EFECTS OF ATRAZINE AND CYANAZINE ON CHLORPYRIFOS TOXICITY IN CHIRONOMUS TENTANS (DIPTERA: CHIRONOMIDAE)

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Abstract—Toxicities of two triazine herbicides (atrazine and cyanazine) and an organophosphate insecticide (chlorpyrifos) were evaluated individually and with each herbicide in binary combination with chlorpyrifos using fourth-instar larvae of the aquatic midge, Chironomus tentans. Chlorpyrifos at 0.25 µg/L resulted in an effect in less than 10% of midges in 48-h acute toxicity bioassays. Neither atrazine nor cyanazine alone at relatively high concentrations (up to 1,000 µg/L) caused significant acute toxicity to C. tentans. However, atrazine and cyanazine caused significant synergistic effects on the toxicity of chlorpyrifos when midges were exposed to mixtures of atrazine or cyanazine (10, 100, 1,000 µg/L) with chlorpyrifos (0.25 µg/L). At fixed concentrations (200 µg/L) of the herbicides, toxicity of chlorpyrifos was enhanced by 1.8- and 2.2-fold by atrazine and cyanazine, respectively, at the 50% effective concentration levels. Although atrazine and cyanazine are not effective inhibitors of acetylcholinesterase (AChE) in vitro, the synergism of the two triazine herbicides with chlorpyrifos was associated with increased in vivo inhibition of AChE in midges. We observed a positive correlation between the degree of inhibition of AChE and the concentration of atrazine or cyanazine in the presence of a fixed concentration of chlorpyrifos. It is possible that these herbicides may affect cytochrome P450 enzymes to confer synergistic effects on the toxicity of chlorpyrifos.

Keywords—Atrazine, Cyanazine, Chlorpyrifos, Acetylcholinesterase, Chironomus tentans

INTRODUCTION

Triazine herbicides such as atrazine and cyanazine are widely used pesticides in agricultural areas in the United States [1] and are among the most commonly detected pesticides in surface and ground waters [2,3]. For example, atrazine concentrations were as high as 2,300 µg/L in surface water and 700 µg/L in ground water; cyanazine concentrations reached 1,300 µg/L in surface water and 3,500 µg/L in ground water [4]. In Kansas agricultural areas, atrazine concentrations commonly exceed 20 µg/L in surface water during crop growing seasons [5,6]. Organophosphate insecticides also are frequently detected in surface water in the United States and other countries [7–9]. For example, maximum concentrations of chlorpyrifos detected in rivers and streams in the Lake Erie basin of Ohio ranged from 0.16 to 3.84 µg/L [9]. In a cornfield spray study, mean concentrations of chlorpyrifos measured in adjacent water bodies ranged from nondetectable levels to 67 µg/L [10].

Many studies have evaluated potential hazards of atrazine in rats, freshwater mollusks, and other animals [11–15]. However, few studies have examined potential interactions between triazine herbicides and insecticide mixtures. For example, synergistic effects of atrazine on the toxicity of several insecticides have been found through studies of the aquatic midge Chironomus tentans [16,17]. Results of such studies suggest that the response-addition model may not always accurately predict toxicity for mixtures of chemicals having different modes of action [16]. Because triazine herbicides and insecticides commonly co-occur in the environment [8,18], it is important to investigate interactions between these two types of pesticides and how they affect nontarget species.

This study aimed to investigate the effects of two triazine herbicides (atrazine and cyanazine) on the toxicity of an organophosphate insecticide (chlorpyrifos) using fourth-instar larvae of C. tentans; the effects of binary combinations of each herbicide in combination with chlorpyrifos on in vivo inhibition of AChE, a critical enzyme involved in nerve impulse transmission in animals [19]; and the in vitro effects of each herbicide and chlorpyrifos-oxon (an oxidative metabolite of chlorpyrifos) on AChE. Results from this study were expected to provide new insights into the interactions of pesticide mixtures leading to synergistic toxicity to aquatic biota, thereby improving the assessment of pesticide effects on aquatic systems.

MATERIALS AND METHODS

Organisms

An aquatic midge (C. tentans) colony, originally obtained from the U.S. EPA Environmental Research Laboratory, Duluth, Minnesota, was provided in October 1999 by the Department of Biological Sciences, Wichita State University, Kansas, USA. The C. tentans colony was cultured in the Department of Entomology at Kansas State University based on U.S. EPA standard procedure [20] with slight modifications described by Pape-Lindstrom and Lydy [16]; the midges were reared in mixed-age brood cultures instead of separating each generation from the egg masses. Fourth-instar midges were harvested directly from the mixed-age cultures and were used for the pesticide bioassays and the AChE activity assays.

Chemicals

Acetone (American Chemical Society certified) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetyl-
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Acetylcholinesterase activity assay

The AChE activity of the fourth-instar midges was measured using the method described by Ellman et al. [24] as modified by Zhu et al. [25], with ATC being used as a substrate. All surviving midges were collected from each beaker as a sample. Each sample was then homogenized in an ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100 at the rate of 100 µL per mudge. Homogenates were centrifuged at 15,000 × g for 15 min at 4°C, and supernatants were transferred to new tubes. Residual AChE activity in the supernatants then was measured using an enzyme kinetic microplate reader (Molecular Devices, Menlo Park, CA, USA) at a wavelength of 405 nm immediately after 100 µL of the mixture of ATC and DTNB was added to 50 µL of the supernatant. The final concentrations of ATC and DTNB in the reaction mixture were 0.25 and 0.40 mM, respectively.

In vitro inhibition of AChE was determined using the method of Gao and Zhu [26]. For this assay, 100 fourth-instar midges were taken directly from the culture and homogenized in 2 mL of ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100 (Boehringer, Indianapolis, IN, USA). After the homogenates were centrifuged at 15,000 × g for 15 min at 4°C, the supernatant was transferred to new tubes. Atrazine, cyanazine, and chlorpyrifos-oxon were dissolved in acetone as stock solutions, which were then diluted with 0.1 M phosphate buffer (pH 7.0). Ten microliters of each concentration of the pesticide was mixed with 10 µL of supernatant and preincubated at 5 min at 25°C. The final concentrations were 10, 100, and 1,000 µg/L for each pesticide and below 1% for acetone. The residual AChE activity was measured using the microplate reader (Molecular Devices) at 405 nm immediately after 180 s of the mixture of ATC and DTNB was added to the inhibition mixture. The final concentrations of ATC and DTNB in the reaction mixture were 0.25 and 0.40 mM, respectively. Percentages of residual AChE activity were calculated after arc sine square root transformations using LSD multiple-comparisons [23].

RESULTS

Synergistic effects of herbicides on the toxicity of chlorpyrifos

Cyanazine and atrazine alone at 1, 10, 100, or 1,000 µg/L was not very toxic to C. tentans in 48-h acute toxicity bioassays; only less than 10% of the larvae were affected (Figs. 1 and 2). With chlorpyrifos alone at 0.25 µg/L, 3 to 10% of midges were affected. However, mixtures of atrazine or cyanazine at 10, 100, or 1,000 µg/L in combination with chlorpyrifos at 0.25 µg/L were much more toxic to the midges (p < 0.05). In the presence of atrazine at 10, 100, and 1,000 µg/L, chlorpyrifos resulted in 24 ± 6.0%, 42 ± 3.7%, and 83 ± 7.0% of midges affected, respectively. Cyanazine at 10, 100, and 1,000 µg/L also was toxic in the presence of chlorpyrifos, with 16 ± 2%, 80 ± 6%, and 96 ± 2% of midges affected, respectively. We observed positive correlations between atrazine or cyanazine concentration and percent midges affected in the presence of 0.25 µg/L of chlorpyrifos in water (Figs. 1 and 2). At fixed concentrations of atrazine and cyanazine (200 µg/L), the toxicity of chlorpyrifos was increased by 1.8- and 2.2-fold, respectively, at the 50% effective concentration level (Table 1).
with chlorpyrifos was nearly parallel to that of chlorpyrifos alone, suggesting that these herbicides do not alter the mechanism of action of chlorpyrifos on *C. tentans*.

**Effects of herbicides on in vivo inhibition of AChE by chlorpyrifos**

At 1,000 μg/L, atrazine and cyanazine alone inhibited AChE activity by 27.7 ± 5.0% and 25.7 ± 5.3%, respectively, compared with controls (Figs. 3A and 4A). In contrast, chlorpyrifos alone at 0.25 μg/L inhibited AChE activity by 32.0 ± 5.9%. However, 75.1 ± 2.8%, 80.7 ± 2.7%, and 85.6 ± 4.0% AChE activity was inhibited in midges treated with the mixtures of chlorpyrifos (0.25 μg/L) and atrazine at 10, 100, 1,000 μg/L, respectively. Cyanazine also exhibited synergistic effects on the inhibition of AChE by chlorpyrifos (0.25 μg/L), with 27.9 ± 1.3%, 63.0 ± 12.0%, and 66.8 ± 2.9% AChE activity inhibited in midges treated with the mixtures of chlorpyrifos and cyanazine at 10, 100, and 1,000 μg/L, respectively. The correlations between atrazine or cyanazine concentration and percentage of AChE inhibition in the presence of 0.25 μg/L of chlorpyrifos in water were significant (Figs. 3B and 4B).

**In vitro inhibition of AChE by herbicides and chlorpyrifos-oxon**

We evaluated midge AChE in vitro to determine whether atrazine, cyanazine, and chlorpyrifos-oxon could inhibit the enzyme directly. As expected, chlorpyrifos-oxon inhibited AChE activity in a dose-dependent manner (Fig. 5). However, neither atrazine nor cyanazine inhibited AChE at the given concentrations (Fig. 5). Thus atrazine and cyanazine apparently do not inhibit AChE from midges in vitro, but can significantly increase chlorpyrifos inhibition of AChE in vivo.

### Table 1. Synergistic effects of atrazine (ATR, 200 μg/L) and cyanazine (CYA, 200 μg/L) on toxicity of chlorpyrifos (CHL) to fourth-instar Chironomus tentans*

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>No.</th>
<th>EC25 (95% CI)</th>
<th>EC50 (95% CI)</th>
<th>EC90 (95% CI)</th>
<th>Slope ± SE</th>
<th>χ² (probability)</th>
<th>Synergism ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHL only</td>
<td>299</td>
<td>0.39 (0.35–0.43)</td>
<td>0.49 (0.46–0.52)</td>
<td>0.74 (0.67–0.86)</td>
<td>1.63 ± 0.14</td>
<td>3.36 (0.50)</td>
<td></td>
</tr>
<tr>
<td>CHL + ATR</td>
<td>346</td>
<td>0.20 (0.18–0.22)</td>
<td>0.28 (0.26–0.30)</td>
<td>0.50 (0.44–0.61)</td>
<td>1.37 ± 0.10</td>
<td>2.21 (0.82)</td>
<td>1.75</td>
</tr>
<tr>
<td>CHL + CYA</td>
<td>248</td>
<td>0.19 (0.18–0.20)</td>
<td>0.22 (0.21–0.24)</td>
<td>0.31 (0.29–0.33)</td>
<td>2.66 ± 0.16</td>
<td>0.65 (0.88)</td>
<td>2.23</td>
</tr>
</tbody>
</table>

* The chlorpyrifos toxicity data are presented as EC25, EC50, and EC90 and their 95% confidence intervals (95% CI) in micrograms per liter (μg/L), the effective concentrations at which 25, 50, and 90% of tested midges were affected, respectively, in a 48-h bioassay.

* Number of midges tested in each bioassay.

* Pearson’s chi-square and the probability of χ². The probability >0.05 indicates that the observed regression model is not significantly different from the expected model (i.e., a significant fit between the observed and expected regression models).

* Synergism ratio was obtained by dividing EC50 of CHL only by that of the CHL + ATR or CHL + CYA mixture. The nonoverlap of the 95% CIs of the EC50 values between CHL only and the CHL + ATR or CHL + CYA mixture indicates a significant synergism of ATR or CYA to CHL (p < 0.05).
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Fig. 3. Effects of atrazine (ATR), chlorpyrifos (CHL), and the ATR and CHL mixtures on acetylcholinesterase (AChE) activity in fourth-instar *Chironomus tentans*. (A) Comparisons of residual AChE activity in surviving midges treated with 0.25 µg/L CHL, various concentrations of ATR, and the mixtures of a fixed concentration (0.25 µg/L) of CHL and various concentrations of ATR for 48 h. Vertical bars indicate standard errors of the mean (n = 5). Different letters on the bars indicate that the means are significantly different among the treatments (p < 0.05) in least significant differences multiple comparison tests [23]. (B) Relationship between the percentage of AChE inhibition and the concentration of ATR in the presence of a 0.25 µg/L fixed concentration of CHL (r² > 0.99, p < 0.05). The dashed line indicates the percentage of AChE inhibition for midges treated with 0.25 µg/L CHL only.

Fig. 4. Effects of cyanazine (CYA), chlorpyrifos (CHL), and the CYA and CHL mixtures on acetylcholinesterase (AChE) activity in fourth-instar *Chironomus tentans*. (A) Comparisons of residual AChE activity in surviving midges treated with 0.25 µg/L CHL, various concentrations of CYA, and the mixtures of a fixed concentration (0.25 µg/L) of CHL and various concentrations of CYA for 48 h. Vertical bars indicate standard errors of the mean (n = 5). Different letters on the bars indicate that the means are significantly different among the treatments (p < 0.05) in least significant differences multiple comparison tests [23]. (B) Relationship between the percentage of AChE inhibition and the concentration of CYA in the presence of a 0.25 µg/L fixed concentration of CHL (r² > 0.91, p < 0.05). The dashed line indicates the percentage of AChE inhibition in the midges treated with 0.25 µg/L CHL only.

DISCUSSION

Pesticides applied to crops, lawns, and animals are commonly found in soil or water, and water contamination is often caused by more than one pesticide at relatively low concentrations [28]. Several studies have investigated the effects of atrazine on nontarget organisms [16,17]. Pape-Lindstrom and Lydy [16] reported that atrazine at 20 mg/L (the water solubility limit) did not affect *C. tentans* mortality in 96-h toxicity tests, suggesting that atrazine itself is not very toxic to *C. tentans*. However, a mixture of atrazine and methyl-parathion was more toxic than either chemical alone [16]. In the present study, we tested the toxicity of atrazine and cyanazine over a wide range of concentrations and did not observe significant toxicity to *C. tentans* for either herbicide at any concentration tested. However, both atrazine and cyanazine at 10, 100, and 1,000 µg/L significantly increased the toxicity of chlorpyrifos to *C. tentans*. Because these herbicide and chlorpyrifos (0.25 µg/L) concentrations were similar to those frequently detected in surface waters, particularly during crop growing seasons [4,9], it is possible that these chemicals pose greater risks to nontarget organisms such as aquatic midges than previously supposed.

Our study further indicated that the synergism noted between the triazine herbicides and chlorpyrifos was associated with an increase in in vivo inhibition of AChE in the midges. The correlation between the degree of AChE inhibition and
the concentration of atrazine or cyanazine in the presence of a fixed concentration (0.25 μg/L) of chlorpyrifos was positive and significant. Although midges treated with atrazine or cyanazine had lower AChE activity than control midges, our in vitro study clearly indicated that atrazine and cyanazine were not effective AChE inhibitors. Therefore, the synergistic toxicity of the herbicide and chlorpyrifos mixtures was not caused by direct inhibition of AChE by herbicides. Instead the herbicides probably enhance the inhibition of AChE by organophosphate compounds through an indirect mechanism.

Pape-Lindstrom and Lydy [16] suggested that induction of cytochrome P450 enzymes by atrazine might be responsible for this synergistic effect. Recent studies show that atrazine not only enhances the uptake of organophosphate insecticides but facilitates the biotransformations of chlorpyrifos to chlorpyrifos-oxon. The induction of chlorpyrifos-oxon is a much more potent AChE inhibitor than its parent compound (i.e., chlorpyrifos), the accelerated activation process for chlorpyrifos-oxon ultimately could result in the increase of in vivo inhibition of AChE, thereby causing greater toxicity to midges, as observed in the current study (Fig. 6). This hypothetical mechanism is supported by the dose-dependent relationship between the herbicide concentration and the percentage of AChE inhibited in the presence of a fixed concentration of chlorpyrifos-oxon; the greater rate of biotransformation of chlorpyrifos to chlorpyrifos-oxon in the presence of concentration of atrazine or cyanazine, and chlorpyrifos mixtures support Pape-Lindstrom and Lydy’s [16] hypothesis and the overall picture [17,29] with C. tentans to date.

Organophosphate insecticides, generally including organophosphorothioates, organophosphorodithioates, and other organophosphates, attack AChE as their primary target, thereby accounting for their toxicity to various animal species [30]. However, organophosphorothioates and organophosphorodithioates require oxidative activation by cytochrome P450 enzymes in living organisms to become oxon-analogs, which are much more potent AChE inhibitors than their parent compounds [31]. Based on our study and on previous research [17,29], atrazine and cyanazine both appear to induce cytochrome P450 enzymes responsible for oxidative activation of certain organophosphates, such as chlorpyrifos (an organophosphorothioate), to chlorpyrifos-oxon. Because chlorpyrifos-oxon is a much more potent AChE inhibitor than its parent compound (i.e., chlorpyrifos), the accelerated activation process for chlorpyrifos-oxon ultimately could result in the increase in vivo inhibition of AChE, thereby causing greater toxicity to midges, as observed in the current study (Fig. 6).

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