Binary combinations of organophosphorus and synthetic pyrethroids are more potent acetylcholinesterase inhibitors than organophosphorus and carbamate mixtures: An *in vitro* assessment

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HIGHLIGHTS

- *In-vitro* mixture toxicity of five organophosphates and two carbamates pesticides in binary combinations was investigated.
- Housefly head acetylcholinesterse based bioassays showed differential toxicity under oxidised and un-oxidised conditions.
- Eight binary combinations exhibited synergistic effects under oxidised conditions
- Triazophos in binary combination with carbaryl and carbofuran exhibited synergistic effects.
- Organophosphate pesticides exhibited greater acetylcholinesterase inhibition in combination with pyrethroids.

ARTICLE INFO

ABSTRACT

Anticholinesterase insecticides such as organophosphorous (OP) and carbamates pesticides (CB); and synthetic pyrethroids (SP) pesticides commonly co-occur in the environment. This raises the possibility of antagonistic, additive, or synergistic neurotoxicity in exposed organisms. Acetylcholinesterase (AChE) inhibition has been demonstrated to be useful as a biomarker for exposure to OP and CBs in many environments. This study investigated the response of housefly (*Musca domestica*) head AChE (HF-AChE) exposed to five OPs; chlorpyrifos (CPF), malathion (MLT), triazophos (TRZ), monocrotophos (MCP) and profenofos (PRF) and two CBs; carbaryl (CRB) and carbofuran (CBF) as individual compounds and as binary mixtures of OPs and CBs under *in vitro* conditions. In addition, the selected OPs and CBs were evaluated for their toxicity in binary combinations with two SPs; deltamethrin (DLT) and cypermethrin (CYP) at fixed concentrations of 0.1 and 10 μg/L. The toxicological interaction of five OPs with two CBs pesticides was evaluated under oxidised and un-oxidised conditions using a toxic unit (TU) approach and a concentration addition (CA) model. Pyrethroid combinations were assessed only under oxidised conditions. Since OPs and CBs act by a similar mechanism of inhibition of AChE, a dose additive effect was expected, but not conclusively found. TRZ with either CBF or CRB exhibited synergism under oxidised and un-oxidised conditions but the degree of synergism was stronger under un-oxidised conditions. Additivity was exhibited by CBF + MCP, CRB + MCP, CRB + MLT and CBF + MCP under un-oxidised conditions and CRB + MCP and CRB + CPF under oxidised conditions. Pyrethorids in combination with OPs (TRZ, MLT and CPF) were highly synergistic. In the present study, we used pure housefly head AChE without any interference of monooxygenase and/or esterase enzyme activities. Therefore these other enzymes were not producing the observed deviations from concentration-addition in the binary combinations between OPs, CBs and SPs. The mechanisms of OP, CB and SP interactions in pesticide mixtures requires further investigation.

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1. Introduction

In India, the total consumption of pesticides during 2009–10 was 52,408 metric tons (MT). This included usage of 46,139 MT of indigenous pesticides and 6269 MT of imported pesticides (Zonal Conference, 2013). Based on 2009–10 survey, among these pesticides, usage of organophosphorus pesticides (OPs) in India varied from 1164 to 1815 MT. Cypermethrin usage was the highest (2473 MT) whereas deltamethrin usage was the lowest (94 MT). In 2011–2012, pesticide usage in India increased to 52,979 MT. Among carbamates (CBs), carbofuran usage is higher than carbaryl, but both these insecticides are being imported.

Recently, OP-pyrethroid combinations such as cypermethrin-ethion, deltamethrin-triazophos, and deltamethrin-chlorpyrifos have been introduced to overcome emerging pyrethroid resistance in agricultural pests (Martin et al., 2003). Fixed dose combinations such as 50% of chlorpyrifos and 5% cypermethrin manufactured in China, 50% profenofos and 4% cypermethrin, 1% delatamethrin in combination with 35% EC triazophos and several other illegall pesticide mixtures are sold in India (Tripathi et al., 2006). Pyrethroids and OPs are extensively used for vector-borne disease control and are particularly important to check the outbreak of malaria. Pesticides can adversely affect vegetables, fruits, animal resources and human health (Amaraneni and Pillala 2001; Kumari et al., 2003; Mandal and Singh, 2010; Gowda and Somashekar 2012; Lari et al., 2014). Residues of OPs, CBs and synthetic pyrethroids were detected in measurable amounts during 1997–1998 market sampling survey of winter vegetables in Haryana, India (Kumari et al., 2003). According to this study, approximately 32% of the vegetable samples were contaminated with OPs and CBs above their respective maximum residue limit (MRL) values. An OP pesticide, chlorpyrifos (CPF) was detected in surface waters in Bhandara and Amravati regions of India at a concentrations up to 0.5 μg/L (Lari et al., 2014). Residues of CPF have been also found in fish in Kolleru Lake at 88,600 μg/kg (Amaraneni and Pillala, 2001).

Traditionally, the evaluation of the quality of the aquatic environment has been undertaken at the chemical level, identifying and quantifying chemical residues in the different compartments of the ecosystem. However, to make an accurate assessment of the environmental risk, the quantification of chemical residues should be complemented with the investigation of biomarkers that serve as early warning signals (Domingues et al., 2010). Acetylcholinesterase (AChE) is an enzyme that has been widely used as a sensitive biomarker of exposure to OP and CB pesticides (Kumar and Chapman 2001; Walker et al., 2001; Laetz et al., 2009, 2013; Arora and Kumar, 2015).

The OPs and CBs inhibits AChE which, in turn, hydrolyses acetylcholine. Acetylcholine is a neurotransmitter at cholinergic nerve synapses. These include the postganglionic parasympathetic and cholinergic sympathetic nerves, and both sympathetic and parasympathetic preganglionic fibres. Binding of OP with AChE leads to phosphorylation of the enzyme and this reaction is not easily reversible (Matsumura, 1985; Fukuto, 1990; Erdman, 2004). The rate of spontaneous reactivation of AChE is very slow with diethyl OPs while it is relatively fast with dimethyl OPs. Further, there is ageing of the phosphorylated enzyme after which the enzyme cannot be reactivated by oximes. The replacement of an oxygen atom in the organophosphorus structure by sulfur leads to the formation of organothiophosphorus compounds such as chlorpyrifos, malathion and parathion, which have a lower lethal potential. However, in vivo metabolisation to the oxon metabolite enhances their toxicity (Erdman, 2004). There is a clear measurable potential for mixtures of OPs and carbamates in agricultural waters to cause AChE inhibition in field-exposed fish (Gruber and Munn, 1998; Kumar and Chapman, 2001). Cypermethrin, a synthetic (Type II) pyrethroid acts on voltage-gated sodium and chloride channels in contrast to Type I pyrethroids that predominantly delay the closure of sodium channels. Pyrethroids are 2250 times more toxic to insects than humans (Bradberry et al., 2005). The mammalian resistance to pyrethroid toxicity is due to their capacity for ester hydrolysis leading to rapid detoxification. Carboxylesterase inhibitors such as OPs may enhance the toxicity of pyrethroid by inhibiting detoxification (Hernandez et al., 2013).

Although OPs share a common mechanism of action, assessing cumulative risk from exposures to OP mixtures remains a challenge (Mileson et al., 1998). In parallel to human health, OP mixture toxicity remains an important consideration for the conservation of non-target, non-mammalian species. Assessing the effects of mixtures of contaminants is an issue of high priority by many governmental agencies around the world (Eggen et al., 2004; Lydy et al., 2004; Woods et al., 2002; Schloz and Hopkins, 2006; Phyu et al., 2011; Laetz et al., 2013). As the potential for multiple chemical exposure increases, the question raised is whether the toxicity of mixtures of chemicals is simply additive or whether there is potentiation of toxicity. The general consensus has been that chemicals interact by concentration addition. However past studies have demonstrated that concentration addition of the components of a mixture does not always reflect the overall interaction of a mixture. The combinations of toxicants that have enhanced toxicity are of greatest concern because the toxicity predicted from the individual components would under estimate the overall toxicity. Consequently combined effects of mixtures have to be considered in ecological and human health risk assessment. In the case of pesticides, the difficulty is due in part to their large numbers that enter aquatic systems via various fate and transport pathways (Barbash, 2003; Schulz, 2004).

In this study, the −purified AChE enzyme from the housefly head was used as an in vitro model (i) to assess the toxicity of five organophosphorous pesticides: chlorpyrifos (CPF), profenofos (PRF), malathion (MLT), monocrotophos (MCP), triazophos (TRZ) in binary combinations with carbaryl (CRB) and carbofuran (CBF) under oxidised and un-oxidised conditions (ii) to assess the toxicity of five OPs and two CBs with the synthetic pyrethroids: cypermethrin (CYP) and deltamethrin (DLT) at fixed concentration in binary combinations under oxidised conditions.

2. Materials and methods

2.1. Chemicals and reagents

Acetylcholinesterase from the housefly (Musca domestica) was purchased from TARI (Taiwan Agricultural Research Institute). All solvents used for stock solutions were analytical grade. Acetylthiocholine iodide (ATCh), 5′,5′-Dithio-bis(2-Nitrobenzoic Acid) (DTNB), p-Nitrophenyl acetate, carbaryl (99.8% purity), chlorpyrifos (99.5% purity), profenofos (98.4% purity), malathion and carbofuran (99.0% purity), monocrotophos, cypermethrin and deltamethrin (99.8% purity) were purchased from Sigma-Aldrich Pvt. Ltd.

Pesticide stock solutions were prepared in acetone (4–5 g/L) and stored at −18 °C. Working stock solutions were prepared freshly in 50% methanol. The measured concentrations of pesticides ranged between 95 and 110% of their nominal values, The concentration range for different OPs used in the in vitro bioassays were as follows: CPF (0.003–3.2 μg/L); MCP (0.1–48000 μg/L); TAZ (1–5 μg/L); PRF (7–8100 μg/L) and MLT (0.2–15 μg/L).

2.2. Enzyme purity and specificity

The lyophilised protein sample was dissolved in 25 mM potassium phosphate (pH 7.2) buffer. The dissolved protein was

analysed by polyacrylamide gel electrophoresis (PAGE) under native conditions. Native PAGE (polyacrylamide gel electrophoresis) was performed as described (Schägger and Jagow, 1991) with the following modifications. Native PAGE was performed at 23 °C using 3–12% polyacrylamide gradient gel for 100 min at 150 V (Native PAGE™, ThermoFisher scientific, USA) as per manufacturer's instructions. The samples were prepared in a NativePAGE™ Sample Buffer and 18.9 μg of protein was loaded. After electrophoresis the gel was stained for the esterase activity as described (Ono et al., 1994). Briefly, the gel was placed into a solution containing a mixture of Fast Blue B dye (Sigma Aldrich, USA) and α-naphthyl acetate solution for 10 min and photographed.

The substrate specificity of the enzyme against choline esters and carboxyl esters was determined by following methods. The specific activity of the enzyme with ATCh was measured at pH 7.5 in 50 mM phosphate buffer by monitoring the increase in absorbance within the linear range at 412 nm. The increase in absorbance results from the generation of 2-nitro-5-thiobenzoate dianion (TNB^{2-}) from DTNB after its reaction with thiocholine. The concentration of TNB^{2-} was calculated by using its molar extinction coefficient, which is reported to be $14150\,M^{-1}cm^{-1}$ elsewhere (Riddles et al., 1979). The carboxylesterase activity of this enzyme against p-nitrophenyl acetate was measured in above mentioned buffer by monitoring the increase in absorbance due to production of p-nitrophenol. As the pH of the reaction mixture was not very favourable for the full deprotonation of p-nitrophenol, the increase in absorbance was monitored within the linear range at its isosbestic point which is reported to be 348 nm (Hotta et al., 2002).

2.3. In-vitro enzyme assay

The in vitro inhibition of AChE was evaluated using housefly-head purified AChE enzyme using a modified version of the method described by Ellman et al. (1961) and validated by Arora and Kumar, 2015. Briefly, test solutions (15 μL) at various concentrations of each pesticide and their binary mixtures were added to each microplate well followed by the addition of AChE enzyme solution. Preliminary experiments were conducted to establish the optimal incubation time for the enzyme reaction. Reaction for 10 min was observed to be optimal incubation time where linear reaction was observed for OPs and CBs, under the present study. The mixture was incubated at room temperature for ten minutes and then the residual AChE activity was determined with the microplate reader (Titerteks Multiskan MC Photometer) at 405 nm for 10 min following addition of mixture of DTNB and ATCh solutions (250 μL). The final concentrations of AChE, ATCh and DTNB were 0.005 μL/mL, 1.5 and 0.22 mM, respectively. The final well concentration of methanol was <0.01%. The kinetics of the 10 min measuring phase was determined (ΔA/T). The results were corrected for the solvents and the reaction mixture with DTNB and ATCh.

All assays were conducted with three replicates and the data from three independent trials were pooled (nine wells were averaged for each pesticide; n = 9) to calculate the median AChE inhibitory concentration (EC_{50}) based on nominal aqueous concentrations. A spreadsheet developed by Barnes et al. (2003) and validated for AChE investigations (Kumar et al., 2010) was used for estimating the concentrations that caused a 20 and 50% effect (EC_{20}, and EC_{50}, respectively) and their 95% confidence intervals. This spread sheet is based on the Logit model.

2.4. Enzyme analyses before and after oxidation

It has been shown that OPs containing a thione group, such as malathion, chlorpyrifos, triazophos, are converted into oxon derivatives by oxidation with diluted bromine water (0.4%). These oxon derivatives are more potent ChE inhibitors. Diluted bromine water (5%) was used for oxidation of OP pesticides as described earlier by Arora and Kumar (2015). The final well concentration for bromine water was 3.33×10^{-6}%. The enzyme inhibition for individual and binary mixtures of the pesticides was assessed before and after oxidation with bromine water. Negative control/blank reactions were also run with AChE and only Br water. No effect was observed in the absence of pesticide in the reaction mixture. We did not measure any other metabolite produced after the reaction with bromine water.

2.5. Binary combinations of pesticide mixtures- experimental design

The concentrations used in the mixture toxicity tests were based on EC_{50} values derived from tests conducted with individual pesticides. The pesticides were added in equitoxic concentrations (identical fractions of their individual EC_{50} values for each pesticide). Six concentrations below the EC_{50} value (1/64 EC_{50}, 1/32 EC_{50}, 1/16 EC_{50}, 1/8 EC_{50}, $^{1}/_{4}$ EC_{50} and $^{1}/_{2}$ EC_{50}), one at the EC_{50} value and three concentrations above the EC_{50} value (2, 4 and 8 x EC_{50}) were established using serial dilutions. Binary mixtures of the pesticides were used for toxicity tests. The nominal amounts of pesticides were pipetted into the 96-well plate. All mixture assays were conducted in three independent triplicate trials.

For binary combinations, EC values were expressed on the basis of individual components in the mixture. For example, in binary combinations CBF + CPF, CBF-Mix represents toxicity of CBF to inhibit AChE as (EC_{10}, EC_{20} and EC_{50}) in the presence of CPF. Similarly, CPF-Mix represents toxicity of CPF to inhibit AChE in the presence of CBF.

For binary combinations of SPs with OPs and CBs, the pyrethroids concentration was kept constant at 0.1 and 10 μg/L and dose response curves were evaluated for EC50 and EC20 values.

2.6. Approaches used for assessing mixture toxicity

2.6.1. Toxic unit approach

The toxic unit (TU) model has been used extensively to determine the overall toxicity of a mixture (Spehar and Fiandt 1986; Forget et al., 1999; Woods et al., 2002). The TU is the sum of the toxic contributions of each component in the mixture. The TU for a binary mixture is given by the following equation:

$$TU = \frac{ECx\ A\ (mix)}{ECx\ A\ (alone)} + \frac{ECx\ B\ (mix)}{ECx\ B\ (alone)}$$

where A and B are chemicals, ECx (mix) is the effect of each component in the binary mix and ECx (alone) is the EC_{50} or EC_{20} of A and B applied as single components. By this model, if TU equals one, the toxicity of the mixture is additive. If TU is greater than one, the toxicity is less than additive (antagonistic) and if the TU is less than one, the toxicity of the mixture is more than additive (synergistic) (Spehar and Fiandt, 1986).

For pyrethorid combinations, the mixture toxicity was evaluated by using similar TU approach where EC_x (mix) and EC_x alone for OPs and CBs were compared at a given pyrethroids concentration.

2.6.2. Concentration addition model

The two most widely used conceptual models for predicting mixture toxicity are concentration addition (CA), introduced by Loewe and Muischnek (1926), and independent action (IA), first described by Bliss (1939).

For this study, we applied the CA equation (equation 1) as implemented by Altenburger et al. (2004) to examine whether the concentration-response relationships of binary combinations

could be predicted based on the range of effects observed in response to single compounds.

$$ECx_{mix} = (\Sigma i = 1 \text{ to } n(p_i/ECx_i))^{-1} \qquad (1)$$

ECx_{mix} is the concentration of the mixture resulting in an effect of $x\%$ relative to control, ECx_i is the concentration of component i resulting in an effect of $x\%$ when applied in isolation, and p_i is the proportion of the total concentration of the mixture (in molar terms) represented by component i. Whether the toxicity of each mixture conformed to CA was determined using a two-step process. First, if the observed mixture toxicity value fell within the 95% confidence intervals of the expected value of a model then the mixture was considered to have conformed to the model. If, however, an observed mixture toxicity value was outside the 95% confidence intervals of the expected value then the mixture potentially may not conform to that model.

To enable a quantitative estimation of the difference in predicted and measured toxicities, the model deviation ratio (MDR) method (Belden et al., 2007) was used. For the CA model, the MDR was derived by dividing the predicted toxicity value (EC_{20}) by the observed toxicity value. The MDR values greater than 1.3 mean that the toxicity of the mixture conforms with synergism while values less than 0.7 are taken to conform to antagonism (Belden et al., 2007) The MDR approach has been successfully applied by other researchers (Phyu et al., 2011; Arora and Kumar, 2015).

3. Results

3.1. Enzyme purity and specificity

Presence of a single band with esterase activity ascertained presence of a single esterase in the enzyme preparation (Fig. 1). The specific activity for this enzyme against ATCh and p-nitrophenyl acetate was measured to be 13535 ± 322 and 1.84 ± 0.05 nmoles $min^{-1} mg^{-1}$ respectively. This several log folds difference in the
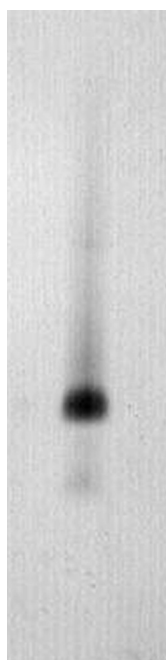

Fig. 1. NATIVE-PAGE stained with α-naphthyl acetate and Fast Blue B dye to show presence of esterases in the enzyme preparation.

specific activities for two different types of esters suggests that the enzyme is AChE.

3.2. AChE inhibition of single pesticides under oxidised and un-oxidised conditions

Among the two CBs selected in this study, CBF was 12 times more toxic than CRB (Table 2). In addition, individual CRB and CBF EC_{50} values did not vary significantly under un-oxidised and oxidised conditions (Tables 1 and 2). With oxidation, the sensitivity of HF-AChE to CPF, TRF and MLT increased significantly as reported earlier (Arora and Kumar, 2015) (Table 2). In contrast, the two OPs (MCP and PRF), did not exhibit any significant differences in toxicity under oxidised and un-oxidised conditions (Tables 1 and 2).

3.3. Binary mixture combinations under un-oxidised and oxidised conditions

Eight possible binary combinations of four selected OPs and 2 CBs (CBF + CPF, CBF + MCP, CBF + PRF, CBF + TRZ, CRB + CPF, CRB + MCP, CRB + PRF, CRB + TRZ) were assessed for their toxicity under oxidised and un-oxidised conditions using HF-AChE during *in vitro* exposures (Tables 1 and 2). Under un-oxidised conditions, CBF + CPF was the most toxic combination with 58.5 µg/L EC_{50} for CPF-mix in comparison to CPF-mix EC_{50} value of 80 µg/L for CRB + CPF (Table 1). The CRB and CBF in combination with CPF exhibited lower EC_{50} values in comparison to the individual CPF EC_{50} value of 113 µg/L (Table 1). Under un-oxidised conditions, the EC_{50} value of TRZ in binary combination with CBF and CRB was reduced by 12 folds and 45 folds, respectively (Table 1). In contrast, TRZ toxicity under oxidised conditions was significantly reduced (from 3.7–4.5 folds) in binary combination with CBF and CRB (Table 2).

3.4. Mixture toxicity assessment

The toxicological interactions of ten OP and CB binary combinations were evaluated using the toxic unit approach (TU, Table 3) and the concentration addition model (CA, Table 4). In the present study, we used pure AChE enzyme resulting in the absence of Phase I biotransformation enzymes in the *in vitro* bioassays. We used bromine water to create oxidised conditions, which mimics the action of these enzymes.

Based on TU and CA models, TRZ with CBF and CRB exhibited synergism under oxidised and un-oxidised conditions but the degree of synergism was stronger under un-oxidised conditions (Tables 3 and 4). The CRB + PRF combination exhibited antagonism under oxidised and un-oxidised conditions (Tables 3 and 4). In contrast, the CA model demonstrated synergism for CRB + MCP and CBF + MCP under un-oxidised conditions and nearly additive under oxidised conditions (Table 4).

Under un-oxidised conditions, the TU approach demonstrated CBF + PRF combination to be antagonistic whereas synergism was established using CA model (Table 3). In contrast, both CA and TU models exhibited synergism for CBF + PRF under oxidised conditions. The TU model confirmed additive responses for CBF + CPF, CBF + MCP, CRB + MCP and CRB + MLT under un-oxidised conditions (Table 4) and CRB + MCP and CRB + CPF under oxidised conditions (Table 3).

In the current study, we also evaluated HF-AChE inhibition for four OPs (MLT, MCP, CPF and TRZ) and one CB (CRB) in binary combination with two SPs (DLT and CYP). In the presence of DLT and CYP at fixed concentration of 0.1 and 10 µg/L, the MLT toxicity was increased 72–77 fold and TRZ toxicity was increased from 6 to 12 fold while CPF and CRB was increased up to two fold only (Table 5). The TU model confirmed strong synergism for CPF, TRZ and MLT in the presence of DLT or CYP (Table 6).

Table 1

EC$_{10}$, EC$_{20}$ and EC$_{50}$ values based on housefly (*Musca domestica*) head acetylcholinesterase (HF-AChE) inhibition during *in vitro* exposures to individual organophosphorus and carbamate pesticides and their mixtures under un-oxidised (un-brominated) conditions. 95% confidence intervals are provided in parenthesis.

Un-oxidised (un-brominated) singles				
	EC$_{10}$ (µg/L)	EC$_{20}$ (µg/L)	EC$_{50}$ (µg/L)	Slope
CPF[a]	32.6 (25–42)	51.7 (42.8–62)	113 (103–124)	−4.06
TRZ[a]	19904 (7093–55847)	43851 (21617–88949)	1,69,199 (128289–223152)	−2.36
PRF[a]	175 (152–201)	320 (288–356)	900 (849–954)	−3.09
MCP[a]	1898 (1668–2159)	3816 (3463–4205)	12591 (11904–13317)	−2.67
MLT[a]	2691 (2219–3263)	5821 (5025–6742)	21764 (20091–23575)	−2.42
CRB	31 (25.8–37.5)	62.2 (54–71.5)	202.7 (188–218)	−2.70
CBF	2.8 (2.46–3.4)	5.4 (4.7–6)	15.6 (14.6–16.7)	−2.99

Un-oxidised (un-brominated) Mixtures				
		EC$_{10}$ (µg/L)	EC$_{20}$ (µg/L)	EC$_{50}$ (µg/L)
CBF + CPF	CBF-Mix	1.5 (1.3–1.8)	2.9 (2.5–3.3)	8.5 (7.9–9)
	CPF-Mix	10.6 (8.9–12.6)	20 (17.5–22.7)	58.5 (54.5–62.8)
CBF + MCP	CBF-Mix	1(0.67–1.5)	1.9(1.4–2.59)	5.5(4.7–6.59)
	MCP-Mix	1071.9 (841–1365)	2198(1835–2634)	7509(6822–8265)
CBF + MLT	CBF-Mix	1.1(0.77–1.68)	2.1(1.6–2.9)	6.5(5.59–7.68)
	MLT-Mix	574(432–764)	1222(987–1513)	4440(3955–4984)
CBF + PRF	CBF-Mix	0.9(0.59–1.36)	1.7(1.2–2.39)	5.4(4.5–6.4)
	PRF-Mix	46(36–58)	94.6(79–113)	324(292–359)
CBF + TRZ	CBF-Mix	0.15(0.036–0.6)	0.38(0.12–1.1)	1.8(1–3.2)
	TRZ-Mix	733(122–4400)	2187(543–8803)	14164(6707–29909)
CRB + CPF	CPF-Mix	17.9(15–21)	31(27.7–35)	79.7(74.8–85)
	CRB-Mix	28(24–32.9)	48.8(43.49–54.8)	124.9(117–133)
CRB + MLT	CRB-Mix	10(8.48–12)	23.6(20.7–26.9)	101(94–108)
	MLT-Mix	1427(1217–1673)	3315(2942–3735)	14003(13107–14960)
CRB + PRF	PRF-Mix	112(88.9–142)	211(177–251)	621(566–682)
	CRB-Mix	19.7(15.5–24.9)	36.9(31–44)	108(98.5–119)
CRB + TRZ	CRB-Mix	0.03(0.001–0.9)	0.19(0.01–3)	4.2(0.6–26)
	TRZ-Mix	22(2–255)	148(21–1036)	3686(1084–12532)
CRB + MCP	CRB-Mix	16.8(13.7–20.5)	30.9(26.5–36)	87.7(80.8–95)
	MCP-Mix	5975(5501–6489)	1145(935–1401)	2107(1810–2452)

CBF-carbofuran; CPF-chlorpyrifos; CRB-carbaryl; MLT-malathion; MCP-monocrotophos; PRF-profenofos; TRZ-triazophos.

[a] EC values for individual OPs are based on Arora and Kumar (2015).

4. Discussion

The primary mode of toxicity for OPs and CBs is inhibition of cholinesterase activity. Inhibition of AChE activity results in the build up of acetylcholine (ACh) at cholinergic nerve endings causing continual nerve stimulation (Scholz and Hopkins, 2006). CPF, MLT and TRZ represent examples of the phosphorothioate class of OP insecticides. Oxidative desulfurization by Phase I biotransformation enzymes replaces the sulfur atom with an oxygen atom, resulting in an O-analog metabolite that is a more effective AChE inhibitor than the parent OP (Fukuto, 1990). Generally, the oxon analogues are more potent AChE inhibitors than the parent compound (Kralj et al., 2006). In the present study, carbamate toxicity was similar under oxidised and un-oxidised conditions which is in confirmation with the fact that these pesticides do not require biotransformation to an oxon form in order to inhibit AChE enzymes.

In the present study, HF-AChE inhibition exposed to five OPs in binary mixtures with CBs was investigated under *in vitro* conditions. To examine the effects of oxidation on OP potency in the HF-AChE system, bromine water was used as an oxidising agent. HF-AChE inhibition for CRB and CBF was similar under oxidised and un-oxidised conditions. Individual OPs and CBs under un-oxidised conditions exhibited sensitiviy to AChE inhibition in the following order:

CBF > CPF > CRB > PRF > MCP > MLT > TRZ

Under oxidised conditions, CPF was observed to be the most potent pesticide and HF-AChE inhbition was in the following order:

CPF > TRZ > CBF > MLT > CRB > PRF > MCP

The CA model has recently been suggested as a conservative default assessment tool for aquatic mixtures (not just pesticides), irrespective of modes or mechanisms of action (Kortenkamp et al., 2009). Based on the fact that OPs and CBs act by a similar mechanism of inhibition of AChE, one would expect that the behaviour of a mixture would be a simple dose-additive effect (Deneer, 2000; Kudsk et al., 2005; Belden et al., 2007). In the present study, OP+ CB binary combinations were mainly synergistic under oxidised conditions. Although OPs and CBs have the same mode of action, the principal difference between OP-and carbamate-induced inhibitory action is in the stability of the AChE-OP/carbamate complex. Carbamates are considered reversible AChE inhibitors and OPs as irreversible AChE inhibitors. OPs are able to phosphorylate serine residues of AChE in non-reversible way, whereas the carbamylated serine residue is less stable and the carbamyl moiety can be split from the enzyme by spontaneous hydrolysis (Kuhr and Dorough, 1976; Darvesh et al., 2008).

We have earlier reported antagonistic effects of the binary combination of OPs (CPF + PRF, CPF + MLT, MCP + MLT, PRF + MLT, MLT + TRZ and PRF + TRZ) under oxidised conditions (Arora and Kumar, 2015). This was explained on the basis of dispositional and/or receptor antagonism. We also reported synergistic responses for the majority of the OP binary combinations assayed under un-oxidised conditions (Arora and Kumar, 2015). Triazophos showed very strong synergism in binary combinations with CPF, MCP and PRF under un-oxidised conditions. In the present study, TRZ showed synergistic effects with CBF and CRB. Laetz et al. (2009) observed both concentration additivity and synergism in the binary combinations of carbamates and organophosphates tested, with a greater degree of synergism at higher exposure concentrations. However, the comparison between *in vivo* and *in vitro*

Table 2
EC$_{10}$, EC$_{20}$ and EC$_{50}$ values based on housefly (*Musca domestica*) head acetylcholinesterase (HF-AChE) inhibition during *in vitro* exposures to individual organophosphorus and carbamate pesticides and their binary mixtures under oxidised (brominated) conditions. 95% confidence intervals are provided in parenthesis.

Oxidised (brominated) Singles		EC$_{10}$ (µg/L)	EC$_{20}$ (µg/L)	EC$_{50}$ (µg/L)	Slope
CPF[a]		0.05 (0.04–0.06)	0.09 (0.08–0.1)	0.22 (0.21–0.24)	−3.53
TRZ[a]		0.11 (0.098–0.139)	0.2 (0.18–0.24)	0.59 (0.55–0.65)	−3.10
PRF[a]		256 (225–293)	429 (389–472)	1030 (975–1089)	−3.64
MLT[a]		18 (15–21.9)	35.8 (31–41)	115 (107–124)	−2.74
MCP[a]		2492 (2217–2800)	4515 (4147–4915)	12470 (11895–13071)	−3.14
CRB		49.7 (42.7–57.9)	93 (83.5–104.5)	274 (258–291)	−2.96
CBF		1.4 (0.9–2.0)	3.2 (2.4–4.2)	13.2 (11.5–15.3)	−2.25

Oxidised (brominated) Mixtures		EC$_{10}$ (µg/L)	EC$_{20}$ (µg/L)	EC$_{50}$ (µg/L)
CBF + CPF	CBF-Mix	0.7 (0.4–1.4)	1.6 (0.9–2.7)	6.15 (4.5–8.4)
	CPF-Mix	0.008(0.004–0.017)	0.019(0.01–0.03)	0.07(0.05–1.0)
CBF + MCP	CBF-Mix	0.68 (04–1.1)	1.3 (0.9–1.9)	4.1(3.2–5.2)
	MCP-Mix	401.7 (298–541)	827.9 (660.9–1037)	2850 (2474–3282)
CBF + MLT	CBF-Mix	0.46(0.19–1)	0.95(0.5–1.8)	3.3(2.3–4.8)
	MLT-Mix	1.58(0.96–2.6)	3.9(2.6–5.8)	18.7(14.9–23.4)
CBF + PRF	CBF-Mix	0.3(0.14–0.8)	0.8(0.4–1.59)	3.4(2.3–5)
	PRF-Mix	16(10–24.5)	40(28.9–56)	194.7(159–237)
CBF + TRZ	CBF-Mix	0.6(0.37–0.9)	1.18(0.8–1.7)	3.7(3–4.5)
	TRZ-Mix	0.02(0.01–0.03)	0.04(0.03–0.06)	0.13(0.1–0.16)
CRB + CPF	CPF-Mix	0.038(0.029–0.051)	0.06(0.05–0.07)	0.14(0.12–0.16)
	CRB-Mix	25.4(18–35)	41(32–52)	93.7(81.5–107.8)
CRB + MLT	CRB-Mix	17.6(13–23.9)	30(24--37.5)	74.7(65.5–85)
	MLT-Mix	8(5.9–10.9)	13.7(10.9–17)	34(29.9–38.9)
CRB + PRF	PRF-Mix	150.8(105–216)	251(191.7–329)	601(515–701)
	CRB-Mix	39(27–56)	65(49.7–85)	155.8(133.6–181.7)
CRB + TRZ	CRB-Mix	22.5(15.9–31.7)	40(31–51.8)	108(93.9–124.6)
	TRZ-Mix	0.03(0.02–0.04)	0.06(0.04–0.07)	0.16(0.14–0.18)
CRB + MCP	CRB-Mix	23.95 (16.63–34.49)	46.41 (35.47–60.71)	143.81 (124.28–166.41)
	MCP-Mix	418(241–724)	992(652–1510)	4352 (3336–5680)

CBF-carbofuran; CPF-chlorpyrifos; CRB-carbaryl; MLT-malathion; MCP-monocrotophos; PRF-profenofos; TRZ-triazophos.

[a] EC values for individual OPs are based on Arora and Kumar (2015).

results should be used with caution and care should be taken when extrapolating among taxonomic groups.

Kok and Hasirci (2004) carried out *in vitro* studies using a binary mixture of enzymes, namely AChE and choline oxidase, in a biosensor. They evaluated the effect of three CBs, namely aldicarb, carbaryl, and carbofuran and their combinations in binary mixtures on AChE activity. They found that the total inhibition was not simply additive, but was lower than the sum of individual inhibition values. The degree of difference between the predicted and actual inhibition values varied depending on the combinations used and their concentrations in the assay. It was concluded that there was competition between the resulting in a lower combined

inhibition than expected. Where a pesticide with a lower affinity was present in high concentrations, it prevented other pesticides from interacting with the enzyme, thus lowering the overall inhibition.

Interactions between chemicals in a mixture can be concentration dependent, or concentration ratio dependent (Crofton et al., 2005; Laetz et al., 2009). In the present study, the inhibition of AChE with combined pesticide OP and CB exposure during *in vitro* assays was induced by the OP components of the pesticide mixtures. Pesticides also act on biochemical targets other than ChEs. The calcium-dependent A-esterases can catalytically inactivate a number of OPs without being inhibited. Butrylcholinestearse

Table 3
Toxic units (TU) derived from EC$_{20}$ and EC$_{50}$ values of acetylcholinesterase inhibition by 20%, and 50%, respectively for individual and binary combinations of un-oxidised and oxidised organophosphorus and carbamate pesticides (TU <1, = 1 and >1 indicates synergism, additive effect and antagonism, respectively).

	Un-oxidised (un-brominated)			Oxidised (brominated)		
Mixtures	TU-EC$_{20}$	TU-EC$_{50}$	Type of joint action	TU-EC$_{20}$	TU-EC$_{50}$	Type of joint action
CBF + CPF	0.92	1.06	nearly additive	2.4	0.7	antagonism at lower levels and then synergistic at higher
CRB + CPF	1.38	1.32	antagonism	1.11	0.99	nearly additive
CBF + MCP	0.93	0.95	nearly additive	0.43	0.49	synergism
CRB + MCP	1.05	0.91	nearly additive	0.72	0.87	synergism at lower levels and then nearly additive at higher
CBF + MLT	0.60	0.62	synergism	0.29	0.37	strong synergism
CRB + MLT	0.95	1.14	nearly additive	0.71	0.57	synergism
CBF + PRF	0.61	0.71	synergism	0.24	0.41	strong synergism
CRB + PRF	1.25	1.22	antagonism	1.28	1.15	antagonism
CBF + TRZ	0.17	0.20	strong synergism	0.42	0.45	synergism
CRB + TRZ	0.01	0.04	strong synergism	0.73	0.66	synergism

CBF-carbofuran; CPF-chlorpyrifos; CRB-carbaryl; MLT-malathion; MCP-monocrotophos; PRF-profenofos; TRZ-triazophos.

Table 4
Summary of mixture toxicity assessment using the concentration addition model for ten binary organophosphorus and carbamate pesticide mixtures under un-oxidised and oxidised conditions.

Pesticide combination	Un-oxidised (un-brominated) binary combinations				Oxidised (brominated) binary combinations			
	Predicted EC$_{20}$ (95%CI) μg/L	Observed EC$_{20}$ (μg/L)	MDR	type of joint action	Predicted EC$_{20}$ (95%CI) μg/L	Observed EC$_{20}$ (μg/L)	MDR	Type of joint action
CBF + CPF	24.7 (21.1–28.4)	22.9	1.08	additive	2.3 (1.8–2.8)	1.6	1.39	synergism
CRB + CPF	57.6 (47–67)	79	0.73	antagonism	36.2 (32.3–40.4)	41.1	0.88	antagonism
CBF + MCP	2576 (2306–2846)	2199	1.17	synergism	1545 (1236–1895)	829	1.86	synergism
CRB + MCP	2036 (1809–2289)	1176	1.73	synergism	2237 (2031–2475)	1038	2.15	synergism
CBF + MLT	2358 (2046–2665)	1224	1.93	synergism	16.1 (12.7–19.9)	4.85	3.32	synergism
CRB + MLT	3475 (3007–4012)	3339	1.04	additive	61.9 (54.5–70.3)	43.7	1.42	synergism
CBF + PRF	166 (147–184)	248	0.67	antagonism	137 (109–170)	40.8	3.37	synergism
CRB + PRF	198 (175–224)	248	0.80	antagonism	246 (222–274)	316	0.78	antagonism
CBF + TRZ	25078 (15181–37584)	2187	11.5	synergism	2.1 (1.6–2.6)	1.22	1.68	synergism
CRB + TRZ	23785 (14620–35728)	148	160	synergism	55 (49–63)	40.1	1.36	synergism

CBF-carbofuran; CRB-carbaryl; CPF-chlorpyrifos; MLT-malathion; MCP-monocrotophos; PRF-profenofos; TRZ-triazophos.
The model divergence ratio (MDR) is the ratio of the observed and predicted toxicity at EC$_{20}$. An MDR value of 1 indicates adherence to the specified model. MDR values >1 indicates greater than expected toxicity and hence synergism among mixture components. MDR values <1 indicates less than expected toxicity and hence antagonism among mixture components. The classification of type of joint action is based on the observed toxicity being outside the 95% confidence limits of the predicted value.

(BChE) can also bind the oxon metabolites of OP insecticides (Richardson et al., 2001) and the carboxylesterases (CES) participate in stoichiometric binding of OP molecules (Sogorb and Vilanova, 2002). The CES family of enzymes is a key participant in the Phase I drug metabolism process, catalysing the hydrolysis of a wide range of ester- and amide-containing compounds.

Pyrethroids do not have an inhibitory effect on AChE. In the present study, DLT and CYP in combination with OPs (TRZ, MLT and CPF) were highly synergistic. According to Khan et al. (2013), OPs could inhibit the detoxification of SPs and increase the toxicity in binary combinations. It was concluded that the toxicities of bifenthrin, cypermethrin and deltamethrin in the resistant population of house flies could be enhanced by the combination with chlorpyrifos, profenofos, emamectin and fipronil. Earlier

investigations have proposed that OPs, when used in combination with SPs, inhibit the enzymes (monooxygenases and/or esterases) responsible for metabolic detoxification in different insect pests (Martin et al., 2003; Bryne and Devonshire, 1991). In the present study under in vitro conditions, we have used pure housefly-head AChE with no A-esterase, CES and BChE enzyme activity. Therefore these other enzymes are not producing the observed deviations from concentration-addition in the binary combinations between OPs, CBs and SPs. However, A-esterases, CES and BChE were not tested in the present study. This should be further investigated in the future studies. In the present study, the EC$_{20}$ and EC$_{50}$ values of AChE inhibition for CPF, TRZ, MCP and MLT in combination with DLT or CYP did not increase with the increase in PYR concentrations (0.1 and 10 μg/L). We hypothesise that the binary mixture results of OPs and SPs were influenced mainly by the OP component of the mixture.

Although attempts to account for mixtures have been made, these have generally assumed that the toxicity of the mixture conforms to the CA mode of joint action (NEPCC, 1999; ANZECC and ARMCANZ, 2000). A number of meta-analyses of mixture toxicity data have reported that while many mixtures conform to CA, between 5% and 30% of mixtures conform to synergism and therefore assuming CA would underestimate their toxicity (Warne and Hawker, 1995; Deneer, 2000; Belden et al., 2007). Few models predicting toxic effects of mixtures have effectively dealt with the influence of magnitudes of exposure and dependence on the ratios of the component chemicals. Future work determining the effects of these pesticide combinations to non-target organisms at environmentally relevant concentration is needed.

5. Conclusions

We have shown that the joint inhibitory effects of mixtures of OP and CB insecticides on housefly-head AChE activity are not always dose-additive in-vitro. The concentration addition approach could severely underestimate combined toxicity of OPs where their oxon analog (metabolite) is more toxic than their parent compound. The present study has highlighted the differential toxicity of binary combinations of OP and CB pesticides under oxidised and un-oxidised conditions. Pyrethroids in combination with OPs and CBs were highly synergistic. The results from this in vitro study demonstrate environmental concern on the

Table 5
EC$_{50}$ and EC$_{20}$ values based on housefly (Musca domestica) head acetylcholinesterase (HF-AChE) inhibition during in vitro exposures to binary mixtures of organophosphorus and carbamate pesticides with pyrethroids at fixed concentrations under oxidised (brominated) conditions. 95% confidence intervals are provided in parenthesis.

Combination of pesticides	EC$_{20}$ (μg/L)	EC$_{50}$ (μg/L)
CPF + CYP-0.1	0.019 (0.011–0.029)	0.09 (0.07–0.11)
CPF + CYP-10	0.018 (0.010–0.029)	0.08 (0.06–0.10)
CPF + DLT-0.1	0.020 (0.016–0.024)	0.06 (0.06–0.07)
CPF + DLT-10	0.033 (0.014–0.078)	0.16 (0.09–0.27)
TRZ + CYP-0.1	0.008 (0.005–0.11)	0.05 (0.01–0.26)
TRZ + CYP-10	0.007 (0.004–0.11)	0.07 (0.01–0.43)
TRZ + DLT-0.1	0.025 (0.019–0.03)	0.06 (0.055–0.074)
TRZ + DLT-10	0.009 (0.0001–0.717)	0.104 (0.006–1.779)
CRB + CYP-0.1	31 (21–46)	123 (98–155)
CRB + CYP-10	24 (14–43)	109 (80–149)
CRB + DLT-0.1	29 (21–40)	125 (105–150)
CRB + DLT-10	28 (22–37)	145 (112–162)
MCP + CYP-0.1	2789 (1775–4383)	8287 (6586–10428)
MCP + CYP-10	2789.7 (1775–4383)	8288 (6586–10428)
MCP + DLT-0.1	2439 (1899–3132)	7196 (6252–8282)
MCP + DLT- 10	2554 (2032–3208)	7420.1 (6538–8421)
MLT + CYP-0.1	1.2 (0.9–1.58)	1.50(1.37–1.76)
MLT + CYP-10	0.99 (0.34–2.91)	1.57 (1.14–2.17)
MLT + DLT-0.1	1 (0.80–1.36)	1.6 (1.4–1.8)
MLT + DLT-10	1.05 (0.81–1.36)	1.62 (1.42–1.86)

CPF-chlorpyrifos; CRB-carbaryl; CYP-cypermetrhrin; DLT-deltamethrin; MLT-malathion; MCP-monocrotophos; TRZ-triazophos.

Table 6
Toxic units (TU) derived from EC$_{20}$ and EC$_{50}$ values of acetylcholinesterase inhibition by 20 and 50%, respectively for individual and binary combinations of organophosphorus and carbamate pesticides with pyrethroids at fixed concentrations under oxidised (brominated) conditions. 95% confidence intervals are provided in parenthesis. TU <1, = 1 and >1 indicates synergism, additive effect and antagonism, respectively.

Combination of pesticides	TU$_{20}$	TU$_{50}$	Type of joint action
CPF + CYP-0.1	0.26 (0.12–0.32)	0.40 (0.30–0.50)	strong synergism
CPF + CYP-10	0.20 (0.11–0.32)	0.36 (0.27–0.45)	strong synergism
CPF + DLT-0.1	0.22 (0.18–0.27)	0.27 (0.25–0.31)	strong synergism
CPF + DLT-10	0.37 (0.16–0.87)	0.74 (0.44–1.23)	nearly synergism
TRZ + CYP-0.1	0.04 (0.025-–.5)	0.08 (0.02–0.43)	strong synergism
TRZ + CYP-10	0.03(0.02–0.55)	0.11 (0.01–72)	strong synergism
TRZ + DLT-0.1	0.125 (0.095–0.15)	0.10 (0.09–0.12)	strong synergism
TRZ + DLT-10	0.048 (0.0005–3.585)	0.17 (0.01–2.97)	strong synergism
CRB + CYP-0.1	0.33 (0.23–0.49)	0.45 (0.36–0.57)	synergism
CRB + CYP-10	0.26 (0.15–0.46)	0.40 (0.29–0.54)	synergism
CRB + DLT-0.1	0.31 (0.22–0.43)	0.46 (0.38–0.55)	synergism
CRB + DLT-10	0.3 (0.24–0.40)	0.53 (0.41–0.59)	synergism
MCP + CYP-0.1	0.70 (0.44–1.098)	0.74 (0.58–0.93)	nearly synergism
MCP + CYP-10	0.70 (0.44–1.098)	0.74 (0.58–0.93)	nearly synergism
MCP + DLT-0.1	0.61 (0.48–0.78)	0.64 (0.56–0.74)	nearly synergism
MCP + DLT- 10	0.64 (0.51–0.81)	0.66 (0.58–0.75)	nearly synergism
MLT + CYP-0.1	0.034 (0.025–0.044)	0.013 (0.012–0.015)	strong synergism
MLT + CYP-10	0.028 (0.009–0.081)	0.014 (0.010–0.019)	strong synergism
MLT + DLT-0.1	0.028 (0.022–0.038)	0.014 (0.012–0.016)	strong synergism
MLT + DLT-10	0.028 (0.023–0.038)	0.014 (0.012–0.016)	strong synergism

CPF-chlorpyrifos; CRB-carbaryl; CYP-cypermetrhirn; DLT-deltamethrin; MLT-malathion; MCP-monocrotophos; TRZ-triazophos.

use of TRZ and MLT in binary combination with CBs and SPs. Increased potency of these OP, CB and SP in binary combinations could result in deleterious effects on non-target organisms. It is, however, difficult to extrapolate the *in vitro* effects of the single pesticides and pesticide mixtures studies to *in vivo* exposures. Further research examining the effect that low levels of these pesticides during *in vivo* exposures needs to be performed in order for environmental managers to make informed decisions.

Acknowledgements

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