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The response of *Lemna minor* to mixtures of pesticides that are commonly used in Thailand

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Abstract

In the field aquatic organisms are exposed to multiple contaminants rather than to single compounds. It is therefore important to understand the toxic interactions of co-occurring substances in the environment. The aim of the study was to assess for effects of individual herbicides (atrazine, 2,4-D, alachlor and paraquat), that are commonly used in Thailand, and their mixtures on *Lemna minor*. Plants were exposed to individual and binary mixtures for 7 days and effects on plant growth rate were established based on frond area measurements. Experimental observations of mixture toxicity were compared with predictions, based on the single herbicide exposure data using concentration addition and independent action models. The single compound studies showed that paraquat and alachlor were the most toxic to *L. minor* followed by atrazine and then 2,4-D. For the mixtures, atrazine with 2,4-D appeared to act antagonistically whereas alachlor and paraquat showed synergism.

Keywords: Herbicide mixtures, Lemna minor, synegism, antagonism

The U. S. Environmental Protection Agency (EPA) recently estimated that more than 540 million kilograms of pesticides are applied to crops around the world and the most frequently used pesticide class is herbicides (Ecobichon, 2001). The use of herbicides has been continuously increasing year on year. In addition, several reports have highlighted problems associated with pesticide overuse and misuse mainly due to a lack of knowledge. Thailand is known as an agricultural country and all of these agricultural activities require extensive use of pesticides to control pests and weeds. In recent years, the total amount of imported pesticides has dramatically increased. As a result of the increasing use of pesticides, there is an increased likelihood that pesticides may contaminate the Thai environment (Tsuzuki 2006; Sangchan et al. 2014). Pesticides can be released into aquatic systems via spray drift, runoff and leaching from soil (Boxall et al. 2013). Once released into aquatic systems they may then cause unintended adverse health impacts on humans and non-target organisms.

Herbicides will not occur in the natural environment individually but will likely occur alongside with other herbicides and other chemicals used in agriculture. A range of interactions are possible from these mixtures of contaminants including greater than additive toxicity, less than additive toxicity and additive toxicity (Belden and Lydy, 2000). Greater than additive (sometime referred to as synergistic) interactions are of the greatest concern in environmental risk assessments as they result in larger impacts than expected based on the toxicity of individual components of a mixture. To better understand the impacts of pesticides on the aquatic environment, it is therefore important to assess the interactions of pesticides within a mixture.

Two models have been used to assess the ecotoxicological impacts of chemical mixtures: concentration addition (CA) and independent action (IA). CA assumes that the components of mixture have the same molecular site of action and can be regarded as dilutions of one another (Loewe and Muischnek 1926). IA sometimes referred to as response addition, which was introduced by Bliss (1939), is based on the concept of dissimilar modes of action of compounds in a mixture where the individual components interact with different molecular target sites.

Synergism and antagonism have been reported in some instances. For example, Belz et al. (2008) have shown that acifluorfen and mesotrione interacted in an antagonistic manner on the aquatic macrophyte *Lemna minor*. Synergistic interactions have been observed by Cedergreen et al. (2006), who studied the effect of prochloraz, imidazole combined with diquat, azoxystrobin, acifluorfen, dimethoate, chlorfenvinphos and pirimicarb on four aquatic organisms including bacteria, daphnids, algae and *Lemna*. The result showed the combination of prochloraz with azoxystrobin and diquat with esfenvalerate resulted in a synergistic effect on daphnids and that diquat with prochloraz interacted synergistically in algal studies.

In this study we explore the effects of mixtures of four commonly used herbicides, that are atrazine, 2,4-D, alachlor and paraquat, which according to farmer surveys are regularly used in combination in Thailand (Coelho et al., 2012) and there are different mode of toxic action in plant. The aim of the present study was to examine the interactions of these herbicides in binary mixtures on *L. minor*. *L. minor* is widely used as a test organism in the environmental risk assessment and is currently recommended as a regulatory phytotoxicity test to support the registration of pesticides (OECD, 2006). We hypothesize that mixtures of commonly used herbicide in Thailand do cause impacts on aquatic plants. The objectives of this research were (1) to measure the toxicity of four commonly used herbicides individually and in binary mixtures; and (2) to use the results to determine whether the study compounds interacted in an additive, synergistic or antagonistic manner.

Materials and Methods

Atrazine (98.5%purity), 2,4-D (99% purity), alachlor (98% purity), paraquat dichloride (99% purity) were obtained from Sigma Aldrich. The summarize physical-chemical properties and mode of action of four herbicide show at Table 1. *L. minor* were cultured in Swedish media. Cultures were maintained in a Sanyo Environmental test chamber at 20 ^{0}C under continuous illumination at 10,000 Lux. *L. minor* was kept in the logarithmic growth phase by sub-culturing the stocks every 7 days. The single compound studies were based on OECD guideline 221 '*Lemna* sp. Growth Inhibition test' (OECD, 2006) with the study endpoint being frond area given that this has previously been shown to be an endpoint that is sensitive to herbicide exposure. Three replicates of a range of pesticide in seven concentrations were prepared from stock solutions of each study pesticide in acetone. Atrazine concentrations ranged from 0.05 to 0.8 mg/L, 2,4-D ranged from 5 to 100 mg/L, and for alachlor and paraquat the range was 5 to 80 µg/L. The final acetone concentration in each test was kept to less than 0.05% v/v to avoid phytotoxicity of the solvent. Associated control and solvent-control solutions were also prepared in triplicate. ~~*L. minor* were exposed in triplicate to the individual pesticide solutions or controls.~~ For atrazine and 2,4-D, borosilicate glass petri dishes were used in the exposures whereas for alachlor and paraquat plastic petri dishes were used to avoid pesticides adsorption onto the glassware (Yeo, 1967). One *L. minor* colony comprising three fronds was added to each petri dish. Digital photographs were then taken of the *L. minor* from above. The areas of the *L. minor* colonies were then determined using image J (Boxall et al., 2013). Each petri dish was transferred into a Sanyo Environmental test chamber for 7 days at the same conditions as detailed above. After 7d, the dishes were removed and photographed and the areas of the *L. minor* colonies determined. Water samples were obtained and kept at 4^{0}C until analysis with high performance liquid chromatography (HPLC), and pH was measured using a Thermo Orion pH meter.

Table 1 The summarize of physical-chemical and mode of action of four herbicides

Hebicide	Log Kow	Log Koc	Family group	Site of action
Atrazine	2.5	1.73-3.17	Triazine	Inhibitors of photosynthetic electron transport
2,4-D	2.81	0.7-2.3	Phenoxyacetic acid	Disruption of the hormonal equilibrium of the auxin-cytokinin system and inhibits root and shoot growth for both broad-leaved plants and grasses.
Alachlor	3.53	High mobile	Chloroacetanilide	Interfere with biosynthesis of lipid, protein and flavonoids.
Paraquat dichloride	-4.5	Non-mobile	Bipyridilum	Affected on photosynthesis electron transport by redox catalyst at photosystem I

In term of the mixture experiment, during the survey we found that the farmers in Thailand use these two combinations (atrazine with 2,4-D and alachlor with paraquat) in rice fields. Therefore, there is a need to explore the chemical interactions within these two herbicide combinations: atrazine with 2,4-D and alachlor with paraquat. The mixture experiments were conducted following a fixed ratio design on the basis of the EC50s from the single compound experiments (Sorensen et al., 2007). The herbicides were mixed at perceived effective concentration ratios of 100:0%, 83:17%, 63:37%, 50:50%, 37:63%, 17:83% and 0:100% (Norgaard and Cedergreen, 2010) and from these seven chemical dilutions were prepared. *L. minor* were then exposed to these seven concentrations using the same approach as for the individual compound ecotoxicity studies. There were three replicates per concentration and 12 control treatments.

The growth rates of *L. minor* were calculated from the results of the image analysis of *L. minor* frond area in each treatment into the individual and mixture studies. The growth rate was calculated according to equation 1 and, in order to calculate the percentage of growth inhibition, equation 2 was used.

$$ASGR = \frac{\ln(Nj) - \ln(Ni)}{tj - ti} \quad \text{Equation 1}$$

Where ASGR is the average specific growth rate, N_i is the frond area at day 7 and N_j is the frond area at day 0..

$$Ii = \frac{(ASGRc - ASGRt)}{ASGRc} \times 100 \qquad \text{Equation 2}$$

Where Ii is the inhibition of measured endpoint for concentration, $ASGR_c$ is the average specific growth rate of total frond area in the control and $ASGR_t$ is the average specific growth rate of total frond area in the tested sample concentration.

Based on the inhibition of chemicals on *L. minor* from day 0 to day 7, calculation of the effective concentrations resulting in 50% growth inhibition (EC50) was determined using nonlinear curve fitting based on a sigmoid model four-parameter logistic function (equation 3) (Belgers et al., 2009).

$$y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{EC50}\right)^{-Hillslope}} \qquad \text{Equation 3}$$

Where min is the bottom of curve, max is the top of curve while EC50 is the concentration giving a response of 50% and Hillslope characterizes the slope of the curve at its midpoint (Sigmaplot UK).

For mixture modeling, there are various modeling approaches used to predict the mixture toxicity (Syberg et al., 2008). In order to predict the joint effect of herbicides, two models have been suggested for use: independent action (IA) and concentration addition (CA).

The CA-reference model is typically interpreted as being the model that is appropriate for use of compounds of a mixture which have a shared mode of action. The equation can be express as

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad \text{Equation 4}$$

Where c_i gives the concentration of the *i*th component in an *n*-component mixture that provoke x% effect.

The IA-reference model is more appropriate for toxicants with dissimilar modes of action (Syberg et al., 2008). The EC50 data for the individual toxicants are used in the IA model (Equation 5) to estimate the effects of the different pesticide combinations tested in the mixture studies described above.

$$E(c_{mix}) = E(c_1) + E(c_2) - E(c_1)E(c_2) \qquad \text{Equation 5}$$

Where $E(c_1)$ and $E(c_2)$ represent the fractional effects (ranging from 0 to 1) caused by the individual toxicants 1 and 2 in the mixture. This usually requires that the concentration-response curves of the individual chemicals(Backhaus and Faust, 2012). $E(c_{mix})$ is the total effect of the mixture.

The isobologram model is a commonly used and powerful graphical approach for exploring the joint action of chemical mixtures. By comparing the isoboles based on the CA and IA predictions and experimental mixture data, conclusions can be drawn on the type(s) of interaction occurring. When an observation point falls below the model lines, this indicates that synergism is occurring whereas if an experimental point falls above a modelled point, this indicates that antagonism occurs (Machado and Robinson, 1994; Cedergreen, 2014). Isoboles were therefore constructed from the results of the CA and IA modelling and the experimental mixture toxicity data in order to draw conclusions on the mixture interactions of the study compounds.

The concentration of atrazine and 2,4-D were confirmed using a PerkinElmer Flexar HPLC equipped with a Supelco 516 C18-db 5µm x 15 cm x 4.6 mm column. For atrazine a methanol:water (60:40, v/v) mobile phase was used, the flow rate was 1 ml/min and the temperature was set at to 40 ^{0}C. The detection wavelength was 220 nm and the injection volume was 15 µl. The calibrations were done using atrazine standard covering a concentration range with high correlation (r^2= 0.998) and retention times were 6-7 minutes. The limit of detection was 0.02 mg/L and the limit of qualification was 0.04 mg/L. For 2,4-D, a methanol:water with 0.1% formic acid (70:30, v/v) mobile phase was used. The temperature was set to 30 ^{0}C and the detection wavelength was 236 nm (ConnickJr. and Simoneaux, 1982) and calibration was by external standards (r^2= 0.999), with retention times between 3-4 minutes. The limit of detection was 0.02 mg/L and the limit of quantification was 0.08 mg/L.

Alachlor ELISA test kit was purchased from Abraxiskits® (PA, USA) and paraquat analysis, ELISA test kits from EnviroLogix®. For alachlor analysis, water samples were removed from the refrigerator and allowed to attain room temperature. Afterward, 25 µl of standard, control and water sample were added into the 96 well flat-bottomed polystyrene ELISA plate. An enzyme conjugate (50 µl) alachlor antibody solution was then added to each well. Wells were then covered with parafilm to prevent contamination and evaporation and incubated at room temperature for 60 minutes. The plate was washed three times with the diluted wash buffer, and then 150 µl of color solution was then added to each well and the plates then incubated for a further 20 minutes. Finally 100 µl of stopping solution was added to each well. The absorbance was read at 450 nm within 15 minutes after addition of the stopping solution. The limit of detection was 0.08 µg/L and the limit of quantification was 2 µg/L.

For paraquat analysis, ELISA test kits were purchased from US Biocontract® (San Diego, USA). 96-wells microplate coated with anti-paraquat antibody was used. Firstly, add 25 µl of standard and samples of each well, and then 100 µl of Paraquat-Horseradish Peroxidase Conjugate (PRQ-HRP) were added in each well and incubate at room temperature for 30 minutes. After incubation, the plate was washed three times with wash buffer, and then 100 µl TMB substrate was added. Plates were then left at room temperature for 15 minutes after which 100 µl of stopping solution was added to each well and the plate was then read using an absorbance at 450 nm. The limit of detection was 0.01 µg/L and the limit of quantification was 0.01 µg/L.

In order to determine the differences of pH and chemical analysis at the beginning and the end of test, a student t-test was performed by sigma plot 12 software (Systat, Chicago, IL). A Shapiro-Wilk's test was chosen to check the normal distribution of data, if failed the Man-Whitney U test was performed instead.

Results and discussion

The pH of the exposure media for all the treatments increased slightly over the study period but this increase was less than one pH unit (Table 2). During the seven-day test, the concentrations of the study compounds in the single and binary mixture solutions at the end of the study were determined to be within ±20% of the starting concentration. (Table 3).

Table 2 Changes in pH in test media during the 7 days of exposure to the atrazine and 2,4-D (a) and alachlor with paraquat (b). Data represent means ± standard deviation (n=3).

chemical concentration ratio	Atrazine and 24D		Alachlor and paraquat	
	Day0 (±sd)	**Day7 (±sd)**	**Day0 (±sd)**	**Day7 (±sd)**
100_0	6.50(±0.05)	7.43(±0.02)	6.50(±0.00)	7.39(±0.09)
83_17	6.50(±0.03)	7.02(±0.06)	6.50(±0.00)	7.34(±0.07)
63_37	6.50(±0.3)	7.09(±0.11)	6.50(±0.00)	7.40(±0.08)
50_50	6.50(±0.5)	7.04(±0.35)	6.50(±0.00)	7.34(±0.12)
37_63	6.50(±0.91)	6.83(±0.72)	6.50(±0.00)	7.35(±0.07)
17_83	6.50(±1.06)	6.36(±0.98)	6.50(±0.00)	7.31(±0.12)
0_100	5.68(±0.99)	5.68(±1.31)	6.50(±0.00)	7.33(±0.08)

Table 3 Changes in chemical exposure concentration in test media during the 7 days of exposure to the pesticide mixtures. Data present means ± standard deviation (n=3).

Chemical concentration ratio	% recovery			
	atrazine	**2,4-D**	**alachlor**	**paraquat**
100	100.4(±1.13)	100.4(±0.53)	179(±84)	154(±92)
83	104.6(±5.34)	100(±0.70)	87(±2)	143(±72)
63	100(±0.00)	100(±0.81)	130(±130)	135(±36)
50	100(±0.00)	100.6(±1.40)	132(±0)	143(±42)
37	100(±0.00)	100(±1.41)	104(±43)	122(±40)
17	100.3(±0.75)	100(±1.21)	159(±131)	128(±67)

The single compound toxicity test showed that paraquat and alachlor were the most toxic of the four study compounds to *L. minor* followed by atrazine and 2,4-D. The EC50s for the single compound toxicity tests were 15, 15, 170 and 27000 µg/L, for paraquat, alachlor, atrazine and 2,4-D, respectively (Table 4). The results are similar to previous studies on the toxicity of the study compounds to *L. minor* and related macrophytes. Previously reported EC50s for the compound to *L. minor* are: 51 µg/L for paraquat, 198 µg/L for alachlor, 153 µg/L for atrazine and >100,000 µg/L for 2,4-D (Fairchild et al., 1997).

L. minor responds differently to different herbicides, which reflect differences in the physicochemical properties of the study compounds, the degree of translocation into the plant, metabolic degradation and the presence or absence of molecular target sites (Michel et al., 2004). The high toxicity of paraquat is explained by the fact that it is a bipyridylium herbicide that can damage the plant tissue very quickly (Brian, 1976). Under sunny conditions leaf discoloration can occur within an hour of applying paraquat to plants. This likely explains the colour changes that were visible on the *Lemna* fronds in the paraquat treatment. Alachlor is a chloroacetamide or amide pesticide and affects root elongation, RNA, protein synthesis, amylase and proteinase activity (Ashton and Bayer, 1976). In our study exposure to the compound resulted in dwarfish fronds. This observation is in agreement with other studies that have shown that alachlor has an impact on frond size due to a disruption of cell division processes (Drost et al., 2007). Atrazine was moderately toxic in this experiment. Atrazine belongs to the triazine group which is characterised by the photosynthesis inhibition in photosystem II by blocking electron transport, leading to a reduction in photosynthetic oxygen production and finally reducing the relative growth rate.

Exposure to 2,4-D showed limited effects on the plants compared to the other compounds (paraquat, alachlor and atrazine). There are many published studies on the toxicity of 2,4-D on aquatic macrophytes (Fairchild et al., 1997; Michel et al., 2004; Belgers et al., 2009). All of these studies indicate that duckweed is insensitive to or experience moderate toxicity from 2,4-D. Their EC50 values range from 500 to >6000 µg/L (Belgers et al., 2009) and from this present study the EC50 was 27000 µg/L. Others have reported that 2,4-D's toxicity is enhanced specifically in dicotyledonous plants rather than monocotyledons because of their differences in morphology and physiology of the two plant groups.

Table 4. EC50 values with 95% confidence intervals (CI) obtained from four parameters dose response curves for mixture ecotoxicity studies using atrazine and 2,4-D or alachlor and paraquat.

	Atrazine (mg/L)				2,4-D (mg/L)			
Ratio	Observed (CA)		Predicted (IA)		Observed (CA)		Predicted (IA)	
	EC_{50}	95% CI	EC50	95% CI	EC_{50}	95% CI	EC_{50}	95% CI
100:0	0.17	(0.15-0.19)	0.17	(0.15-0.19)	-	-	-	-
83:17	0.22	(0.21-0.23)	0.13	(0.12-0.14)	12.4	(12.3-12.5)	19	(18-20)
63:37	0.17	(0.16-0.18)	0.10	(0.12-0.14)	27	(26.6-27.4)	23	(22-24)
50:50	0.12	(0.10-0.14)	0.07	(0.05-0.09)	33	(32-34)	25	0
37:63	0.06	(0.04-0.07)	0.06	(0.05-0.07)	27	(26-28)	26	0
17:83	0.03	0	0.02	(0.02-0.02)	32	(31-33)	26	0
0:100	-			-	27	(26.98-27.02)	27	(22-29.4)
	Alachlor (µg/L)				Paraquat (µg/L)			
Ratio	Observed (CA)		Predicted (IA)		Observed (CA)		Predicted (IA)	
	EC_{50}	95% CI	EC50	95% CI	EC_{50}	95% CI	EC_{50}	95% CI
100:0	15	(13.5-15.5)	15	(12.5-15.5)	-	-	-	-
83:17	8.5	(6.9-10)	10.5	(9.2-11.9)	1.2	(0.1-1.4)	4.6	(4.42-4.81)
63:37	6.7	(5.5-7.8)	7	(6-8.1)	2.7	(2.2-3.1)	7.7	(7.3-8.1)
50:50	5.7	(4.8-6.7)	4	(3.1-4.9)	3.7	(3.1-4.3)	10.3	(9.7-11)
37:63	3.4	(3-4)	3	(2.5-3.5)	4	(3.5-4.6)	11.6	(11-12.5)
17:83	2.3	(2-2.7)	0.78	0	7.3	(6.3-8.3)	13.8	(12.9-14.1)
0:100	-	-	-	-	15	(12.4-18.5)	15	(12.4-17.6)

[a] 95% lower confidence interval [b] 95% upper confidence interval

In terms of mixture toxicity, EC50s for the different mixtures are shown in Table 4. Use of isoboles for comparing the experimental observation with predictions using the CA and IA models showed that the predictions using the IA model were closed to experimental observations for mixtures of atrazine and 2,4-D while both models worked similarly for modelling the effects of paraquat and alachlor (Figures 3a and b). The better performance of the IA model is expected given that the study herbicides all have different modes of action.

While, the IA model performed better, it did not fully explain the experimental observations suggesting that some interactions were occurring. The results indicate that the interaction between the herbicides were occurring. For atrazine and 2,4-D the interaction appeared to be antagonistic (Figure 3a). There is no literature data on atrazine and 2,4-D mixture toxicity to organisms but there are ecotoxicity data for closely related chemicals and organisms. For example, Bisewska *et al.* (2012) examined the toxic interactions of two herbicides, MCPA (2-methyl-4-chlorophenoxyacetic acid) and chloridazone, to the green microalgae and duckweed *L. minor*. Like 2,4-D, MCPA is a chlorophenoxy herbicide. Like atrazine, chloridazone inhibits photosynthesis system II by blocking the electron transport from quinone b(Qb) to plastoquinone (PQ) in the PSII reaction center. The two compounds were found to interact antagonistically in studies with *Lemna.*

For this work, the results of our experiment agree with those previously reported by other researchers that antagonistism is the most common form of herbicide mixture interaction. For example, Belden and Lydy (2000) stated that the variety of joint actions produced by atrazine mixed with other compounds indicates that the effect of atrazine on an organism is dependent on the species, co-contaminant, and levels of atrazine used. In addition, the key factors which lead to decreased or increased antagonism on plants include the herbicide ratios, mode of action, plant species, formulation, adjuvants, timing, stage of growth and the environment (Green, 1989). Antagonism has been found to occur frequently in other studies using mixtures of herbicides belonging to different chemical groups and monocot species (Damalas, 2004). Furthermore, the most common antagonism is when post emergence grass herbicides are mixed with post emergence broadleaf herbicides (Bradford et al., 1989). In terms of the biochemistry when exposing two herbicides on plant, atrazine has been reported to affect oxidative phosphorylation and decrease net photosynthesis by CO_2 uptake. The phenoxy herbicide 2,4-D also decreases net photosynthesis of plants but higher concentrations are needed (Van Oorschot, 1976).

Alachlor and paraquat showed greater than additive toxicity (synergism) when experimental observations were compared to predictions based on the IA and CA model (Figure 3b). Alachlor is a seedling growth inhibitor and is active at two main sites of the developing shoot and roots. This herbicide inhibits the dividing of plant cells, which interrupts shoot elongation and lateral root formation (Minton et al., 1989; Tomlin, 1997). There is evidence to suggest that these herbicides can affect multiple sites within a plant. Similarly, paraquat dichloride is activated by exposure to sunlight to form oxygen compounds such as hydrogen peroxide destroy plant tissues by rupturing plant cell membranes (Van Oorschot, 1976). Among the report on pesticide mixture, they found little evidence of synergism. However, according to earlier reviews, there synergistic interactions have been reported for pesticide with low doses in chemical mixtures (Dennis et al., 2012). In this study the concentration of alachlor and paraquat tested were low. Many studies have been attempted to identify the mechanisms behind the observed synergy in ecotoxity studies but the reasons are still not well understood. Cedergreen (2014) described that the mechanisms causing synergistic interaction can basically affect six processes leading toxic on organism including bioavailability, uptake, internal transportation, metabolization, binding at the target site and excretion.

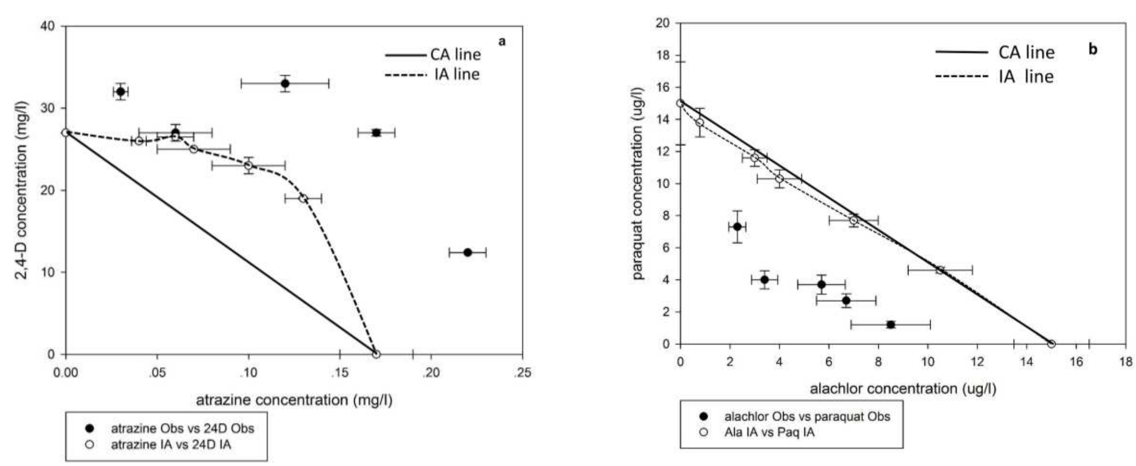

Fig 3. Isobole at the EC_{50} level for the seven mixtures for (a) atrazine and 2, 4-D and (b) alachlor and paraquat obtained either by experimentation or using the independent action model. Points

represent concentrations where 50% reduction in growth was observed and error bar represent the associated 95% CIs.

It has been suggested that the success of the reference models such as IA or CA in predicting effects of mixtures depends on the number of mixture components, the concentration ratio, the steepness of individual concentration response curves and the regression models (Faust et al., 2001). Alahclor and paraquat are classified by different activities. Alachlor is classified as systemic herbicide, which translocate through the plant either from foliar application down to roots or from soil application up to leaves but paraquat is non-systemic or contact herbicide which absorbed by external tissue of plants such as leaves, stems and root (Tomlin, 1997).

From the results of this study the IA model appears to perform better than the CA model for estimating the combined effects of the two pairs of herbicides. For atrazine and 2,4-D, the use of this model would provide a conservative estimation of effects whereas for paraquat and alachlor it would underestimate effects.

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