



# 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 the 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 the effects on plant growth rate were assessed based on frond area measurements. Experimental observations of mixture toxicity were compared with predictions based on single herbicide exposure data using concentration addition and independent action models. The single compound studies showed that paraquat and alachlor were 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* · Synergism · 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. 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 a higher 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 (Schulz 2004). Once released into aquatic systems, they may cause unintended adverse health impacts on humans and non-target organisms.

In agricultural stream, herbicides will not occur individually but alongside other herbicides and other chemicals used in agriculture. A range of interactions are possible from these mixtures of contaminants including greater than additive, less than additive and additive toxicity (Belden and Lydy 2000). Greater than additive (sometimes 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 toxic interactions of pesticides.

Two models have frequently 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. Hence, 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*.

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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 on algae. Given the possibility of synergism and antagonism, there is a need to explore the effects on local species of pesticide mixtures commonly used by farmers in Thailand. Such information is currently very limited.

In this study, we explore the effects of mixtures of four commonly used herbicides—atrazine, 2,4-D, alachlor and paraquat. These herbicides are, according to farmer surveys, regularly used in combination in Thailand (Coelho et al. 2012) and target different modes of toxic action in plants. 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 environmental risk assessments and is currently recommended as a regulatory phytotoxicity test to support the registration of pesticides (OECD 2006). 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 summarized physical–chemical properties and modes of action of the four herbicides are shown in Table 1. *L. minor* were cultured in Swedish media. Cultures were maintained in a Sanyo Environmental test chamber at 20°C under continuous illumination at 10,000 lx. *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 each pesticide in seven concentration ratios were prepared from the stock solutions of each 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 5–80 µg/L. The final acetone concentration in each test was kept to <0.05% v/v to avoid phytotoxicity of the solvent. Associated control and solvent-control solutions were also prepared in triplicate. 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 pesticide 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 under the same conditions as detailed above. After 7 days, the dishes were removed and photographed and the areas of the *L. minor* colonies determined. Water samples were obtained and kept at 4°C until analysis with high performance liquid chromatography (HPLC), and pH was measured using a Thermo Orion pH meter.

In terms of the mixture experiment, during the survey we found that farmers in Thailand use these two combinations, namely, 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. The mixture experiments were conducted following a fixed ratio design on the basis of the EC<sub>50</sub>s 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 in the individual compound ecotoxicity studies. There were three replicates per treatment concentration and 12 control treatments so as to avoid contamination from control.

**Table 1** Summary of physical–chemical properties and mode of action of four herbicides (Tomlin 1997; Cavanaugh et al. 1998)

| 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  | Bipyridylum        | Affected on photosynthesis electron transport by redox catalyst at photo-system I  |

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

$$ASGR = \frac{\ln(N_0) - \ln(N_7)}{t_7} \quad (1)$$

where ASGR is the average specific growth rate,  $N_7$  is the frond area at day 7,  $N_0$  is the frond area at day 0 and  $t$  is 7-day time period of this experiment.

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

where  $I_i$  is the growth inhibition for concentration  $i$ ,  $ASGR_c$  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 concentration.

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

$$y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{EC_{50}}\right)^{-Hillslope}} \quad (3)$$

where  $\min$  is the bottom of curve,  $\max$  is the top of curve, while  $EC_{50}$  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 used: IA and CA.

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

$$\sum_{i=1}^n \frac{c_i}{EC_{xi}} = 1 \quad (4)$$

where  $c_i$  gives the concentration of the  $i$ th component in an  $n$ -component mixture that provoked  $x\%$  effect.

The IA-reference model is more appropriate for toxicants with dissimilar modes of action (Syberg et al. 2008). The  $EC_{50}$  data for the individual toxicants are used in the IA model (Eq. 5) to estimate the effects of the different pesticide combinations tested in the mixture studies described above.

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

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.  $E(c_{\text{mix}})$  is the total effect of the mixture (Backhaus and Faust 2012).

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 including the confidence interval (95% level) falls below the model lines, this indicates that synergism is occurring, whereas if an observation including the confidence interval falls above the model, 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 5  $\mu\text{m} \times 15 \text{ cm} \times 4.6 \text{ mm}$  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 to 40°C. The detection wavelength was 220 nm and the injection volume was 15  $\mu\text{L}$ . The calibrations were done using atrazine standard covering a concentration range with high correlation ( $r^2 = 0.998$ ), and retention times were 6–7 min. The limit of detection was 0.02 mg/L and the limit of quantitation 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°C and the detection wavelength was 236 nm (Connick et al. 1982) and calibration was by external standards ( $r^2 = 0.999$ ), with retention times between 3 and 4 min. The limit of detection was 0.02 mg/L and the limit of quantitation was 0.08 mg/L.

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

For paraquat analysis, ELISA test kits were purchased from US Biocontract® (San Diego, USA). A 96-well microplate coated with anti-paraquat antibody was used. First, 25 µL of standard and 25 µL of sample were added to each well, and then 100 µL of Paraquat-Horseradish Peroxidase Conjugate (PRQ-HRP) were added to each well and incubated at room temperature of 15–25°C for 30 min. After incubation, the plate was washed three times with wash buffer, and then 100 µL TMB substrate was added. The plate was then left at room temperature for 15 min after which 100 µL of stopping solution was added to each well. The plate was then read using an absorbance at 450 nm. The limit of detection was 0.01 µg/L and the limit of quantitation was 0.02 µg/L.

In order to determine the differences of pH and chemical analysis result at the beginning and the end of test, the Student's *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 this failed, the Mann–Whitney U test was performed instead.

### Results and Discussion

The pH of the exposure media for all treatments increased slightly over the study period, but this increase was < 1 pH unit (Table 2). Scherholz and Curtis (2013) and Chi et al. (2011) have explained that in experimental media culture, pH increases as the dissolved CO<sub>2</sub> is removed from the water through photosynthesis.

During the 7-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).

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 EC<sub>50</sub>s for the single compound toxicity tests were 15, 15, 170 and 27,000 µg/L, for paraquat, alachlor, atrazine and 2,4-D, respectively (Table 4). The results are generally

**Table 3** Changes in chemical exposure concentration in test media during the 7 days of exposure to pesticide mixtures

| 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) |

Data present means ± standard deviation (n = 3)

similar to previous studies on the toxicity of these compounds to *L. minor* and related macrophytes. Previously reported EC<sub>50</sub>s of the compounds 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). That some of the reported EC<sub>50</sub> values differ from those of this study may be due to the use of different test media (Seibert et al. 2002).

*Lemna minor* responds differently to different herbicides, which reflect the differences in the physicochemical property 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 bipyridylum herbicide that can damage plant tissue very quickly (Brian 1976). Under sunny conditions, leaf discoloration can occur within an hour of application (Cavanaugh et al. 1998). This likely explains the color 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 which have shown that alachlor has an impact on frond size due to a disruption of cell division processes (Drost et al. 2007). Atrazine was

**Table 2** Changes in pH in test media during the 7 days of exposure to atrazine with 2,4-D (a) and alachlor with paraquat (b)

| Chemical concentration ratio | Atrazine and 2,4-D |              | Alachlor and paraquat |              |
|------------------------------|--------------------|--------------|-----------------------|--------------|
|                              | Day 0 (± SD)       | Day 7 (± SD) | Day 0 (± SD)          | Day 7 (± 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) |

Data represent means ± standard deviation (n = 3)

**Table 4** EC<sub>50</sub> values with 95% confidence intervals (CI) obtained from four parameters dose response curves for mixture ecotoxicity studies using atrazine with 2,4-D or alachlor with paraquat

| Ratio | Atrazine (mg/L)  |             |                  |             | 2,4-D (mg/L)     |               |                  |             |
|-------|------------------|-------------|------------------|-------------|------------------|---------------|------------------|-------------|
|       | Observed (CA)    |             | Predicted (IA)   |             | Observed (CA)    |               | Predicted (IA)   |             |
|       | EC <sub>50</sub> | 95% CI      | EC <sub>50</sub> | 95% CI      | EC <sub>50</sub> | 95% CI        | EC <sub>50</sub> | 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 <sub>50</sub> | 95% CI      | EC <sub>50</sub> | 95% CI      | EC <sub>50</sub> | 95% CI        | EC <sub>50</sub> | 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) |

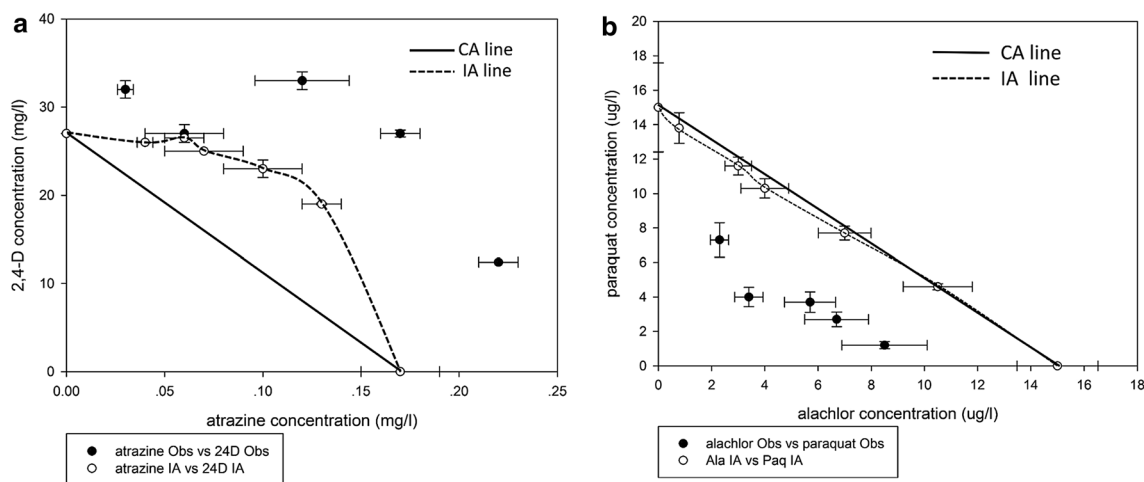
moderately toxic in this experiment. Atrazine belongs to the triazine group which is characterised by the photosynthesis inhibition in photosystem II by blocking electron transport, leading to a reduction in photosynthetic oxygen production and finally reducing the relative growth rate. Exposure to 2,4-D 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 EC<sub>50</sub> values range from 500 to > 6000 µg/L (Belgers et al. 2009) and > 100,000 µg/L for 2,4-D (Fairchild et al. 1997) and in this present study the EC<sub>50</sub> was 27,000 µg/L. Others have reported that 2,4-D's toxicity is enhanced specifically in dicotyledonous plants rather than monocotyledons because of the morphological and physiological differences between the two plant groups (Song 2014).

In terms of mixture toxicity, the EC<sub>50</sub>s for the different mixtures are shown in Table 4. Use of isoboles for comparing the experimental observations with predictions using the CA and IA models showed that the predictions from the IA model were closer to the experimental observations for the mixture of atrazine and 2,4-D, while both models worked similarly for the effects of the mixture of paraquat and alachlor (Fig. 1a, b). The better performance of the IA model

is expected given that the studied herbicides have different modes of action (Table 1).

That the IA model did not fully explain the experimental observations suggests that some interactions occurred. For atrazine and 2,4-D, the interaction appeared to be antagonistic (Fig. 1a). 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 between 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. Similar to this study, MCPA and chloridazone were found to interact antagonistically in studies with *Lemna*.

The results of our experiment agree with those previously reported by other researchers, that antagonism is the most common for herbicide mixtures. 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 concentration ratios, mode of



**Fig. 1** Isobole at the EC50 level for the seven mixtures for **a** atrazine and 2, 4-D mixtures and **b** alachlor and paraquat obtained either by experimentation or using the IA model. Points represent the concen-

trations where 50% reduction in growth was observed and error bar represents the associated 95% CIs

action, plant species, formulation, adjuvants, timing, stage of growth and the environment (Green 1989). Furthermore, the most common antagonism occurs when post emergence grass herbicides are mixed with post emergence broadleaf herbicides (Bradford et al. 1989). In terms of biochemistry, when exposing two herbicides on plants, atrazine has been reported to affect oxidative phosphorylation and decrease net photosynthesis by  $\text{CO}_2$  uptake. This is because atrazine, as a photosynthesis inhibitor, inhibited  $\text{CO}_2$  fixation by inducing stomata closure which causes the internal  $\text{CO}_2$  level to rise (Fedtke 1982). The phenoxy herbicide 2,4-D also decreases net photosynthesis of plants, but higher concentrations are needed (Van Oorschot 1976).

Alachlor and paraquat mixture showed greater than additive toxicity (synergism) when experimental observations were compared to model predictions based on the IA and CA (Fig. 1b). 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). Similarly, paraquat dichloride is activated by exposure to sunlight to form oxygen compounds such as hydrogen peroxide and destroys plant tissues by rupturing plant cell membranes (Van Oorschot 1976). Among the reports on the pesticide mixture, little evidence of synergism can be found. However, according to earlier reviews, their synergistic interactions have been reported for the low doses of the pesticides in chemical mixtures (Dennis et al. 2012). In this study, the concentrations of alachlor and paraquat tested were low. Many ecotoxicity studies have attempted to identify the mechanisms behind the observed synergy, but the reasons are still not well understood. Cedergreen

(2014) described how the mechanisms causing synergistic interaction basically involve six processes leading to toxic effects on organisms. These processes include bioavailability, uptake, internal transportation, metabolism, binding at the target site and excretion, but these mechanisms, though they require further uncovering, will not be covered here since they are beyond the scope of this study.

Based on the IA and CA model, the two mixtures produced different effects on the aquatic plant *L. minor*, and the effects of pesticide mixture depend on the number of mixture components and the concentration ratio (Faust et al. 2001). This study bears relevance to the Thai environment as Thai farmers use these four pesticides together in varying concentrations (Panuwet et al. 2008). While data on the occurrence of pesticides in Thailand's surface waters indicate that concentrations in rivers are much lower than the concentrations examined in this study, the available data are limited. Furthermore, based the results of this study, alachlor–paraquat mixtures are likely to pose serious threats to organisms in aquatic systems in Thailand. Given that the four study herbicides are sold and used in larger quantities than other herbicides in Thailand (Panuwet et al. 2012), while the environmental monitoring that has been done is limited, it is possible that contamination to surface water and ground water might be greater in reality than the data may imply. We hope that this report provides a way of predicting the toxicity level of chemicals and contributes to the public understanding of how some of the most commonly used pesticides in Thailand interact and what effects should be expected of their mixtures. By looking at pesticides in mixtures, the results of this research become much more relevant to pesticide exposure scenarios in the field.

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