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MIXTURE TOXICITY OF PHOSTEBUPIRIM AND CYFLUTHRIN: SPECIES-SPECIFIC RESPONSES

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Abstract: Currently, the potential impact of insecticide mixtures to nontarget organisms is largely unknown, and additional study is needed. The present study investigated the mixture toxicity of the organophosphate insecticide phostebupirim and the pyrethroid insecticide cyfluthrin using 4 nontarget species including *Daphnia magna*, *Hyalella azteca*, *Pimephales promelas* (fathead minnow), and *Danio rerio* (zebrafish). For each species, the toxicity of equipotent mixtures was compared with the expected toxicity estimated using the independent action (IA) and concentration addition (CA) models. Lethal and sublethal responses to *D. magna* and *H. azteca* were best described with the IA model. For both fish species, mixture toxicity was significantly higher than that estimated using either mixture model. The synergism noted in fish exposed to the combination of phostebupirim and cyfluthrin was confirmed by exposing *P. promelas* larvae to a nontoxic dose of phostebupirim and a range of toxic cyfluthrin concentrations, and vice versa. Sublethal and lethal concentrations to fish were up to 7 times lower for the mixture than in concurrently run individual compound exposures. Potential mechanisms for the synergistic responses found in fish are presented. *Environ Toxicol Chem* 2017;36:1947–1954. © 2016 SETAC

Keywords: Organophosphate Pyrethroid Additivity Potentiation Toxicity models

INTRODUCTION

Although the application of insecticides to corn, excluding seed-coating insecticides, has decreased in past years in Illinois (USA), formulations containing phostebupirim, an organophosphate insecticide, and cyfluthrin, a pyrethroid insecticide, are still applied to corn in large amounts, with a total of 70 tons and 4 tons applied in 2010, respectively [1]. The organophosphates and pyrethroids are commonly used in combination in formulations to prevent pest resistance [2].

Few ecotoxicological data are available for cyfluthrin and phostebupirim [2–8], and to our knowledge no data exist for mixtures of the 2 compounds. Phostebupirim has been shown to pose an acute risk above the concern levels of the US Environmental Protection Agency (USEPA) to small birds, amphibians, reptiles, mammals, fish, and crustaceans [2]. Likewise, cyfluthrin is highly toxic to nontarget terrestrial invertebrates, as well as aquatic vertebrates and invertebrates [3]. Both insecticides are neurotoxins, although they have different mechanisms of toxic action. Phostebupirim is an acetylcholinesterase inhibitor that blocks the degradation of the neurotransmitter acetylcholine at nerve synapses, which causes hyperexcitation of the central nervous system [2]. Cyfluthrin is a type II synthetic pyrethroid that acts on nerve axons by inhibiting neurotransmitter delivery via inhibition of the calcium ion channels coupled with a stimulatory effect on the sodium ion channels, affecting both the peripheral and central nervous systems [3].

Mixture toxicity can be predicted by different models, including independent action (IA) and concentration addition (CA). These models have been extensively described in the literature [9–11]. Briefly, the IA model predicts the toxicity of a mixture of compounds based on individual toxicities, assuming completely independent modes of toxic action [9]; this model is based on the hypothesis that the same effect caused by several compounds should be accounted for only once. The CA model is based on addition of concentrations of individual compounds, converted to a similar unit. Effects can be estimated from the summed concentrations using a joint concentration–response relationship. It assumes a similar mode of action of the 2 compounds, and no overlap of their effects. For 2 compounds with different modes of toxic action, it is often assumed that joint toxicity will be best described using the IA model. However, Cedergreen et al. [10] showed that less than 50% of all the mixtures they reviewed with different modes of toxic action could actually be predicted by the IA model. In some cases, mixture toxicity does not follow either of these models. For example, mixture toxicity may be significantly higher than expected, that is, when the toxic effects are greater than expected from individual compound toxicities; this is called synergism. Increased toxicity has been noted for other organophosphate and pyrethroid mixtures for several species, including target insects and nontarget organisms [12–14]. Similarly, if the mixture toxicity is lower than expected from individual compound toxicities, the mixture is considered antagonistic. When a compound at nontoxic levels increases the toxicity of another compound, this specific type of synergism is called potentiation. Zhang et al. [15] showed that the mixture effect of a 50:50 binary combination of organophosphates and pyrethroids to zebrafish may not only be synergistic, but also additive, or even antagonistic, depending on the organophosphate–pyrethroid combination tested. Similarly, Belden and Lydy [11] exposed *Pimephales promelas* and *Chironomus tentans* to a mixture of chlorpyrifos and esfenvalerate, and found that the mixture toxicity was additive or synergistic based on the species tested.

The objective of the present study was to investigate the mixture toxicity of phostebupirim and cyfluthrin to 4 different

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species of aquatic nontarget organisms, *Daphnia magna, Hyalella azteca, Danio rerio,* and *P. promelas.* Equipotent mixtures were used, and comparisons of the mixture toxicity with the IA and CA models were made to determine whether the mixture toxicity was following either model, or was synergistic or antagonistic. The *D. rerio* and *P. promelas* were exposed to a range of concentrations of 1 compound in the presence and in the absence of nontoxic doses of the other compound, to determine whether potentiation occurred in addition to the synergism observed at high concentrations.

MATERIALS AND METHODS

Bioassays

To assess the risk caused by the insecticides individually and as a mixture, acute toxicity bioassays were performed on *D. magna, H. azteca, D. rerio,* and *P. promelas* following protocols adapted from the Institutional Animal Care and Use Committee and the USEPA guidelines [16–18]. Detailed test conditions are given in Supplemental Data, Table S1. Bioassays were conducted in static mode without additional aeration of the media. The *H. azteca* were fed 0.2 mL/beaker of a yeast, cerophyl, and trout chow solution at the beginning of the tests, right after addition of animals into the beakers, and every 48 h until the end of the bioassay. The other test species were not fed during the bioassays, as directed by the guidelines. Moderately hard reconstituted water (500 mL [19]) was added to each beaker for the water tests, with the exception of the *D. magna* bioassay, which used 200 mL of moderately hard reconstituted water. Pesticide-grade acetone (Fisher Scientific) was used as the carrier solvent (50 μL, i.e., 0.01% v/v for all water tests, except *D. magna* which was 0.025% v/v) to enrich the water with insecticides in each beaker, and a set of solvent controls was included with each bioassay. A set of negative controls was also included with each test, with water free of any solvent and target insecticides. Each spiking level and the controls were conducted in triplicate, and each replicate contained 10 organisms. For each species, range-finding preliminary bioassays were performed with exposure to individual insecticides, and then mixture bioassays were performed simultaneously with individual compound exposures to compare individual and mixture toxicities with a test conducted at the same time and under the same conditions. Definitive tests used 7 dosing levels for each individual compound and equipotent mixtures. The levels were chosen so as to include expected toxicant threshold concentrations/effective concentrations (LC1s/EC1s), lethal concentrations to half of the population/effective concentrations to half of the population (LC50s/EC50s), and lethal concentrations/effective concentrations to 99% of the population (LC99s/EC99s) from the preliminary test, assuming the toxicity followed either the IA or the CA model. When the toxicity did not follow the models, additional bioassays were run until adequate concentrations were used, and a dose–response curve was obtained. Measured concentrations of all 7 levels for individual compound tests, and equipotent tests are given in the Supplemental Data, Table S2. For both fish species, 2 additional bioassays were performed using 7 dosing levels for 1 of the compounds, and 1 level of the other compound at a concentration below the no-observed-effect concentration (Supplemental Data, Table S3).

Endpoints included lethality, difficulty swimming, and/or lack of erratic movements. The LC50s and EC50s were calculated from measured concentrations of the media using SPSS (IBM, 20.0) probit regression after log transformations of concentrations.

Insecticide analyses

Insecticide analyses were performed at the lower, median, and upper spiking levels to check water immediately before addition of the animals, and at the end of each bioassay. Mean media concentrations were calculated by averaging concentrations at the beginning and end of the bioassays, and by interpolation of concentrations at levels in between the ones analyzed, assuming a linear curve. Samples were extracted directly after collection, or stored in the freezer at –18 °C until extraction. Phostebupirim and cyfluthrin were extracted simultaneously from mixture exposure samples using methods developed in our previous study [2]. Additional details are provided in the Supplemental Data.

Analytes were quantified in negative chemical ionization (NCI) mode on an Agilent Technologies 6850 gas chromatograph coupled with a 5975C inert XL EI/CI MS detector. The system was equipped with an HP-5MS Agilent column (30 m × 0.25 mm × 0.25 μm). The total run time was less than 25 min, starting with an oven temperature of 90 °C, increasing to 275 °C at 15 °C/min, then to 285 °C at 2 °C/min, and finally to 300 °C at 10 °C/min, and then held at 300 °C for 6 min. The mass spectrometer detector was operated in the selected ion monitoring mode with a quadrupole temperature of 150 °C and source temperatures of 150 °C. Each analyte was searched using a quantitation ion and 2 confirmation ions, that is, m/z = 183, 167/275 for phostebupirim, and m/z = 207, 209/171 for cyfluthrin. Cyfluthrin existed as a mixture of 8 possible isomers, which produced 4 different peaks (for the 4 pairs of diastereoisomers) on the chromatogram. These peaks were not well resolved, especially the third and fourth peaks that coeluted; therefore, the 4 peaks were integrated together, and total cyfluthrin was reported.

Method detection limits in water samples were 4.0 ng/L and 3.2 ng/L, for phostebupirim and cyfluthrin, respectively [20].

Mixture toxicity models

Two mixture models, the IA and CA, were used to predict the toxicity of binary mixtures of the insecticides [21]. The IA model assumes that each compound acts independently and that the same effect as a result of the 2 compounds can be accounted for only once. The IA model is based on binary data, and the effect of the total mixture concentration can be predicted by the expected effect of each component, as shown in Equation 1.

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \quad (1)$$

where $E(c_{mix})$ is the predicted effect of the mixture, and $E(c_i)$ is the effect expected from component i.

The CA model was based on the assumption that each compound contributes to the toxicity of the mixture, and the toxicity of the mixture was predicted using Equation 2.

$$ECx_{mix} = \left(\sum_{i=1}^{n}\frac{p_i}{ECx_i}\right)^{-1} \quad (2)$$

where ECx_{mix} is the total concentration of the mixture that causes x effect, p_i is the proportion of component i in the

mixture, and ECx_i is the concentration of component i that would cause x effect.

Model deviation ratios (MDRs) were calculated from equipotent mixture experiments using Equation 3.

$$\mathrm{MDR} = \mathrm{EC50_{model}}/\mathrm{EC50_{observed}} \quad (3)$$

where $\mathrm{EC50_{model}}$ is the EC50 predicted by the IA or CA model, and $\mathrm{EC50_{observed}}$ is the EC50 observed experimentally. The EC50 values were calculated based on the sum of both insecticide concentrations in the mixture. If the lethal endpoint was utilized, $\mathrm{LC50_{model}}$ and $\mathrm{LC50_{observed}}$ were used instead of $\mathrm{EC50_{model}}$ and $\mathrm{EC50_{observed}}$, respectively. A MDR value greater than 1 indicated higher toxicity of the mixture of compounds compared with the mixture models. Similarly, a MDR value lower than 1 indicated lower toxicity of the observed mixture compared with what would be predicted by the mixture models. However, as suggested by Belden et al. [21], synergistic or antagonistic effects were only considered significant if the MDR values were greater than 2 or lower than 0.5, or if the 95% confidence interval of the EC50s did not overlap.

In the case of the mixture bioassays, where 1 of the compounds was spiked at a nontoxic concentration, synergistic ratios (SRs) were calculated using Equation 4.

$$\mathrm{SR} = \mathrm{EC50_{individual}}/\mathrm{EC50_{mixture}} \quad (4)$$

where $\mathrm{EC50_{individual}}$ is the EC50 measured for organisms exposed to 1 insecticide, and $\mathrm{EC50_{mixture}}$ is the EC50 measured for the same insecticide, but this time the organisms were also exposed to nontoxic levels of the other insecticide. If the lethal endpoint was utilized, $\mathrm{LC50_{individual}}$ and $\mathrm{LC50_{mixture}}$ were used instead of $\mathrm{EC50_{individual}}$ and $\mathrm{EC50_{mixture}}$, respectively. Similarly to the MDR, a SR value greater than 1 indicated potentiation of the nontoxic insecticide on the other insecticide, whereas a SR value lower than 1 indicated an antagonistic effect. Significance of the SRs was determined in the same manner as that of the MDR.

RESULTS AND DISCUSSION

Toxicity bioassays: Individual insecticides

All toxicity results reported in the present study had satisfactory survivorship in the negative and solvent controls ($\geq$80% survival or no effect). Sublethal (EC50s) and lethal (LC50s) concentrations along with slopes and intercepts of the probit dose–response curves are shown in Tables 1 and 2. For both insecticides, the ratios of LC50s to EC50s for the same contaminant/species ranged from 1.1 to 2.0, meaning that lethality occurred at concentrations close to where sublethal effects were detected.

Aquatic invertebrates were several orders of magnitude more sensitive to phostebupirim and cyfluthrin than the 2 fish species tested (Figure 1), and the difference was greater for phostebupirim than cyfluthrin. Phostebupirim LC50s were approximately 22 000-fold and 4000-fold lower for *D. magna* and *H. azteca* than for *P. promelas*, respectively. Cyfluthrin LC50s were approximately 1300-fold and 50-fold lower for *D. magna* and *H. azteca* than for *P. promelas*, respectively. Burkepile et al. [22] noticed the same trend with diazinon, a similar organophosphate to phostebupirim, with LC50s approximately 6700-fold and 1100-fold lower for *D. magna* and *H. azteca* than for *P. promelas*, respectively. This higher toxicity noted for aquatic invertebrates compared with fish was expected, because insecticides are usually selected for their low toxicity to vertebrates. In addition, *H. azteca* is known to be more sensitive to pyrethroids than other invertebrate species [4,23]. The different organisms selected in the present study greatly differ in physiology, and the lower toxicity of both insecticides to fish larvae may suggest either a more potent toxicity mechanism in aquatic invertebrates or better defense mechanisms in fish, especially for phostebupirim.

Daphnia magna was particularly sensitive to phostebupirim, with the lowest EC50 and LC50 for this compound being 51 ng/L and 100 ng/L, respectively. This was in the range of toxicity values previously reported for other organophosphates for the same species and organism age [24]. In previous studies, *D. magna* (<48 h old) and *H. azteca* (7–10 d old) were shown to possess cytochrome P450 monooxygenases capable of activating organophosphates by forming extremely potent acetylcholinesterase inhibitors from phosphorothioate insecticides [25–27]. Fish embryos and larvae were also shown to be capable of bioactivating organophosphates into more potent oxons [12,28,29]. In addition, cytochrome P450 enzymes may be involved in defense mechanisms via insecticide oxidation [30], and these enzymes may be induced by xenobiotics, resulting in the increased biotransformation of these xenobiotics [31–35]. Therefore, the variation in susceptibility of different species to phostebupirim may be driven by the balance of different phostebupirim-induced cytochrome P450 enzymes, some involved in the transformation of phostebupirim into its oxon form, and others involved in its metabolic detoxification (Figure 2). Livingstone [31] found that levels of total cytochrome P450 enzymes were higher in fish than in aquatic invertebrates. Higher levels of detoxifying cytochrome P450 enzymes would explain the lower sensitivity noted in the present study for fish to either insecticide, compared with the aquatic invertebrates.

Several authors have suggested that the toxic mechanism of phosphorothioates depends on the ability of the organisms to transform organophosphates into their oxon form, but also the capability of the oxons to inhibit acetylcholinesterase [28], which both depend on many factors, such as species and organism age (Figure 2) [31,32,35]. Acetylcholinesterase inhibition may also be influenced by the presence of carboxylesterase enzymes, which, because of structural similarities, may associate irreversibly with organophosphates and render them less available for binding with acetylcholinesterase [12,36,37]. When an organophosphate molecule is bound to carboxylesterases, it can be hydrolyzed, and is unable to bind and inhibit acetylcholinesterase (Figure 2). The toxicity as a result of acetylcholinesterase inhibition is thus reduced, and, because carboxylesterase inhibition was not shown to cause lethal effects, carboxylesterase inhibition by organophosphates may be considered a detoxification process [37]. If fish larvae have higher levels of carboxylesterases than the aquatic invertebrates, this could explain the lower sensitivity of fish to phostebupirim (Figure 2).

Dose–response curves were steeper for *D. rerio* when exposed to phostebupirim, as confirmed by slopes 2 to 6 times higher than for other species (Tables 1 and 2). The steep slopes may be because of more rapid onset of effects possibly as a result of increased biotransformation of the parent to the oxon form, or more rapid uptake. Even though both fish species were less sensitive than the invertebrates to the target insecticides, zebrafish showed much steeper slopes then fathead minnows when exposed to phostebupirim. The different biochemistry, mainly in term of esterases and cytochrome P450 enzymes, may

Table 1. Slopes (a) and intercepts (b) of probit dose–response curves, and following sublethal concentrations (median effect concentrations [EC50s]) to half of the test organisms (95% confidence intervals in parentheses) of individual insecticides given to reference nontarget organisms[a]

			Equipotent mixture							
	Units		Phostebupirim	Cyfluthrin	Experimental	CA model	IA model	Phos/Cyf (%)	MDR CA	MDR IA
Hyalella azteca	µg/L	a	7.559	5.105	12.548	6.517	8.030	99.92/0.08		
		b	2.289	15.845	3.383	3.114	2.581			
		EC50	0.498	0.8×10^{-3}	0.538	0.334	0.488		0.62	0.91
Daphnia magna	µg/L	a	5.113	6.943	6.167	5.748	7.351	69.94/30.06		
		b	6.628	10.606	7.526	7.939	8.822			
		EC50	0.051	0.030	0.060	0.042	0.067		0.70	1.12
Pimephales promelas	µg/L	a	8.805	12.315	10.611	9.757	6.278	99.96/0.04		
		b	–27.041	0.465	–26.017	–28.166	–15.499			
		EC50	1178	0.917	283	771	1179		2.7	4.2
Danio rerio	µg/L	a	33.027	7.204	9.938	10.364	7.271	99.92/0.08		
		b	–82.958	5.666	–15.829	–21.736	–16.832			
		EC50	324	0.163	39	127	208		3.3	5.3

[a]Equipotent mixture results are also presented. The proportion of each compound in the mixture is given (Phos/Cyf), as well as the MDRs.
CA = concentration addition; IA = independent addition; MDR = model deviation ratio.

be the cause of the differences in the toxic effects observed. A better knowledge of levels of carboxylesterases, acetylcholinesterases, and cytochrome P450 enzymes in neonates of both species would likely help to answer this research question.

In the case of cyfluthrin, similar esterase and cytochrome P450 enzyme detoxification processes may be involved, but pyrethroids do not need to be bioactivated by cytochrome P450 enzymes to express their toxicity, and they have a different mechanism of toxic action (Figure 2). This may explain the lower effective concentrations found for cyfluthrin for all species when compared with phostebupirim. Indeed, EC50s were generally several orders of magnitude lower for cyfluthrin than for phostebupirim (note the difference in scaling in Figure 1), with the only exception being *D. magna*, whose sensitivity to cyfluthrin was in the same range as for phostebupirim. This was likely because of the particular sensitivity of *D. magna* for organophosphates.

Pyrethroid toxicity is also known to be temperature dependent, with higher toxicity at lower temperatures [38,39]. In the present study, only *D. magna* bioassays were run at lower temperatures (20 °C vs 23 °C for the other species). Cyfluthrin toxicity to *D. magna* was lower than for *H. azteca*; therefore, the higher temperature did not explain the higher toxicity to *H. azteca*. The same temperature was used for both fish species and did not explain the higher toxicity to *D. rerio*.

Toxicity bioassays: Equipotent mixtures

The slopes of individual dose–response curves ranged from 5.1 to 33.0 for the target compounds and sublethal and lethal endpoints, and across all species (Tables 1 and 2). For these steep curves, the CA models were more conservative than the IA models. In each of our equipotent toxicity mixture tests, the dose–response curve predicted by the CA model occurred at lower concentrations than the 1 predicted by the IA model (Figure 3). Drescher and Boedeker [10] previously showed that for a normally distributed population (probit model) at high mixture concentrations, the CA model predicted effects at lower concentrations than the IA model and both models were similar at low concentrations. The dose–response curves predicted by the CA and IA models were generally parallel with the IA curve shifted toward higher concentrations with ratios of EC50s and LC50s between the 2 models ranging from 1.3 to 1.7.

For *D. magna* and *H. azteca*, equipotent mixture effects are better described with the IA model than with the CA model (Figure 3A and B). For *D. magna*, the experimental model practically overlaid the IA model curve for both sublethal and

Table 2. Slopes (a) and intercepts (b) of probit dose–response curves, and lethal concentrations (LC50s) to half of the test organisms (95% confidence intervals into parentheses) of individual insecticides given to reference nontarget organisms[a]

			Equipotent mixture							
	Units		Phostebupirim	Cyfluthrin	Experimental	CA model	IA model	Phos/Cyf (%)	MDR CA	MDR IA
Hyalella azteca	µg/L	a	8.033	3.898	18.365	6.326	8.033	99.92/0.08		
		b	2.057	11.225	4.581	2.442	2.164			
		LC50	0.555	1.3×10^{-3}	0.563	0.419	0.549		0.74	0.98
Daphnia magna	µg/L	a	2.503	3.758	4.097	3.078	3.970	69.94/30.06		
		b	2.499	5.558	4.505	3.741	4.374			
		LC50	0.100	0.033	0.080	0.062	0.082		0.78	1.03
Pimephales promelas	µg/L	a	4.170	5.127	7.218	6.279	4.468	99.96/0.04		
		b	–14.006	–1.176	–19.729	–15.499	–14.146		2.7	4.0
		LC50	2283	1.696	541	1470	2161			
Danio rerio	µg/L	a	14.133	15.643	7.283	14.944	6.411	99.92/0.08		
		b	–37.637	8.906	–12.735	–34.286	–12.275		3.5	6.1
		LC50	460	0.270	56	196	341			

[a]Equipotent mixture results are also presented. The proportion of each compound in the mixture is given (Phos/Cyf), as well as the MDRs.
CA = concentration addition; IA = independent addition; MDR = model deviation ratio.

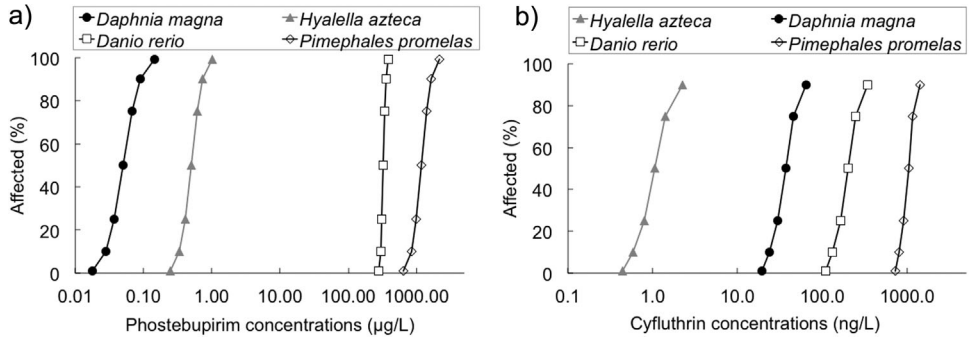

Figure 1. Modeled dose–response curves of sublethal effects for all species to either (**a**) or (**b**) cyflurthrin individually.

lethal endpoints, and MDRs were 1.12 and 1.03, respectively. For *H. azteca*, the experimental and IA model curves were further apart, but the 95% confidence intervals on the experimental model overlapped with the IA model for most concentrations (Figure 3B). The experimental and IA curves were close to one another around the EC50 and LC50, which are the levels where the MDRs were calculated, because the median concentrations are the most statistically reliable values [40]. Therefore, the deviations from the IA models at low and high ends of the curves are less statistically reliable than at the EC50 and LC50 levels. The MDRs for *H. azteca* were close to 1 for the IA model, with values of 0.91 and 0.98 for sublethal and lethal endpoints, respectively (Tables 1 and 2). Therefore, phostebupirim and cyfluthrin appeared to act independently, because IA was the best model for describing the mixture data for both of the aquatic invertebrates.

For the fish species, which required insecticide concentrations several orders of magnitude higher than for the aquatic invertebrates to observe the same level of effect, synergism was clearly observed between phostebupirim and cyfluthrin (Figure 3C and D). There was no overlap between the experimental model and either the IA or CA curves, and the experimental dose–response curves were at much lower concentrations of the mixture than either of the model curves (Figure 3C and D). The MDR values were as high as 6.1, which suggested a significant synergism for both fish species (Tables 1 and 2).

The mixture toxicity of the target insecticides was therefore highly species dependent, and the effects noted were likely because of different enzymes involved in bioactivation of phostebupirim and/or detoxification of either or both insecticides. In the case of the synergism observed in fish, 1 or several of the following mechanisms may be occurring. As previously discussed, phostebupirim bioactivation to the oxon is required for toxic effects, and the presence of cyfluthrin may have induced cytochrome P450 enzyme production, causing a greater amount of phostebupirim-oxon to be found in the fish, and therefore higher toxicity (Figure 2). The presence of cyfluthrin may also have decreased detoxification of phostebupirim by competition of the 2 insecticides for carboxylesterases. However, because cyfluthrin was shown to be much more potent than phostebupirim for all the species studied, the opposite mechanism was more likely: the high amount of phostebupirim may have inhibited all carboxylesterases, or possibly other enzymes, otherwise available for cyfluthrin detoxification, increasing the toxicity as a result of the pyrethroid (Figure 2).

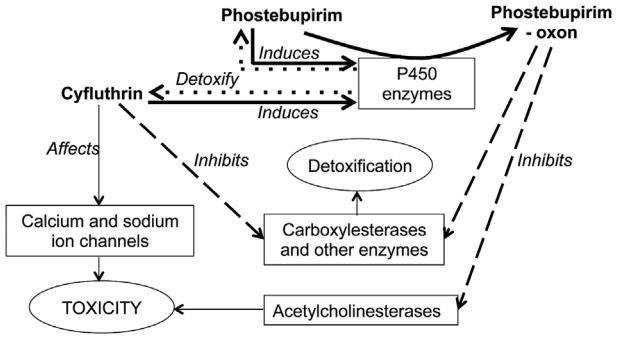

Figure 2. Proposed mechanisms of toxic effects to fish neonates of both phostebupirim and cyfluthrin when simultaneously present.

In the case of resistant target insects, an additional mechanism has been proposed to describe the greater than additive response found for organophosphate and pyrethroid mixtures [29]. It has been suggested that organophosphates and pyrethroids may act as competitive substrates for cytochrome P450 enzymes, which may not be able to biotransform pyrethroids when they are involved in organophosphate bioactivation [14]. Similarly, Baerg et al. [41] showed that some organophosphates were able to inhibit P450 activities in maize, which then prevented hydroxylation of herbicides, and thus increased injury in the plant. Therefore, the oxidation required for phostebupirim to be toxic performed by P450 enzymes may reduce hydroxylation and detoxification of cyfluthrin (Figure 2).

One could argue that all mechanisms actually occur simultaneously, and are linked (Figure 2). Cyfluthrin (and maybe even phostebupirim), may induce more cytochrome P450 enzymes, which can convert phostebupirim to the toxic oxon form, which inhibits both carboxylesterase and acetylcholinesterase, the latter causing toxicity. Carboxylesterase inhibition by phostebupirim would then prevent detoxification of pyrethroids, and cause additional toxicity from cyfluthrin. Concurrently, competition of cytochrome P450 enzymes between activation of phostebupirim and detoxification of cyfluthrin and phostebupirim may increase the mixture toxicity.

Toxicity bioassays: Potentiation

To better understand the mechanisms involved in the synergistic effects noted for phostebupirim and cyfluthrin in fish, the toxicity of 1 compound was compared with the toxicity of the other compound at concentrations causing no observable effect. *Pimephales promelas* was exposed to a range of cyfluthrin concentrations in the absence and in the presence of phostebupirim at 38 µg/L (~EC5, or 11% of the EC50). A control beaker at this latter concentration of

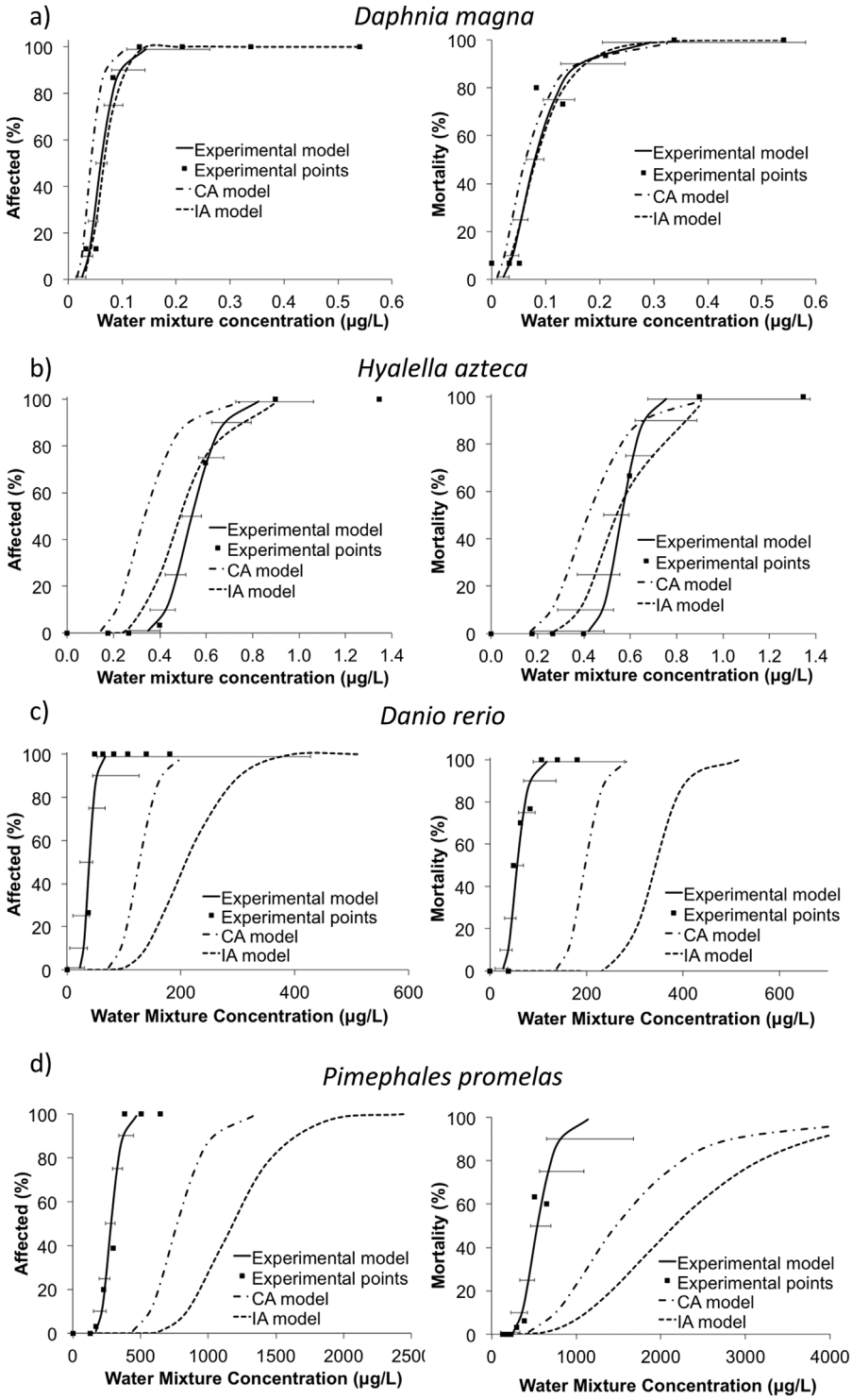

Figure 3. Experimental and model-derived dose–response curves of mixtures of phostebupirim and cyfluthrin to (**a**) *Daphnia magna,* (**b**) *Hyalella azteca,* (**c**) *Dania rerio,* and (**d**) *Pimephales promelas*. Horizontal error bars represent 95% confidence intervals on the modeled experimental data. CA = concentration addition; IA = independent action.

phostebupirim showed no sublethal or lethal effects to fish. Figure 4A shows that the dose–response curves for both sublethal and lethal effects were shifted toward lower cyfluthrin concentrations in the presence of phostebupirim. The 95% confidence intervals at the EC50 and LC50 values did not overlap, showing a potentiation of cyfluthrin toxicity by phostebupirim, with SRs of 7.3 and 2.8 for sublethal and lethal endpoints, respectively. Similar results were obtained for comparable experiments with *D. rerio* that were exposed to a range of cyfluthrin concentrations and 108 µg/L of phostebupirim (<EC1, or 8% of the EC50, results not shown because similar trends as for *P. promelas* were observed). The 95% confidence intervals at the EC50 and LC50 values did not overlap, indicating a potentiation of cyfluthrin toxicity by phostebupirim, with SRs of 2.7 and 1.6 for sublethal and lethal endpoints, respectively. These experiments supported the hypothesis that phostebupirim enhanced cyfluthrin toxicity, even at low concentrations. This potentiation was likely

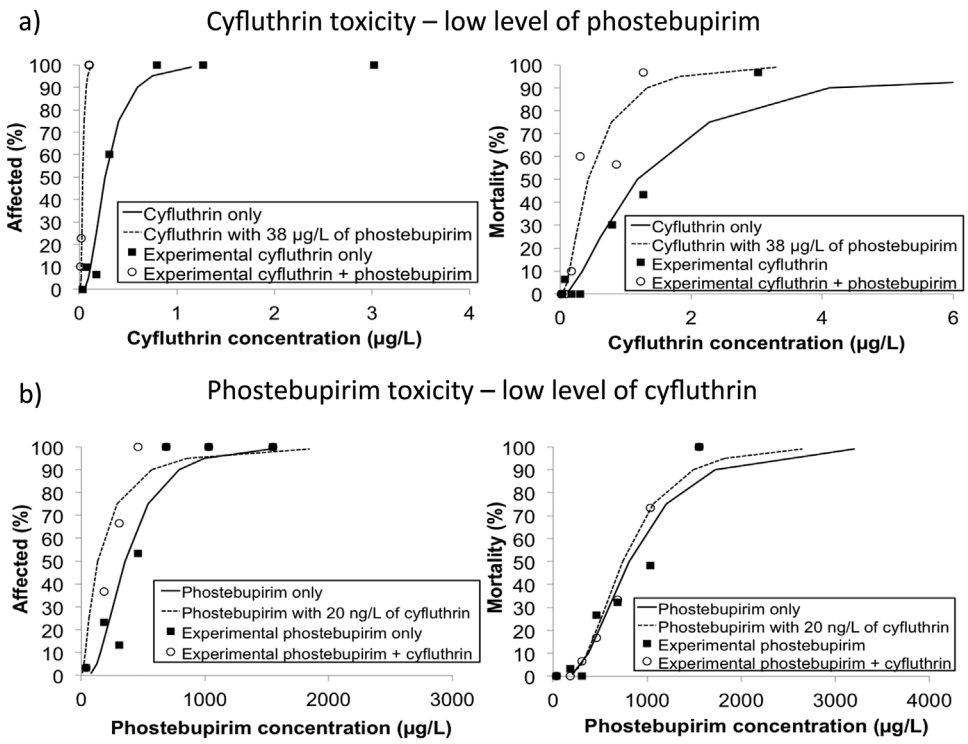

Figure 4. Experimental dose–response curves for *Pimephales promelas* exposed to (**a**) a range of cyfluthrin and 1 level of phostebupirim; and (**b**) a range of phostebupirim and 1 level of cyfluthrin.

because of inhibition of the carboxylesterases or other enzymes involved in cyfluthrin detoxification by phostebupirim, and/or the decreased detoxification of cyfluthrin by P450 enzymes already involved in biotransformation of phostebupirim into its toxic oxon form (Figure 2).

Another complementary experiment was performed with *P. promelas* exposed to a range of phostebupirim concentrations in the absence and in the presence of cyfluthrin at 22 ng/L (<EC1, 8% of the EC50). A control beaker at this latter concentration of cyfluthrin showed no sublethal or lethal effect to fish. Figure 4B shows the results for sublethal and lethal endpoints. Potential potentiation was observed for sublethal effects with a SR of 2.6, but 95% confidence intervals were not available for the sublethal endpoint. There was no potentiation observed for lethality, with the 2 dose–response curves almost overlapping, and the SR was 1.1. The first potentiation experiment with nontoxic doses of phostebupirim also showed a higher SR for sublethal than for lethal effects, the latter probably demanding more of the synergistic compound to get the same SR. Similar experiments conducted with *D. rerio* showed no significant potentiation of phostebupirim toxicity by cyfluthrin (34 ng/L, <EC1, 9% of the EC50), as the 95% confidence intervals overlapped, and the SR was 1.1 for the sublethal endpoint. The range of phostebupirim concentrations used was adjusted for accurate sublethal toxicity assessment only, and mortality was equal to that seen in the controls in all beakers, regardless of the presence of cyfluthrin. *Pimephales promelas* may have been a little more sensitive to potentiation of phostebupirim toxicity by cyfluthrin than *D. rerio*, but the lack of confidence intervals for the sublethal effects observed in *P. promelas* prevented a more definitive conclusion. Overall, the effect of cyfluthrin on phostebupirim toxicity was less pronounced than the effect of phostebupirim on cyfluthrin toxicity for both fish species.

CONCLUSIONS

The toxicity of mixtures of phostebupirim and cyfluthrin was assessed in 4 nontarget species, which all were affected by the compounds differently. Phostebupirim and cyfluthrin seemed to act independently on *D. magna* and *H. azteca* as the experimental data could be predicted well by the IA model. In contrast, both fish species were subject to significant synergism, suggesting a close interaction between the 2 insecticides. The present study showed the difficulty in predicting the toxicity of binary mixtures of organophosphates and pyrethroids. Because of the dissimilar modes of action of the 2 insecticides, the IA model was expected to more accurately predict the mixture toxicity than the CA model, and, this was the case for the 2 aquatic invertebrate species *D. magna* and *H. azteca*. However, the synergism observed in the 2 fish species may also have been expected, because of the known effect of organophosphates inhibiting enzymes responsible for pyrethroid detoxification. Toxic effects caused by organophosphate and pyrethroid binary mixtures are therefore species dependent, and also depend on organism life stage and the physical and chemical properties of the insecticide, as previously described in the literature. A better understanding of both the biochemistry of the species at different life stages and the mechanisms of toxic action of each compound is critical in understanding the impacts of pesticide mixtures.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3724

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Disclaimer—Use of a company or product name does not imply approval or recommendation of the product by Southern Illinois University or the Howard G. Buffett Foundation.

Data Availability—The toxicity input files are on OpenSIUC, available through http://opensiuc.lib.siu.edu/communities.html and accessed through the Fisheries and Illinois Aquaculture Center community. The direct link is http://opensiuc.lib.siu.edu/fiaq_reports/6

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