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

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8 **Abstract**

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

20 ***Keywords: Herbicide mixtures, Lemna minor, synergism, antagonism***

21 The U. S. Environmental Protection Agency (EPA) recently estimated that more than 540 million
22 kilograms of pesticides are applied to crops around the world and the most frequently used
23 pesticide class is herbicides (Ecobichon, 2001). The use of herbicides has been continuously
24 increasing year on year. In addition, several reports have highlighted problems associated with
25 pesticide overuse and misuse mainly due to a lack of knowledge. Thailand is known as an
26 agricultural country and all of these agricultural activities require extensive use of pesticides to
27 control pests and weeds. In recent years, the total amount of imported pesticides has dramatically
28 increased. As a result of the increasing use of pesticides, there is an increased likelihood that
29 pesticides may contaminate the Thai environment (Tsuzuki 2006; Sangchan et al. 2014). Pesticides
30 can be released into aquatic systems via spray drift, runoff and leaching from soil (Boxall et al.
31 2013). Once released into aquatic systems they may then cause unintended adverse health impacts
32 on humans and non-target organisms.

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

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

47 Synergism and antagonism have been reported in some instances. For example, Belz et al. (2008)
48 have shown that acifluorfen and mesotrione interacted in an antagonistic manner on the aquatic
49 macrophyte *Lemna minor*. Synergistic interactions have been observed by Cedergreen et al.
50 (2006), who studied the effect of prochloraz, imidazole combined with diquat, azoxystrobin,
51 acifluorfen, dimethoate, chlorfenvinphos and pirimicarb on four aquatic organisms including
52 bacteria, daphnids, algae and *Lemna*. The result showed the combination of prochloraz with
53 azoxystrobin and diquat with esfenvalerate resulted in a synergistic effect on daphnids and that
54 diquat with prochloraz interacted synergistically in algal studies.

55 In this study we explore the effects of mixtures of four commonly used herbicides, that are atrazine,
56 2,4-D, alachlor and paraquat, which according to farmer surveys are regularly used in combination
57 in Thailand (Coelho et al., 2012) and there are different mode of toxic action in plant. The aim of
58 the present study was to examine the interactions of these herbicides in binary mixtures on *L.*
59 *minor*. *L. minor* is widely used as a test organism in the environmental risk assessment and is
60 currently recommended as a regulatory phytotoxicity test to support the registration of pesticides
61 (OECD, 2006). We hypothesize that mixtures of commonly used herbicide in Thailand do cause
62 impacts on aquatic plants. The objectives of this research were (1) to measure the toxicity of four
63 commonly used herbicides individually and in binary mixtures; and (2) to use the results to
64 determine whether the study compounds interacted in an additive, synergistic or antagonistic
65 manner.

66 **Materials and Methods**

67 Atrazine (98.5% purity), 2,4-D (99% purity), alachlor (98% purity), paraquat dichloride (99%
68 purity) were obtained from Sigma Aldrich. The summarize physical-chemical properties and mode
69 of action of four herbicide show at Table 1. *L. minor* were cultured in Swedish media. Cultures
70 were maintained in a Sanyo Environmental test chamber at 20 °C under continuous illumination
71 at 10,000 Lux. *L. minor* was kept in the logarithmic growth phase by sub-culturing the stocks every
72 7 days. The single compound studies were based on OECD guideline 221 '*Lemna* sp. Growth
73 Inhibition test' (OECD, 2006) with the study endpoint being frond area given that this has
74 previously been shown to be an endpoint that is sensitive to herbicide exposure. Three replicates
75 of a range of pesticide in seven concentrations were prepared from stock solutions of each study
76 pesticide in acetone. Atrazine concentrations ranged from 0.05 to 0.8 mg/L, 2,4-D ranged from 5
77 to 100 mg/L, and for alachlor and paraquat the range was 5 to 80 µg/L. The final acetone
78 concentration in each test was kept to less than 0.05% v/v to avoid phytotoxicity of the solvent.
79 Associated control and solvent-control solutions were also prepared in triplicate. ~~*L. minor* were~~
80 ~~exposed in triplicate to the individual pesticide solutions or controls.~~ For atrazine and 2,4-D,
81 borosilicate glass petri dishes were used in the exposures whereas for alachlor and paraquat plastic
82 petri dishes were used to avoid pesticides adsorption onto the glassware (Yeo, 1967). One *L. minor*
83 colony comprising three fronds was added to each petri dish. Digital photographs were then taken
84 of the *L. minor* from above. The areas of the *L. minor* colonies were then determined using image
85 J (Boxall et al., 2013). Each petri dish was transferred into a Sanyo Environmental test chamber

86 for 7 days at the same conditions as detailed above. After 7d, the dishes were removed and
 87 photographed and the areas of the *L. minor* colonies determined. Water samples were obtained and
 88 kept at 4°C until analysis with high performance liquid chromatography (HPLC), and pH was
 89 measured using a Thermo Orion pH meter.

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

Herbicide	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

91 In term of the mixture experiment, during the survey we found that the farmers in Thailand use
 92 these two combinations (atrazine with 2,4-D and alachlor with paraquat) in rice fields. Therefore,
 93 there is a need to explore the chemical interactions within these two herbicide combinations:
 94 atrazine with 2,4-D and alachlor with paraquat. The mixture experiments were conducted
 95 following a fixed ratio design on the basis of the EC50s from the single compound experiments
 96 (Sorensen et al., 2007). The herbicides were mixed at perceived effective concentration ratios of
 97 100:0%, 83:17%, 63:37%, 50:50%, 37:63%, 17:83% and 0:100% (Norgaard and Cedergreen,
 98 2010) and from these seven chemical dilutions were prepared. *L. minor* were then exposed to these
 99 seven concentrations using the same approach as for the individual compound ecotoxicity studies.
 100 There were three replicates per concentration and 12 control treatments.
 101 The growth rates of *L. minor* were calculated from the results of the image analysis of *L. minor*
 102 frond area in each treatment into the individual and mixture studies. The growth rate was calculated
 103 according to equation 1 and, in order to calculate the percentage of growth inhibition, equation 2
 104 was used.

$$105 \quad ASGR = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i} \quad \text{Equation 1}$$

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

$$109 \quad I_i = \frac{(ASGR_c - ASGR_t)}{ASGR_c} \times 100 \quad \text{Equation 2}$$

110
 111 Where I_i is the inhibition of measured endpoint for concentration, $ASGR_c$ is the average specific
 112 growth rate of total frond area in the control and $ASGR_t$ is the average specific growth rate of total
 113 frond area in the tested sample concentration.

114 Based on the inhibition of chemicals on *L. minor* from day 0 to day 7, calculation of the effective
 115 concentrations resulting in 50% growth inhibition (EC50) was determined using nonlinear curve
 116 fitting based on a sigmoid model four-parameter logistic function (equation 3) (Belgers et al.,
 117 2009).

$$118 \quad y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{EC50}\right)^{-Hillslope}} \quad \text{Equation 3}$$

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

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

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

$$127 \quad \sum_{i=1}^n \frac{c_i}{EC_{xi}} = 1 \quad \text{Equation 4}$$

128 Where c_i gives the concentration of the i th component in an n -component mixture that provoke
 129 $x\%$ effect.

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

$$134 \quad E(c_{mix}) = E(c_1) + E(c_2) - E(c_1)E(c_2) \quad \text{Equation 5}$$

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

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

145 from the results of the CA and IA modelling and the experimental mixture toxicity data in order
146 to draw conclusions on the mixture interactions of the study compounds.

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

158 Alachlor ELISA test kit was purchased from Abraxiskits® (PA, USA) and paraquat analysis,
159 ELISA test kits from EnviroLogix®. For alachlor analysis, water samples were removed from the
160 refrigerator and allowed to attain room temperature. Afterward, 25 μ l of standard, control and
161 water sample were added into the 96 well flat-bottomed polystyrene ELISA plate. An enzyme
162 conjugate (50 μ l) alachlor antibody solution was then added to each well. Wells were then covered
163 with parafilm to prevent contamination and evaporation and incubated at room temperature for 60
164 minutes. The plate was washed three times with the diluted wash buffer, and then 150 μ l of color
165 solution was then added to each well and the plates then incubated for a further 20 minutes. Finally
166 100 μ l of stopping solution was added to each well. The absorbance was read at 450 nm within 15
167 minutes after addition of the stopping solution. The limit of detection was 0.08 μ g/L and the limit
168 of quantification was 2 μ g/L.

169

170 For paraquat analysis, ELISA test kits were purchased from US Biocontract® (San Diego, USA).
171 96-wells microplate coated with anti-paraquat antibody was used. Firstly, add 25 μ l of standard
172 and samples of each well, and then 100 μ l of Paraquat-Horseradish Peroxidase Conjugate (PRQ-
173 HRP) were added in each well and incubate at room temperature for 30 minutes. After incubation,
174 the plate was washed three times with wash buffer, and then 100 μ l TMB substrate was added.
175 Plates were then left at room temperature for 15 minutes after which 100 μ l of stopping solution
176 was added to each well and the plate was then read using an absorbance at 450 nm. The limit of
177 detection was 0.01 μ g/L and the limit of quantification was 0.01 μ g/L.

178

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

183 **Results and discussion**

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

188
 189 **Table 2** Changes in pH in test media during the 7 days of exposure to the atrazine and 2,4-D (a)
 190 and alachlor with paraquat (b). Data represent means \pm standard deviation (n=3).
 191

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

192 **Table 3** Changes in chemical exposure concentration in test media during the 7 days of exposure
 193 to the pesticide mixtures. Data present means \pm standard deviation (n=3).
 194

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

195
 196 The single compound toxicity test showed that paraquat and alachlor were the most toxic of the
 197 four study compounds to *L. minor* followed by atrazine and 2,4-D. The EC50s for the single
 198 compound toxicity tests were 15, 15, 170 and 27000 μ g/L, for paraquat, alachlor, atrazine and 2,4-
 199 D, respectively (Table 4). The results are similar to previous studies on the toxicity of the study
 200 compounds to *L. minor* and related macrophytes. Previously reported EC50s for the compound to
 201 *L. minor* are: 51 μ g/L for paraquat, 198 μ g/L for alachlor, 153 μ g/L for atrazine and >100,000
 202 μ g/L for 2,4-D (Fairchild et al., 1997).

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

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

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

Ratio	Atrazine (mg/L)				2,4-D (mg/L)			
	Observed (CA)		Predicted (IA)		Observed (CA)		Predicted (IA)	
	EC ₅₀	95% CI	EC ₅₀	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)
Ratio	Alachlor (µg/L)				Paraquat (µg/L)			
	Observed (CA)		Predicted (IA)		Observed (CA)		Predicted (IA)	
	EC ₅₀	95% CI	EC ₅₀	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)

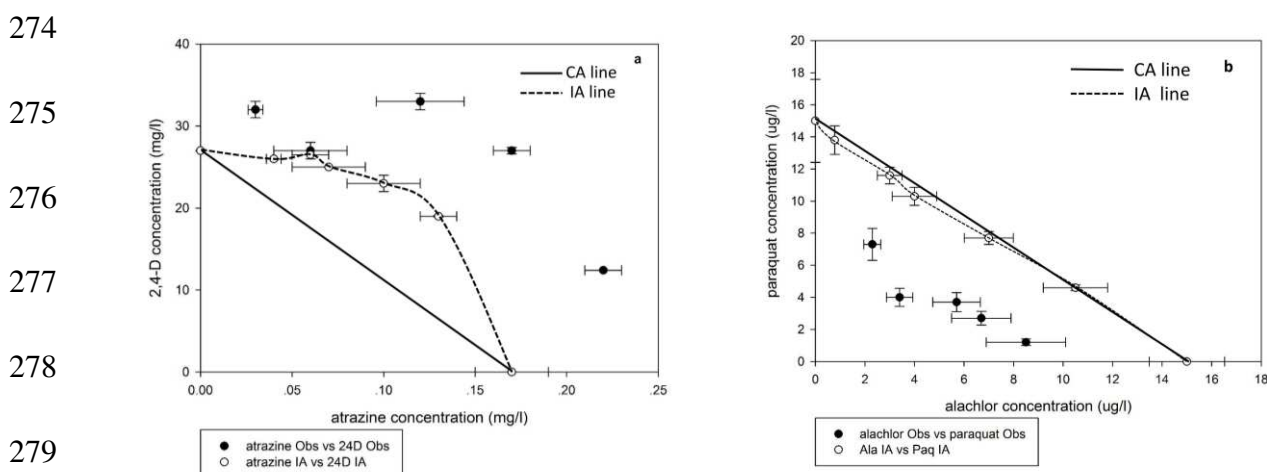
227 ^a 95% lower confidence interval ^b 95% upper confidence interval

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

234 While, the IA model performed better, it did not fully explain the experimental observations
 235 suggesting that some interactions were occurring. The results indicate that the interaction between
 236 the herbicides were occurring. For atrazine and 2,4-D the interaction appeared to be antagonistic
 237 (Figure 3a). There is no literature data on atrazine and 2,4-D mixture toxicity to organisms but
 238 there are ecotoxicity data for closely related chemicals and organisms. For example, Bisewska *et*
 239 *al.* (2012) examined the toxic interactions of two herbicides, MCPA (2-methyl-4-
 240 chlorophenoxyacetic acid) and chloridazone, to the green microalgae and duckweed *L. minor*. Like
 241 2,4-D, MCPA is a chlorophenoxy herbicide. Like atrazine, chloridazone inhibits photosynthesis
 242 system II by blocking the electron transport from quinone b(Qb) to plastoquinone (PQ) in the PSII
 243 reaction center. The two compounds were found to interact antagonistically in studies with *Lemna*.

244 For this work, the results of our experiment agree with those previously reported by other
 245 researchers that antagonism is the most common form of herbicide mixture interaction. For
 246 example, Belden and Lydy (2000) stated that the variety of joint actions produced by atrazine
 247 mixed with other compounds indicates that the effect of atrazine on an organism is dependent on
 248 the species, co-contaminant, and levels of atrazine used. In addition, the key factors which lead to
 249 decreased or increased antagonism on plants include the herbicide ratios, mode of action, plant
 250 species, formulation, adjuvants, timing, stage of growth and the environment (Green, 1989).
 251 Antagonism has been found to occur frequently in other studies using mixtures of herbicides
 252 belonging to different chemical groups and monocot species (Damalas, 2004). Furthermore, the
 253 most common antagonism is when post emergence grass herbicides are mixed with post emergence
 254 broadleaf herbicides (Bradford et al., 1989). In terms of the biochemistry when exposing two
 255 herbicides on plant, atrazine has been reported to affect oxidative phosphorylation and decrease
 256 net photosynthesis by CO₂ uptake. The phenoxy herbicide 2,4-D also decreases net photosynthesis
 257 of plants but higher concentrations are needed (Van Oorschot, 1976).

258 Alachlor and paraquat showed greater than additive toxicity (synergism) when experimental
 259 observations were compared to predictions based on the IA and CA model (Figure 3b). Alachlor
 260 is a seedling growth inhibitor and is active at two main sites of the developing shoot and roots.
 261 This herbicide inhibits the dividing of plant cells, which interrupts shoot elongation and lateral
 262 root formation (Minton et al., 1989; Tomlin, 1997). There is evidence to suggest that these
 263 herbicides can affect multiple sites within a plant. Similarly, paraquat dichloride is activated by
 264 exposure to sunlight to form oxygen compounds such as hydrogen peroxide destroy plant tissues
 265 by rupturing plant cell membranes (Van Oorschot, 1976). Among the report on pesticide mixture,
 266 they found little evidence of synergism. However, according to earlier reviews, there synergistic
 267 interactions have been reported for pesticide with low doses in chemical mixtures (Dennis et al.,
 268 2012). In this study the concentration of alachlor and paraquat tested were low. Many studies have
 269 been attempted to identify the mechanisms behind the observed synergy in ecotoxicity studies but
 270 the reasons are still not well understood. Cedergreen (2014) described that the mechanisms causing
 271 synergistic interaction can basically affect six processes leading toxic on organism including
 272 bioavailability, uptake, internal transportation, metabolization, binding at the target site and
 273 excretion.



280 Fig 3. Isobole at the EC₅₀ level for the seven mixtures for (a) atrazine and 2, 4-D and (b) alachlor
 281 and paraquat obtained either by experimentation or using the independent action model. Points

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

284 It has been suggested that the success of the reference models such as IA or CA in predicting
285 effects of mixtures depends on the number of mixture components, the concentration ratio, the
286 steepness of individual concentration response curves and the regression models (Faust et al.,
287 2001). Alachlor and paraquat are classified by different activities. Alachlor is classified as systemic
288 herbicide, which translocate through the plant either from foliar application down to roots or from
289 soil application up to leaves but paraquat is non-systemic or contact herbicide which absorbed by
290 external tissue of plants such as leaves, stems and root (Tomlin, 1997).

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

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