Qualitative identification was based upon retention times within 0.50 % of standards, whereas quantitation was based upon peak area utilizing external standards. Extraction efficiencies and standard deviations ($n = 12$) of the analytes extracted from moderately hard water were as follows: chlorpyrifos, 91.0 ± 6.5; atrazine, 91.0 ± 12.8; methyl parathion, 93.8 ± 6.8; malathion, 95.7 ± 6.1; and diazinon, 93.1 ± 9.4.

Tier II—Determination of uptake and oxygen consumption

Uptake experiments were performed to obtain initial estimates of accumulation kinetics for chlorpyrifos from midges previously exposed to 200 μg/L of atrazine as compared to controls exposed to acetone only. Atrazine was added to 12 beakers each containing 10 midges, whereas acetone only was added to an additional 12 beakers that served as controls. The test system was the same as previously described in tier I toxicity testing. After 48 h of pre-exposure, ^{14}C-chlorpyrifos was added at a sublethal concentration (0.17 μg/L) and uptake was measured over 20, 40, and 80 min. Midges from four beakers from each treatment were then collected at each time point, blotted dry on filter paper, and weighed to the nearest 0.1 mg. Preliminary experiments indicated that no notable biotransformation occurred during the 80 min of the uptake experiment. Therefore, the midges were placed directly into scintillation cocktail (ScintiSafe Plus 50%, Fisher Scientific, Pittsburgh, PA, USA), sonicated for 30 s with a Tekmar Sonic Disruptor (Cincinnati, OH, USA), and counted by liquid scintillation counting using a Packard 1900 TR scintillation counter (Meriden, CT, USA). All scintillation counting was performed using sample counts that were corrected for background and quench using the external standards ratio method [15,16]. Pesticide water concentrations remained constant over the entire uptake period. However, an assumption was made that no elimination occurred during the uptake experiment. Because uptake curves were linear through 80 min, this assumption seems reasonable. Homogeneity of slopes test [17] was utilized to determine statistical differences between uptake rates.

Differences in oxygen consumption were also measured between midges previously exposed to atrazine at 200 μg/L as compared to controls exposed to acetone only. Increased oxygen consumption is indicative of increased respiration rates and respiration rate is an important factor in determining the uptake rate of a chemical. Atrazine (200 μg/L) was added to 10 beakers each containing 10 midges, whereas acetone only was added to an additional 10 beakers that served as controls. The test system was the same as previously described in tier I toxicity testing. After 48 h of pre-exposure, midges were randomly assigned to 300-ml biological oxygen demand bottles at a rate of 15 midges per bottle with five replicates being used. Each bottle was filled completely with properly dosed (acetone or atrazine at 200 μg/L) moderately hard reconstituted water that was previously aerated to saturation. Forty grams of fine silica sand was added to prevent cannibalism. Zero head space was allowed in the bottles, which were stored in the dark at 20°C. After 24 h, the bottles were opened, the dissolved oxygen was measured using a YSI™ Model 55 dissolved oxygen meter (Yellow Springs, OH, USA), and midges were weighed to the nearest 0.1 mg. The amount of oxygen consumed per time per midge mass (O_2 mg/h/g midge) was calculated. Controls consisting of acetone and atrazine-dosed water without midges were also run ($n = 5$). A Student's t test was used to statistically compare the atrazine treatment with the control [17].

Tier III—In vivo biotransformation

The test system used for the in vivo experiments was the same as previously described in tier I toxicity testing with the exception that 10 replicate beakers, instead of 3, were used for each treatment. Treatments consisted of the following combinations: chlorpyrifos (EC1); chlorpyrifos (EC1) and atrazine (200 μg/L); chlorpyrifos (EC1) and PBO (1,000 μg/L); and chlorpyrifos (EC1), atrazine (200 μg/L), and PBO (1,000 μg/L). Dosage levels were chosen from tier I results so that large differences in acute toxicity were present between treatments; therefore, differences in body residue could be more easily determined. Two beakers (with five replicates each) were evaluated at a time. Midges collected from each set of beakers were combined and then randomly divided into two groups. The first group was used for total radioactivity determinations by liquid scintillation counting. The second group was used for qualitative identification of chlorpyrifos and metabolites by thin-layer chromatography followed by liquid scintillation counting. All midges capable of movement (unaffected) after the 96-h dosing period were collected, blotted dry with filter paper, and weighed. The first group of midges was placed directly into scintillation cocktail (ScintiSafe Plus 50%, Fisher Scientific), sonicated for 30 s, and then counted by liquid scintillation counting. The second group of midges was extracted using 10 ml of acetonitrile and sonicated for 30 s. The extract was then dried by filtering through a column of sodium sulfate. The final volume of the extract was reduced to 0.2 ml under a direct stream of nitrogen and then spotted on a thin-layer chromatography plate that was developed using a mobile phase benzene:hexane:acetone, 50:45:5 v/v/v. Regions of the plate corresponding to cold standards (chlorpyrifos, chlorpyrifos-*O*-analog, and TCP) were determined by visualization of the fluorescent backing of the thin-layer chromatography plates using incident ultraviolet light at 254 nm. Each known region,

as well as a region surrounding the plate's origin, was removed, placed into scintillation cocktail, and counted by liquid scintillation counting. The region that corresponded to TCP and material that did not migrate from the origin was considered collectively for calculations as polar metabolites. Analysis of variance [17] was used to determine if mean body residues and percent effect differed among treatments. Means were separated using Tukey's Studentized mean test ($p < 0.05$).

To verify that water concentrations remained stable throughout the test, 1-ml water samples were taken before and after the 96-h testing period for total radioactivity determinations. In addition, 250 ml of water was also collected after testing and extracted from the beakers by solid-phase extraction techniques as previously described. The extract was then analyzed using thin-layer chromatography and liquid scintillation counting. This second analytical procedure allowed for the determination of metabolite concentrations in the water. Control beakers, consisting of dosed water without midges, were also analyzed to determine if chlorpyrifos transformation was occurring in the water.

Tier IV—In vitro biotransformation

Midges were exposed to atrazine at 2,000 μg/L, atrazine at 200 μg/L, or a solvent control for 48 h using the same testing system as previously described in tier I. Four replicates, each replicate consisting of eight beakers that contained 10 midges each (total of 80 midges), were used for each treatment. After exposure, each set of midges was blotted dry on filter paper and weighed to the nearest 0.1 mg. Whole-body homogenates were prepared in 150 mM potassium phosphate buffer and 50 mM sucrose (pH 7.6), using a teflon glass Potter-Elvehjem–type homogenizer (Wheaton, Millville, NJ, USA). The homogenates were centrifuged at 10,000 *g* for 20 min in a Sorvall RC2-B™ centrifuge (Sorvall, Newtown, CT, USA) at 2°C. The supernatant was further centrifuged at 100,000 *g* for 1 h in a Beckman TL 90™ ultracentrifuge (Beckman Instruments, Fullerton, CA, USA) at 2°C. The remaining pellet was reconstituted in 0.5 ml of the same buffer. Protein concentration was determined using the biuret method. Total Protein Standard was purchased from Sigma and serum albumin was used as a standard protein.

Assays were performed similarly to the methods described by Siegfried and Mullin [20] for the determination of aldrin epoxidase activity with the exception that [^{14}C] chlorpyrifos was added instead of aldrin. Optimization of aldrin epoxidase assays has been previously reported for *C. tentans* [5] and *C. riparius* [12]. Parameters for the current study used the results of these studies. Accordingly, all assays were conducted at pH 7.6 and 31°C for 45 min. Nicotinamide adenine dinucleotide phosphate (NADPH; 1.0 mM) was added to satisfy cofactor requirements along with an NADPH-regenerating system consisting of glucose-6-phosphate (4 mM) and 1 unit of glucose-6-phosphate dehydrogenase. With the addition of 150 μl of enzyme extract (approximately 0.20 mg of protein), the final volume of the incubation mix was 300 μl. All steps within the procedure were performed at 4°C until incubation began. After incubation, 200 μl of methanol:water:acetic acid, 50:45:5 v/v/v, was added to reduce the pH, thereby stopping the reaction. Samples were immediately frozen at −5°C until analysis. Assays lacking NADPH were run to determine the dependence of the reaction on this cofactor. In addition, assays containing PBO were conducted to characterize the nature of the protein extract.

Separation of chlorpyrifos and metabolites was accomplished using a Hewlett Packard 1100 Series™ high-perfomance liquid chromatograph attached to a Foxy Junior Fraction Collector™ (Isco, Lincoln, NE, USA). The analytical column was a 25-cm C_{18} Hypersil™ (Hewlett Packard), whereas the mobile phase consisted of a binary solvent system. The solvent gradient used started at 100% solvent A (5% methanol, 0.1% acetic acid in deionized water) and immediately began a linear shift to 100% solvent B (acetonitrile) at 17 min, where it held steady for 3 min. Baseline separation of TCP, chlorpyrifos-*O*-analog, and chlorpyrifos was obtained with retention times of 11.6, 13.7, and 17.0 min, respectively. Detection of cold compounds was accomplished using a Hewlett Packard 1100 ultraviolet detector set at wavelength 230 nm. Each assay mixture was thawed before injection and a 20-μl sample was injected. Fractions were collected by timed events allowing windows of 0.50 min before and after each peak of interest. Fractions not correlating with specific analytes were also collected. Scintillation cocktail was added to each fraction and radioactivity was determined by liquid scintillation counting. Statistical differences were determined by ANOVA and Tukey's mean separation test ($p < 0.05$) [17].

RESULTS

Test conditions

Water parameters were similar for all tests in this study. Temperature was maintained at 20 ± 1°C. Dissolved oxygen levels were >70% in all toxicity tests. Conductivity remained between 320 and 350 μS/cm and pH ranged from 7.3 to 7.8.

The stability of the parent compound in water was consistent for all treatments. All initial water concentrations were within 10% of nominal concentrations for atrazine and OP treatments, whereas posttest atrazine concentrations were within 15% for all tests. The OP concentrations dropped during testing; however, the rate of decrease was consistent among groups. Posttest concentrations ranged from 76 to 85% of initial values for chlorpyrifos, 81 to 89% for methyl parathion, 74 to 84% for malathion, and 81 to 98% for diazinon. Although the pesticide stability within the test system was adequate, nominal concentrations were considered more accurate because of the error introduced by the extraction process; therefore, all calculations for acute toxicity tests were performed using nominal values.

Tier I—Acute toxicity test

Atrazine was not acutely toxic at 10,000 μg/L, which is near the water solubility limits. At this atrazine level, less than 3% effect was observed which was similar to controls. Individual OP toxicity data are shown in Table 1. Chlorpyrifos was the most toxic OP tested, followed by malathion, diazinon, and finally methyl parathion. Although atrazine was not found to be acutely toxic, the presence of atrazine at levels as low as 40 μg/L significantly increased the toxicity of chlorpyrifos, methyl parathion and diazinon. Analysis of variance results indicate that significant differences were present for both treatment classes for atrazine + chlorpyrifos mixtures ($p < 0.0001$, $df = 4$, $F = 146$ for atrazine and $p < 0.0001$, $df = 3$, $F = 109$ for chlorpyrifos; Table 2), atrazine + methyl parathion mixtures ($p < 0.0001$, $df = 4$, $F = 16.4$ for atrazine and $p < 0.0001$, $df = 3$, $F = 77.0$ for methyl parathion; Table 2), and atrazine + diazinon mixtures ($p < 0.0001$, $df = 4$, $F = 18.2$ for atrazine and $p < 0.0001$, $df = 3$, $F = 68.0$ for diazinon;

Table 1. Acute toxicity of organophosphorus insecticides dosed individually in preliminary tier I testing. Effective concentration (EC; μg/L) determined for each percent effect is shown with 95% confidence intervals shown in parentheses

	Chlorpyrifos	Methyl parathion	Diazinon	Malathion
EC1	0.12 (0.08–0.17)	7.2 (3.5–11)	4.4 (2.0–7.1)	0.26 (0.13–0.40)
EC5	0.17 (0.12–0.22)	11 (6.4–15)	7.7 (4.2–11)	0.44 (0.26–0.61)
EC15	0.23 (0.18–0.28)	16 (11–21)	13 (8.2–17)	0.70 (0.48–0.90)
EC50	0.39 (0.33–0.45)	32 (25–38)	30 (24–36)	1.5 (1.2–1.9)

Table 2). Interaction terms were not significant for atrazine + methyl parathion and atrazine + diazinon tests; however, a significant interaction was found for the atrazine + chlorpyrifos test ($p = 0.002$, $df = 12$, $F = 2.94$). The positive interaction term is likely due to the mortality rate of the higher atrazine-treated groups reaching 100%. At these mortality levels, differences among treatments are no longer possible, thereby skewing the distribution of the data. Tukey's test results indicate that for chlorpyrifos and methyl parathion, the addition of larger concentrations of atrazine significantly increased the greater than additive response ($p < 0.05$). Larger concentrations of atrazine also increased the average percent effect for diazinon treatment, but the increases were not statistically significant. All three compounds were significantly affected by atrazine at 40 μg/L ($p < 0.05$). Additional Tukey's tests were performed to obtain comparisons among atrazine treatments for each OP level. These tests illustrated that atrazine affected the toxicity of chlorpyrifos, methyl parathion, and diazinon, at each level of OP tested (Table 2). The presence of atrazine did not influence malathion toxicity at the levels used in this study (Table 2).

In order to provide information on the magnitude of the increased OP toxicity, data obtained from the previously discussed factorial experiment were used to estimate EC50 values for all atrazine levels for each OP. Only four data points were used for each estimate and some data sets did not have percent effect data points both above and below 50%. However, each data set did have nonsignificant chi-square values ($p > 0.2$) and at least two partial mortalities. Although not all recommendations for probit analysis were satisfied [15], good estimates of EC50 values were possible for most treatments. The EC50 values and 95% confidence intervals are provided in Table 3 with the corresponding SRs. Because of the factorial method design used within this study, statistical determination of differences among treatments was accomplished, as previously discussed, by analysis of variance; 95% confidence intervals are only provided as a measure of confidence in SR calculations. Atrazine demonstrated the greatest effect on chlorpyrifos toxicity with SRs of 1.84 at 40 μg/L and 4.00 at 200 μg/L. Diazinon and methyl parathion also had a large shift in EC50 values, with SRs of 2.62 and 1.90, respectively, at atrazine at 200 μg/L (Table 3).

Tier II—Determination of uptake rate and oxygen consumption

Midges treated with atrazine at 200 μg/L showed statistically significant increases in uptake rates of chlorpyrifos as compared to control midges ($F = 45.49$, $df = 2$, $p < 0.0001$).

Table 2. Acute toxicity of organophosphorus insecticides (OPs) and atrazine mixtures. Each cell represents the average and standard error of the percent effects found for that mixture. Totals represent the average and standard error for all samples evaluated within a treatment class. Cell, atrazine, or chlorpyrifos values that are not significantly different, as indicated by Tukey's test, are marked with the same lowercase letter, number, or uppercase letter, respectively. Each row (OP treatments) was considered independently. In all cases, a significant difference occurred if $p < 0.05$

	Atrazine (μg/L)					
OP (μg/L)	0	10	40	80	200	Total
Chlorpyrifos						
0.12	0 (0)[a]	2.0 (2.0)[a]	6.4 (4.4)[ab]	26.0 (7.5)[b]	60.1 (3.2)[c]	18.9 (4.9)[A]
0.17	2.0 (2.0)[a]	2.2 (2.2)[a]	30.8 (5.0)[b]	63.0 (5.4)[c]	86.0 (4.0)[d]	36.8 (7.0)[B]
0.23	8.4 (3.8)[a]	14.0 (5.1)[a]	40.2 (5.7)[b]	78.8 (4.8)[c]	98.0 (2.0)[d]	47.8 (7.4)[C]
0.39	39.6 (3.7)[a]	45.8 (7.7)[a]	87.6 (5.1)[b]	96.0 (2.4)[b]	99.9 (0.0)[b]	73.8 (5.6)[D]
Total	12.5 (3.9)[1]	16.0 (4.7)[1]	41.3 (7.1)[2]	65.9 (6.4)[3]	86.0 (3.9)[4]	
Methyl parathion						
7.2	0 (0)[a]	2.2 (2.2)[a]	8.4 (2.1)[ab]	12.2 (3.7)[ab]	24.0 (6.6)[b]	9.4 (2.7)[A]
32	6.0 (4.0)[a]	8.2 (3.8)[ab]	13.4 (5.5)[ab]	18.6 (1.9)[ab]	27.0 (2.5)[b]	14.6 (2.2)[A]
16	14.2 (4.0)[a]	14.6 (2.2)[a]	18.6 (4.2)[a]	26.6 (4.9)[ab]	39.2 (5.5)[b]	22.6 (2.6)[B]
32	42.8 (5.8)[a]	46.6 (7.2)[ab]	65.6 (6.6)[ab]	70.6 (6.6)[ab]	73.8 (7.6)[b]	59.9 (3.8)[C]
Total	15.8 (4.2)[1]	19.7 (4.4)[1,2]	25.5 (5.7)[2,3]	32.0 (5.7)[3,4]	41.0 (5.6)[4]	
Diazinon						
4.4	4.2 (2.4)[ab]	0 (0)[a]	10.8 (0.6)[ab]	17.8 (6.6)[b]	19.2 (7.1)[b]	10.4 (2.4)[A]
7.7	4.4 (2.7)[a]	4.2 (2.6)[a]	16.2 (5.5)[ab]	21.0 (7.3)[ab]	27.2 (6.1)[b]	14.6 (2.8)[A]
12.7	12.2 (4.9)[a]	14.6 (5.2)[ab]	33.0 (4.9)[ab]	35.2 (6.0)[ab]	39.0 (7.0)[b]	26.8 (3.2)[B]
29.7	43.8 (4.4)[a]	46.2 (5.2)[a]	63.8 (5.3)[ab]	68.2 (4.7)[b]	76.4 (4.5)[b]	59.7 (3.3)[C]
Total	16.2 (4.1)[1]	16.3 (4.5)[1]	31.0 (5.2)[2]	35.6 (5.4)[2]	40.5 (5.8)[2]	
Malathion						
0.26	4.0 (2.2)[a]	0 (0)[a]	0 (0)[a]	8.4 (3.6)[a]	6.4 (3.8)[a]	10.4 (2.4)[A]
0.44	6.2 (2.3)[a]	4.2 (2.3)[a]	7.6 (4.5)[a]	12.6 (3.5)[a]	13.6 (5.7)[a]	14.6 (2.8)[A]
0.70	22.6 (7.8)[a]	26.8 (5.1)[a]	24.0 (6.7)[a]	26.0 (4.0)[a]	25.4 (6.5)[a]	26.8 (3.2)[B]
1.5	45.4 (5.7)[a]	47.2 (4.9)[a]	60.2 (6.2)[a]	46.0 (11.2)[a]	47.8 (6.6)[a]	59.7 (3.3)[C]
Total	19.6 (4.6)[1]	19.6 (4.7)[1]	23.6 (5.8)[1]	23.3 (4.7)[1]	23.3 (4.7)[1]	

Table 3. Summary of EC50 values (μg/L) estimated for each organophosphorus insecticide (OP) at the level of atrazine exposure indicated[a]

OP		Atrazine exposure (μg/L)				
		0	10	40	80	200
Chlorpyrifos	EC50	0.44 (0.38–0.57)	0.42 (0.36–0.56)	0.24 (0.21–0.26)	0.16 (0.14–0.17)	0.11 (0.09–0.12)
	SR	—	1	1.83	2.75	4.00
Methyl parathion	EC50	35 (28–50)	34 (27–50)	26 (22–34)	23 (19–30)	18 (15–25)
	SR	—	1	1.35	1.52	1.94
Malathion	EC50	1.7 (1.3–2.8)	1.5 (1.2–2.1)	1.3 (1.0–1.7)	1.7 (1.2–3.6)	1.65 (1.2–3.2)
	SR	—	1	1	1	1
Diazinon	EC50	38 (27–74)	31 (24–46)	21 (16–31)	18 (14–27)	14 (11–19)
	SR	—	1	1.81	2.11	2.71

[a] SR = synergistic ratio calculated by SR = $EC50_{control}/EC50_{treatment}$. The SR values were not calculated if no significant difference ($p < 0.05$) in the atrazine treatment was found as compared to the control (no atrazine treatment) in the two-way analysis of variance and Tukey means separation test.

The chlorpyrifos uptake rate for the control was 0.284 nmole/kg/min, whereas atrazine-treated midges had a 42% higher uptake rate (0.404 nmole/kg/min). Oxygen consumption was also significantly increased in atrazine-dosed organisms as compared to control organisms ($t = 3.92$, $df = 8$, $p = 0.0044$). A 31% increase in oxygen consumption was noted (27.0 and 35.3 mg O_2/mg midge for control and atrazine-treated midges, respectively) and this value was similar in magnitude to the atrazine-induced increase in chlorpyrifos uptake rate. Thus, atrazine levels of 200 μg/L are stressful enough to the midge to increase the respiration rate, resulting in an increased uptake of chlorpyrifos.

Tier III—In vivo biotransformation

Initially, midges were scored to determine percent effect and values obtained were similar to what was expected based upon tier I results. Treatment with atrazine at 200 μg/L and chlorpyrifos at an EC1 level resulted in 45.6% effect, whereas treatment with the EC1 did not cause an effect. Treatment with PBO reduced the percent effect of the atrazine + chlorpyrifos mixture to 6.1% (Fig. 1).

Total body residue levels (total radioactivity) were significantly different among treatments ($F = 30.29$, $df = 3$, $p < 0.0001$). Atrazine + chlorpyrifos–treated midges contained significantly larger amounts of total radioactivity than chlorpyrifos only treatments ($p < 0.05$). The PBO-treated midges had the highest levels ($p < 0.05$); however, the PBO + chlorpyrifos treatment was not significantly different from the PBO + chlorpyrifos + atrazine treatment (Fig. 1). The body residues of parent compound were also significantly different among treatments ($F = 73.02$, $df = 3$, $p < 0.0001$). The PBO treatments were not significantly different from each other; however, PBO treatments were significantly different from treatments without PBO ($p < 0.05$). No significant difference in parent compound concentration was found between chlorpyrifos and chlorpyrifos + atrazine treatments (Fig. 1). Body residues of chlorpyrifos-*O*-analog were present at low concentrations and therefore reliable quantitation was more difficult for this analyte. However, trends indicated that this toxic metabolite was at highest concentration in the chlorpyrifos + atrazine treatment and was at lowest concentration in the PBO treatments ($F = 10.18$, $df = 3$, $p < 0.0005$; Fig. 1). Body residues of polar metabolites were significantly higher ($F = 15.68$, $df = 3$, $p < 0.0001$) in the chlorpyrifos + atrazine treatment, whereas no significant differences were found among other treatments (Fig. 1).

Analysis of water samples collected during tier III testing indicated that the polar metabolite concentration in posttest water for the atrazine and chlorpyrifos treatment containing midges (mean = 9.8 ng/L, SE = 0.2 ng/L) was significantly higher than chlorpyrifos-only treatments with midges (mean = 4.1 ng/L, SE = 0.3 ng/L; $F = 12.71$, $df = 5$, $p = 0.0002$), indicating an increase in biotransformation of chlorpyrifos. Polar metabolite levels for the atrazine + chlorpyrifos treatment that did not contain midges (mean = 3.5 ng/L, SE = 0.6 ng/L) were not significantly different from chlorpyrifos-only treatments with (mean = 4.1ng/L, SE = 0.3 ng/L) or without midges (mean = 3.1 ng/L, SE = 0.7 ng/L; Tukey's $p < 0.05$). Chlorpyrifos concentrations were not significantly different in posttest water for any treatment with means ranging from 105 ng/L (SE = 9 ng/L) for chlorpyrifos-only treatment with midges to 81 ng/L (SE = 9 ng/L) for chlorpyrifos and atrazine treatments with midges. Chlorpyrifos concentrations in treatments without midges were 104 ng/L (SE = 1 ng/L) for chlorpyrifos only and 108 ng/L (SE = 8 ng/L) for the chlorpyrifos and atrazine treatments.

Tier IV—In vitro biotransformation

Preliminary tests demonstrated that the activity of the assay was linear from 0.07 to 0.28 mg of protein per assay. Protein analysis of the extracts indicated that all assays were conducted between 0.14 and 0.22 mg per assay. More than 85% of the radioactivity that was not attributable to the parent compound was found to be either TCP or chlorpyrifos-*O*-analog. Fractions corresponding to short retention times (before TCP) contained only background levels of radioactivity, indicating that phase II biotransformation was not an important factor in the assay. Assays that lacked the cofactor NADPH had less than 15% activity as compared to controls, indicating that the enzymatic reaction is NADPH dependent. The PBO-dosed assays had less than 10% of the activity of the controls. These results provide evidence that the activity being measured is the result of NADPH-dependent cytochrome P450 monooxygenases, because PBO is a selective inhibitor of mixed-function oxidases. Atrazine treatment had a significant effect on the specific activity of the enzymes as measured by *O*-analog formation ($F = 8.59$, $df = 2$, $p = 0.0082$). The atrazine 2,000 μg/L treatment was significantly different from the other treatments ($p < 0.05$); however, the atrazine 200 μg/L treatment was not significantly different from the control (Fig. 2). Significant differences among treatments also existed when measuring TCP formation and total metabolite formation ($F = 5.10$, $df = 2$, $p = 0.0330$ and $F = 5.37$, $df = 2$, $p = 0.0291$, respectively); however, the differences were found to be between atrazine

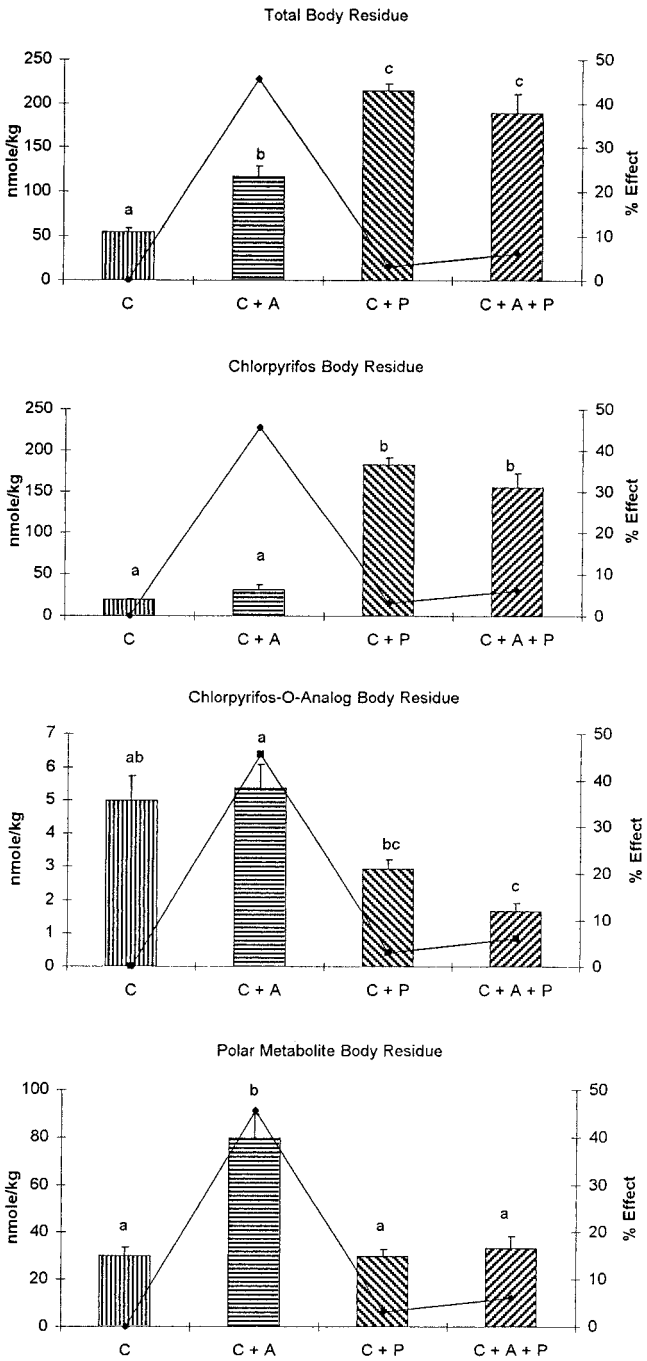

Fig. 1. Body residue of midges exposed to toxicant mixtures for 96 h. Concentrations are indicated by the bars + 1 SE. Treatments marked with the same letter are not significantly different (Tukey's $p < 0.05$). Secondary *y* axis and line illustrate the percent effect found for each treatment group. C = chlorpyrifos; A = atrazine; P = piperonyl butoxide.

treatments only. Both treatments were not significantly different from the control ($p > 0.05$).

DISCUSSION

Tier I experiments indicate that environmentally relevant concentrations of atrazine (40 μg/L) increased the acute toxicity of chlorpyrifos, methyl parathion, and diazinon, but had no effect on malathion. These results add to a growing body of work regarding the joint toxicity of atrazine. Several other investigators have reported synergistic or greater than additive

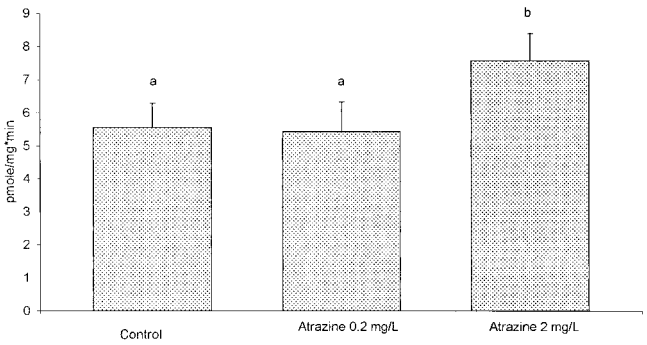

Fig. 2. Specific activities of microsomal enzymes as measured by the formation of chlorpyrifos-*O*-analog are indicated by each bar. Lines indicate + 1 SE. Midges in each treatment were exposed to the indicated concentration of atrazine 48 h before extraction of enzymes. Treatments marked with the same letter are not significantly different (Tukey's $p < 0.05$).

toxicity involving atrazine mixtures. For example, greater than additive (synergistic) toxicity has been noted for mixtures of atrazine and alachlor in amphibians [21], whereas atrazine and parathion mixtures have been shown to cause greater than additive (synergistic) toxicity in mosquito larvae [6]. Also, as previously discussed, atrazine in binary combination with chlorpyrifos, methyl parathion, trichlorfon, and malathion showed greater than additive (synergistic) toxicity in *C. tentans*. However, not all studies have demonstrated that atrazine mixtures will cause greater than additive toxicity. For example, atrazine and carbofuran (carbamate insecticide) mixtures were found to be additive in *C. tentans* using 10-d sediment testing and high enough levels of atrazine to cause direct toxicity (5,000–20,000 μg/kg [22]). However, other investigations have indicated that atrazine in binary combination with mevinophos (OP, phosphate) and methoxychlor (organochlorine) exhibited less than additive (antagonistic) toxicity to *C. tentans* [7]. The variety of joint actions produced by atrazine mixtures indicates that the effect of atrazine on an organism is dependent on the species, cocontaminant, and levels of atrazine used. Also apparent is that atrazine may cause a specific, sublethal effect that only influences the toxicity of some contaminants. For example, if the increased toxicity found in the current study was due to a general decrease in the animal's fitness, then the greater than additive effect would occur for all toxicants used on this organism. However, as previously noted, this is not the case for atrazine in combination with methoxychlor, mevinophos, or carbofuran. Therefore, a specific mechanism likely is responsible for the increased toxicity.

The differences in joint action noted among malathion and the other OPs tested in the present study may be explained by differences in chemical structure. All three compounds that showed greater than additive toxicity are phosphorothioates and have an aromatic side chain. Malathion is a phosphorodithioate and has an aliphatic side chain. Although previous investigators have reported that high levels of atrazine (10,000 μg/L) produced a greater than additive (synergistic) response with malathion [7], the differences in malathion's chemical structure may result in slightly different biotransformation pathways that reduce the influence of atrazine, unless atrazine is present at very high levels.

The current study used much lower concentrations of atrazine (10–200 μg/L) than were used in most of the previous studies (>500 μg/L). Not only are these lower concentrations more relevant to levels that occur in the environment, the lower

levels also change how the greater than additive toxicity is interpreted. For example, atrazine at 200 μg/L increased the toxicity of chlorpyrifos by about a factor of four. This is a substantial increase when considering that levels of 50 times this amount of atrazine did not cause acute toxicity by itself. The most likely explanation for this type of interaction is that atrazine affects the organism in a way that increases the toxic action of chlorpyrifos, methyl parathion, and diazinon. Because the primary metabolite for each of these compounds is more toxic (i.e., stronger acetylcholinesterase inhibitor) than the parent compound, likely mechanisms involve an increase in this metabolite. In this study, two main parameters were investigated as possible mechanisms that could result in synergistic toxicity: increased uptake of chlorpyrifos from the environment and increased biotransformation of chlorpyrifos to chlorpyrifos-*O*-analog.

Tier II results indicated that the uptake rate of chlorpyrifos was elevated by 42% after exposure to atrazine at 200 μg/L (Fig. 1). This increase in uptake is not large enough to account for the fourfold increase in toxicity demonstrated in tier I testing. Assuming that all uptake is the result of first-order kinetics with constant, passive infusion, dose becomes simply the uptake rate multiplied by time. Additionally, a specific dose is assumed to result in a specific percent effect. Then, in two systems of equal exposure time, the EC50 values can be reasoned to differ proportionately to the difference in uptake rates. Therefore, an increase in uptake rate of 42% accounts for approximately one tenth of the 400% increase in toxicity observed.

Increased oxygen consumption was also noted in atrazine-treated midges as compared to control groups. Sublethal stress can cause increased respiration, resulting in increased uptake and toxicity. In fish, uptake of hydrophobic chemicals (log K_{ow} 3–6) has been shown to be limited by the flow of water over breathing surfaces [23,24]. Therefore, chlorpyrifos (log K_{ow} = 4.7) uptake is likely to increase as respiration increases.

The results of tier III and tier IV taken collectively demonstrate that an increase in biotransformation rate occurred in atrazine-treated midges. Body residue analysis of organisms treated in vivo with atrazine had higher levels of polar metabolites, indicating that more biotransformation had occurred (Fig. 1). However, chlorpyrifos-*O*-analog levels were not higher in atrazine treatments, probably because of the rapid hydrolysis of the *O*-analog that occurs within the organism (Fig. 1). In insects, chlorpyrifos-*O*-analog is an intermediate metabolite in the formation of most of the TCP found in an organism. The TCP is then further transformed by phase II reactions (conjugation with a polar organic). Because the concentration for polar metabolites is the collective value for unknown polar metabolites (material that did not leave the origin of the thin-layer chromatography plate) and TCP, an increase in polar metabolites is a good indicator that more chlorpyrifos-*O*-analog was produced within the organism and subsequently further transformed. In vitro assays did measure an increase in chlorpyrifos-*O*-analog (Fig. 2). However, the atrazine concentration necessary to elicit this response was 10 times higher than the concentrations necessary to increase in vivo biotransformation. The bioassay has a great deal of variability associated with it; therefore, small increases may not be detectable using this technique. Even a small level of induction may result in increased biotransformation and toxicity. Because induction was measurably increased at the higher atrazine levels, assuming that lower levels of atrazine produce some induction is reasonable, as is indicated by the in vivo results. Chlorpyrifos-*O*-analog is a very potent acetylcholinesterase inhibitor; therefore, even a slight increase in concentration may result in increased toxicity as demonstrated in tier I testing. In addition, PBO treatments reduced toxicity and reduced formation of metabolites, indicating that cytochrome P450 monooxygenases are involved in the biotransformation process. Other investigators have also demonstrated that atrazine induces mixed-function oxidases. For example, atrazine has been shown to induce cytochrome P450 and general esterase activity in southern armyworm [4], midges [5], and gypsy moths [3].

Trends in toxicity data, specifically for *C. tentans* experiments, indicate that compounds that had greater than additive toxicity when applied in binary combination with atrazine, also had metabolites that are more toxic than the parent compound (phosphorothioate and phosphordithioate OPs). Compounds that have less toxic metabolites (phosphate OPs, organochlorines, and carbamates) were found to have additive relationships with atrazine. Furthermore, the results of tier III and tier IV in the current study indicate that atrazine increased biotransformation rates of chlorpyrifos and specifically formation of its *O*-analog. Taken collectively, this information strongly suggests that the specific sublethal mechanism resulting in synergistic toxicity of some atrazine mixtures is the induction of cytochrome-P450 enzymes, resulting in formation of more *O*-analog and therefore greater toxicity.

Atrazine levels used within this paper are much lower than generally used for acute toxicity studies, because using levels that directly caused acute toxicity was not necessary. Although atrazine rarely exceeds 20 μg/L in most watersheds, lower-order streams receive pulses greater than this level during the primary application period in late spring [2]. These pulses have been reported to commonly reach 30 to 40 μg/L, with a maximum reported value of 108 μg/L [25]. In addition, the species used in this study primarily dwells in sediment that may have higher levels of atrazine than are commonly found in the water column. The duration of exposure for the tests used in this study was either 48 or 96 h. Although lower atrazine levels are more likely to occur in the environment, the duration of exposure would usually be much longer; therefore, this longer exposure may reduce the levels of atrazine necessary to cause increased toxicity of the OPs.

Most midwestern streams are not routinely monitored for insecticides. However, a recent study reported that chlorpyrifos, malathion, and diazinon were three of the most frequently detected insecticides in urban areas, with detection rates of greater than 10, 20, and 60%, respectively. These compounds were also frequently detected in agricultural areas. The same study reported that atrazine was detected in greater than 60% of samples from urban and agricultural areas [26]. The widespread occurrence of these compounds insures that mixtures will co-occur in the environment and are likely to occur at high enough concentrations to cause a toxic effect.

The results of this paper indicate that common environmental mixtures can cause greater than additive toxicity. Estimated SR values indicate that the presence of atrazine can increase the toxicity of chlorpyrifos by a factor of four times the expected value and the toxicity of diazinon and methyl parathion by a factor of two. Further study using additional triazine herbicides, triazine metabolites, and additional species need to be conducted to evaluate the possible environmental risk posed by these mixtures. In addition, this paper demonstrates that atrazine can cause sublethal effects at environ-

mentally relevant concentrations; therefore, additional study is also necessary to evaluate the effects of chronic exposure to atrazine.

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