Advances in Life Sciences 2015, 5(2): 27-38
DOI: 10.5923/j.als.20150502.01

Prediction of Phenolic Compounds and Formulated Glyphosate Toxicity in Binary Mixtures Using *Rhizobium* Species Dehydrogenase Activity

C. O. Nweke[1,*], J. C. Orji[1], N. C. Ahumibe[2]

[1]Department of Microbiology, Federal University of Technology, Owerri, Nigeria
[2]Department of Biotechnology, Federal University of Technology, Owerri, Nigeria

Abstract The toxicities of binary mixtures of formulated glyphosate and phenols (2,4-dichlorophenol, 4-chlorophenol or phenol) were assessed on the basis of inhibition of INT-dehydrogenase activity in *Rhizobium* species. The binary mixtures were studied along fixed ratio rays. The effective doses (IC_{50s}) were estimated using monotonic logistic and hormesis dose-response models. The joint action of the mixtures on test organism predicted with concentration addition (CA) and independent action (IA) models showed varying degrees of accuracy when compared with the experimentally-determined values. The overestimation, underestimation and accurate prediction of the mixture toxicities indicate possible synergistic, antagonistic and additive action of the mixtures. The model deviation ratios (MDR) between predicted and experimentally observed effect concentrations for most mixtures lie between 0.5 and 2.0. Thus, the joint action of the mixtures were considered additive.

Keywords Concentration addition model, Independent action model, Roundup®, Phenols, Mixture toxicity

1. Introduction

Glyphosate [N-(phosphonomethyl)glycine], a post-emergence herbicide that inhibits the photosynthetic processes in plants is one of the most commonly used herbicide in agriculture. Glyphosate is the active ingredient in Roundup®, a popular herbicide formulation containing isopropylamine (IPA) salt of glyphosate (36% glyphosate) and a surfactant, polyoxyethyleneamine (POEA). In order to improve herbicide action, glyphosate is often used in combination with other herbicides including 2,4-dichlorophenoxyacetic acid (2,4-D) [1-3].

In soils, these herbicides are liable to biodegradation by microorganisms and other biological factors. Glyphosate is moderately persistent in soil with half-life ranging from 2 to 197 days [4-7]. The amine salts and esters of 2,4-D with half-life ranging from 1 to 14 days are not persistent under most environmental conditions [8]. In the environment, 2,4-D is degraded to 2,4-dichlorophenol [9] which can further be degraded to 4-chlorophenol and phenol [10-12].

The phenolic intermediates of 2,4-D biodegradation and glyphosate could interact in the environment where mixture of the herbicides were applied. It is therefore necessary to evaluate the toxic effects of the interaction of glyphosate with 2,4-dichlorophenol, 4-chlorophenol and phenol. There is paucity of information about joint toxicity of glyphosate and phenolic compounds. Nweke *et al*. [13] evaluated the joint action of binary mixtures of Roundup® and these phenolic intermediates of 2,4-D biodegradation against dehydrogenase activity in *Rhizobium* species isolated from the root nodule of a leguminous plant. In the work, Brain and Cousens [14] hormesis model as reparameterized by Schabenberger *et al*. [15] (Eq. 1) was used to predict the effective doses of the individual chemicals and their binary mixtures. Isobolographic analysis, Toxic Unit (TU) and Toxic Index (TI) models were used to evaluate the joint action of the mixtures against the dehydrogenase activity of the test organism.

$$\mathrm{E}[\mathrm{Y}] = y_o + \frac{a - y_o + fx}{1 + \left[\frac{p}{100-p} + \left\{\left(\frac{100}{100-p}\right)\frac{fIC_p}{a-y_o}\right\}\right]\cdot\left(\frac{x}{IC_p}\right)^b} \quad (1)$$

Where x is the concentration of the chemical, y_o is the response at infinite x in a descending dose-response curve, a is the maximum response (of untreated control), f is the parameter describing the degree of hormetic response, p is the percentage decrease in the response, a - y_o, ICp is the concentration of the toxicant at a given p, b is a parameter related to the slope at IC_{50} [16].

* Corresponding author:
xrisokey@yahoo.com (C. O. Nweke)
Published online at http://journal.sapub.org/als

In the present study, using our previous data, the joint action of the glyphosate and phenolic compounds were further analyzed using concentration addition (CA) and independent action (IA) models. The CA and IA models are commonly used to evaluate mixture effect of non-interacting chemicals. The CA model assumes that the chemicals have same or similar mode of action while IA model is based on the assumption that the mixture components have different mechanisms of action. The effective concentrations (IC_{50s}) were predicted with CA and IA models and compared with the values estimated from the experimental data by fitting to dose-response models.

2. Materials and Methods

2.1. Test Organism

Rhizobium species was isolated from the root nodules of *Vigna unguiculata* and prepared for toxicity assay as described by Nweke *et al*. [13].

2.2. Binary Mixture Ratios

The binary mixtures of 2,4-DCP, 4-CP and phenol with glyphosate (as active ingredient in formulated glyphosate pesticide, Roundup®) were studied along fixed ratio rays as described by Nweke *et al*. [13]. The bioassays were aimed at determining the toxicity of each of the three phenolic compounds mentioned above in binary mixture with glyphosate as a function of the following weight to weight ratios: p (%) = 100, 80, 60, 50, 20, and 0 of phenolic compound and 100-p (%) of glyphosate, corresponding to phenolic compound: glyphosate ratios of: 100:0 %, 80:20%, 60:40 %, 50:50 %, 20:80 % and 0:100 % respectively.

2.3. Dehydrogenase Activity Assay

Assay of dehydrogenase activity inhibition in suspended cells of *Rhizobium* species exposed to varying concentrations of the phenolic compounds and glyphosate as individual chemicals and mixtures was done as described by Nweke et al. [13]. The assay was based on inhibition of iodonitrotetrazolium formazan (INTF) production by the bacterial cells in response to the test chemicals. The INTF was extracted with amyl alcohol and the absorbance of the extract determined in a spectrophotometer.

2.4. Data Analysis

The dehydrogenase activities at varying concentrations of the individual component and binary mixtures of glyphosate with 2,4-dichlorophenol, 4-chlorophenol or phenol were calculated relative to the control as shown in Eq. 2 to normalize data to percent inhibition scale. The relative responses were generated as mean and their standard deviations from triplicate determinations.

$$\mathrm{E}[\mathrm{Y}]\,(\%) = \frac{C_A - T_A}{C_A} \times 100 \qquad (2)$$

Where, E[Y] is the relative response (dehydrogenase activity), C_A is the absorbance of INTF extract in the control (without toxicants); T_A is the absorbance of INTF extract in the test with different concentrations of toxicants as individual or their mixtures.

Using the ascending dose-response curves, the inhibition data were tested with 4-parameter logistic model (Eq. 3).

$$\mathrm{E}[\mathrm{Y}]\,(\%) = 100 - \left[y_o + \frac{a - y_o}{1 + \left(\frac{x}{IC_{50}} \right)^b} \right] \qquad (3)$$

Where x is the concentration of the chemical, IC_{50} is the concentration of chemical that caused a 50% response, y_o is the minimum E[Y], a is the maximum response, b is parameter determining the relative slope at IC_{50}. The value, 100 was used to convert descending dose-response curve to ascending dose response curve. By restricting the minimum (y_o) and maximum (a) response to 0 and 100 respectively, Eq. 3 translated to the 2-parameter monotonic logistic model (Eq. 4).

$$\mathrm{E}[\mathrm{Y}]\,(\%) = 100 - \frac{100}{1 + \left(\frac{x}{IC_{50}} \right)^b} \qquad (4)$$

In order to predict stimulation of dehydrogenase activity at low doses of glyphosate or its mixtures with 2,4-DCP, 4-CP or phenol, the dose-response data were fitted into hormesis model (Eq. 5) of Schabenberger *et al*. [15].

$$\mathrm{E}[\mathrm{Y}] = 100 - \left[y_o + \frac{a - y_o + fx}{1 + \left[\frac{p}{100 - p} + \left\{ \left(\frac{100}{100 - p} \right) \frac{fIC_p}{a - y_o} \right\} \right] \cdot \left(\frac{x}{IC_p} \right)^b} \right] \qquad (5)$$

Where the parameters are as described in Eq. 1.

Similarly, by restricting the minimum (y_o) and maximum (a) response to 0 and 100 respectively, Eq. 5 became the 3-parameter hormesis model (Eq.6).

$$\mathrm{E}[\mathrm{Y}] = 100 - \frac{100 + fx}{1 + \left[\frac{p}{100 - p} + \left\{ \left(\frac{100}{100 - p} \right) \frac{fIC_p}{100} \right\} \right] \left(\frac{x}{IC_p} \right)^b} \qquad (6)$$

The dose-response data were fitted with the 2-parameter monotonic logistic model (Eq. 4) and the 3-parameter hormesis model (Eq. 6). Thus, concentration–response curves were fitted with no hormetic effect ($f = 0$) in order to compare the joint-action analyses with and without hormesis. The parameters of the models were estimated using least squares approach implemented with TableCurve 2D v5.01.

The Anova and Duncan post-hoc tests were done using IBM SPSS Statistics 21.

2.5. Prediction of Mixture Toxicities

2.5.1. Concentration Addition Model

The concept of concentration addition is based on the assumption that the components of a given mixture act similarly. If the effect of the mixture is additive (concentration addition), the effective concentration of the mixture ($IC_{x(mix)}$) can be predicted from the equation:

$$IC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{IC_{xi}} \right)^{-1} \quad (7)$$

Where n is the number of chemicals, π_i is the proportion of ith chemical in the mixture, such that the sum of $\pi_i = 1$, IC_x is the concentration of ith chemical that gave x effect when tested as an individual. In a mixture of chemicals with n components, Eq. (7) for an IC_{50i} can be substituted into Eq. (4) to give:

$$\mathrm{E(Y)} = 100 - \frac{100}{1 + \left(\frac{x}{\left(\sum_{i=1}^{n} \frac{\pi_i}{IC_{50i}} \right)^{-1}} \right)^{b}} \quad (8)$$

In Eq. (8), x is the total concentration of all the components in the mixture and b is the average slope of the individual components at IC_{50}. Eq. (8) can be rearranged to give:

$$\mathrm{E(Y)} = 100 - \frac{100}{1 + \left(\sum_{i=1}^{n} \frac{\pi_i x}{IC_{50i}} \right)^{b}} \quad (9)$$

The term $\pi_i x$ represent the concentration of ith chemical in the mixture. After the determination of the individual IC_{50}, the CA model (Eq. 9) was used to predict the joint effect of the binary mixtures. By substituting Eq. 7 into Eq. 6, the concentration addition (CA) model based on the hormesis model for IC_{50} (p =50) can be written as:

$$\mathrm{E[Y]} = 100 - \frac{100 + fx}{1 + \left[1 + \left(\frac{2f}{100 \left(\sum_{i=1}^{n} \frac{\pi_i}{IC_{50i}} \right)} \right) \right] \cdot \left(\sum_{i=1}^{n} \frac{\pi_i x}{IC_{pi}} \right)^{b}} \quad (10)$$

The size of hormetic response f was predicted from the relative proportion of each compound in the mixture as described by Belz et al. [17]. If two compounds A and B are mixed with a proportion p of A and (100-p) of compound B, then the expected f at p (f_p) is given by:

$$f_p = f_A \frac{p}{100} + f_B \frac{100 - p}{100} \quad (11)$$

Using the predicted f and average b value, the effect of the binary mixture on stimulation of dehydrogenase activity was predicted on the basis of concentration addition (CA) using the hormesis model (Eq. 10). In each case, the predicted IC_{50} was compared with the experimental IC_{50}.

2.5.2. Independent Action (Response Addition) Model

The concept of independent action or response addition is based on the assumption that the components of a given mixture act dissimilarly. The mathematical expression is as follows:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \quad (12)$$

Where $E(c_{mix})$ represents the total effect or response (scaled from 0 – 1) of an n-compound mixture, c_i is the concentration of the ith compound and $E(c_i)$ is the effect or response of the individual component.

Once the responses of the individual chemicals were established, the toxic effects of the binary mixtures were subsequently predicted using the CA and IA models. The experimentally derived effects were then compared with the predicted effects. The model deviation ratios (MDR) were calculated as the ratio between the predicted effect concentration and the experimentally-observed effect concentration at IC_{50}. A MDR of greater than 1 indicated that the model underestimated toxicity, while a value of less than 1 indicated that the model overestimated toxicity. MDR values ranging from 0.5 to 2 ($0.5 \leq \mathrm{MDR} \leq 2$) indicated that the mixture was most likely to be additive [18, 19].

3. Results

3.1. Toxicity of Single Chemicals

The inhibition of dehydrogenase activity in *Rhizobium* species by glyphosate, 2,4-dichlorophenol, 4-chlorophenol and phenol as single compound as well as the fit of the logistic and hormesis models are shown in Figure 1. All the compounds presented biphasic dose response curves characterized by stimulation of dehydrogenase activity at low doses and inhibition at high doses. However, 4-chlorophenol and glyphosate presented marginal hormetic effects at concentrations ranging from 20 to 60 mg/l and 20 to 400 mg/l respectively. Relatively, 2,4-DCP and phenol were found to have a more significant stimulatory effect on the dehydrogenase activity in *Rhizobium* species with stimulation peak value been 6.9% and 10.3% respectively. At concentrations above the stimulatory levels, glyphosate and phenolic compounds increasingly inhibited dehydrogenase activity as the concentration increased,

reaching saturation at values below 100%. The maximum inhibition of dehydrogenase activity ranged from 88.37% for phenol to 99.14% for formulated glyphosate. The IC_{50s} of the individual compounds varied significantly ($p < 0.05$).

The decreasing order of toxicity was 2,4-DCP > 4-CP > glyphosate > phenol.

3.2. Toxicity of Binary Mixtures of Chemicals

The dose response plots, the model fits for the chemical mixtures and the toxicities of the mixtures predicted from the CA and IA models are shown in Figures 2 – 4. The binary mixtures of phenolic compounds with glyphosate as with the individual compounds were found to be stimulatory to the dehydrogenase activity at low doses and inhibitory at high doses. This stimulatory effect was higher with phenol and glyphosate mixtures. The peak stimulation values were 24.02%, 22.7%, 24.4% and 23.2% for mixtures containing 20%, 50%, 60% and 80% phenol respectively (Fig. 4). As with the individual compounds, the mixtures showed saturation of inhibitory effect. The maximum inhibition of dehydrogenase activity ranged from 94.40% to 99.77% in the mixtures. The experimental data were adequately described by the logistic and hormesis models.

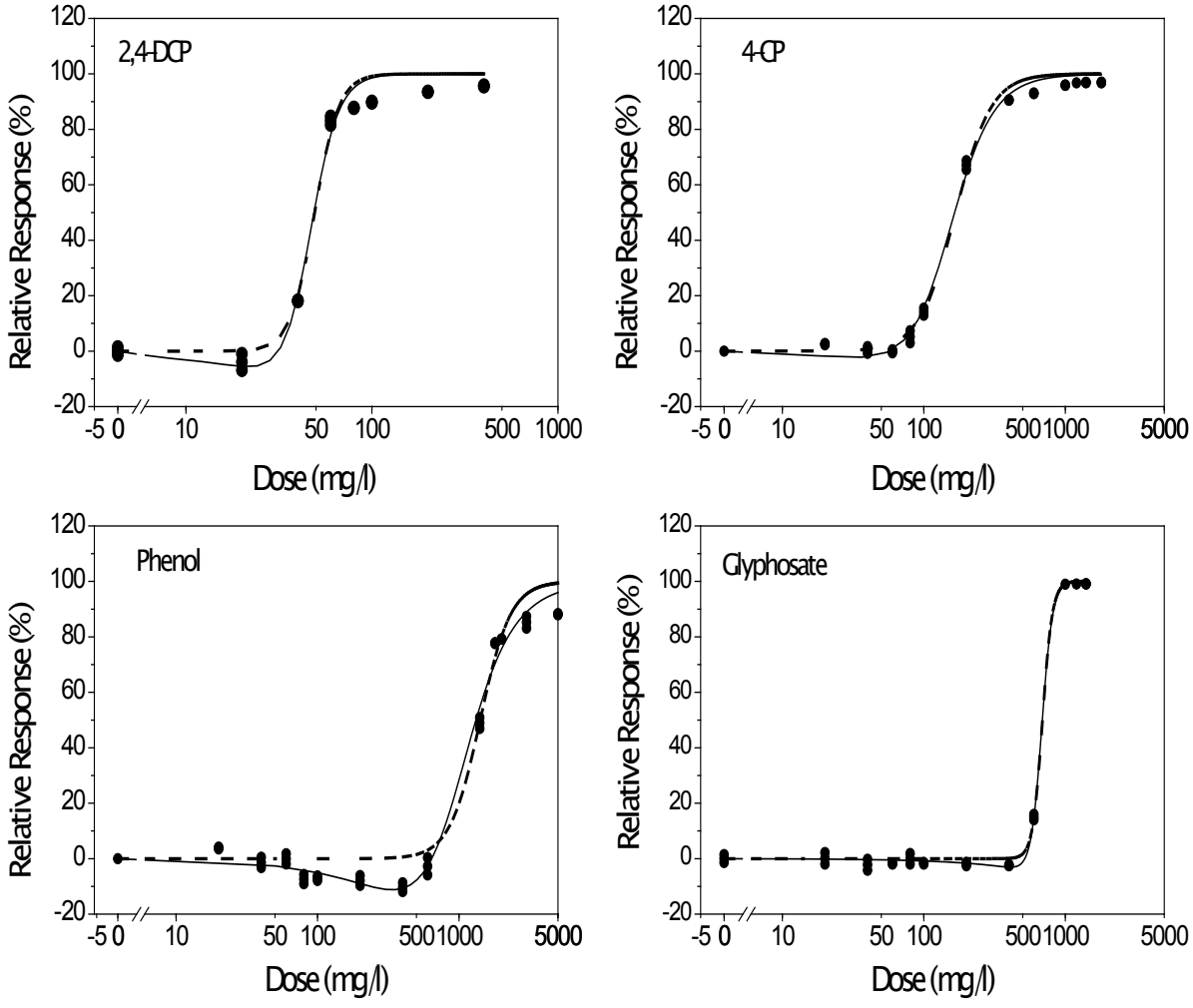

Figure 1. Relative responses of *Rhizobium* species dehydrogenase activity to 2,4-dichlorophenol (2,4-DCP), 4-chlorophenol (4-CP), phenol and formulated glyphosate applied singly. The symbols (closed circle) represent experimental data. Lines represent predicted inhibitions from the monotonic logistic model (Eq. 4) [dashed line] and the hormesis model (Eq. 6) [solid line]

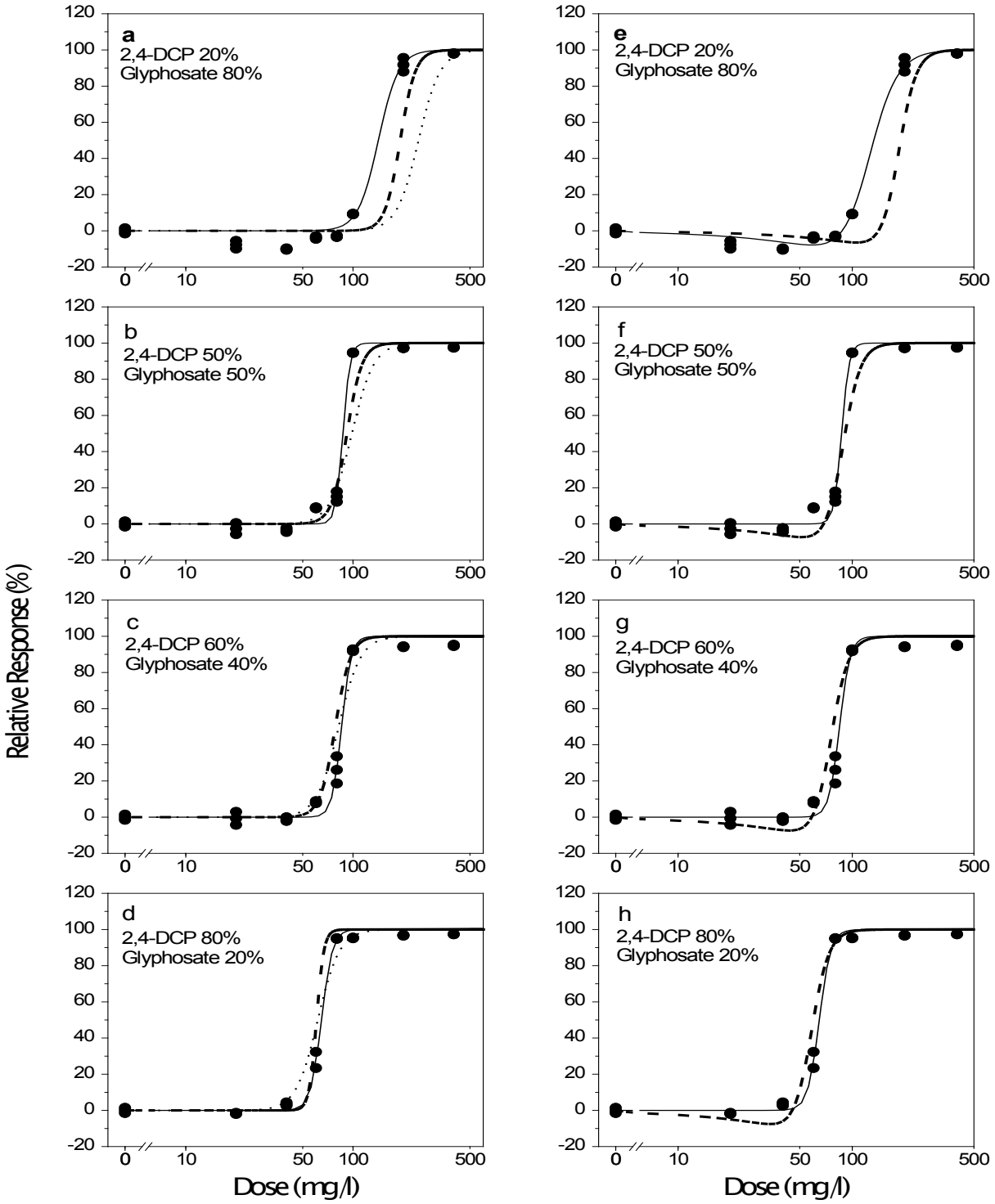

Figure 2. Experimental (data points) and model-predicted toxicity (lines) of binary mixtures of glyphosate and 2.4-dichlorophenol (2,4-DCP) to the dehydrogenase activity of *Rhizobium* species with and without hormesis. The solid lines are the toxicities predicted from the monotonic logistic model (Eq. 4) [a-d] and the hormesis model (Eq. 6) [e-h]. The dashed lines represent values predicted from the CA model and dotted lines represent values predicted from the IA model

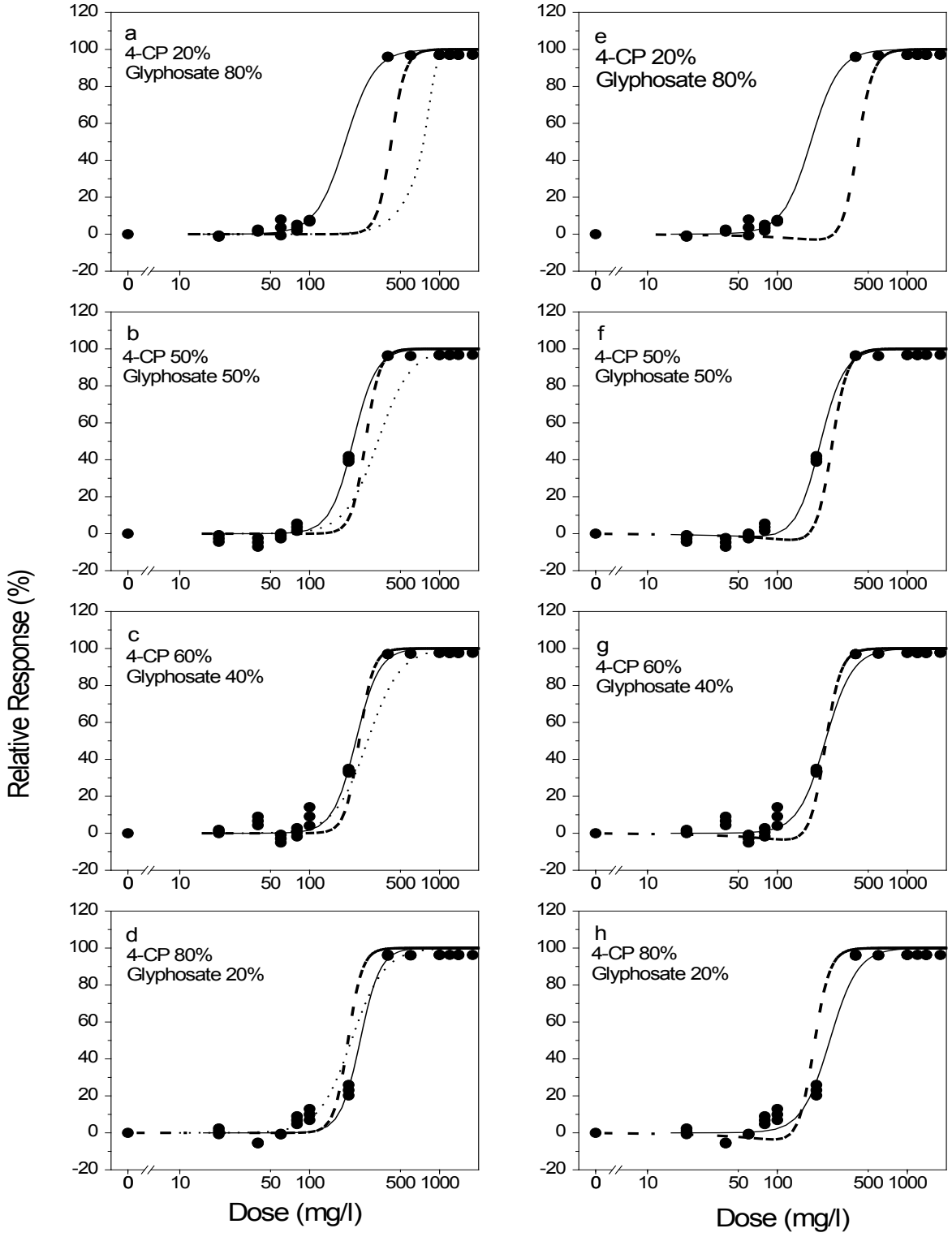

Figure 3. Experimental (data points) and model-predicted toxicities (lines) of binary mixtures of glyphosate and 4-chlorophenol (4-CP) to the dehydrogenase activity of *Rhizobium* species with and without hormesis. The solid lines are the toxicities predicted from the monotonic logistic model (Eq. 4) [a-d] and the hormesis model (Eq. 6) [e-h]. The dashed lines represent values predicted from the CA model and dotted lines represent values predicted from the IA model

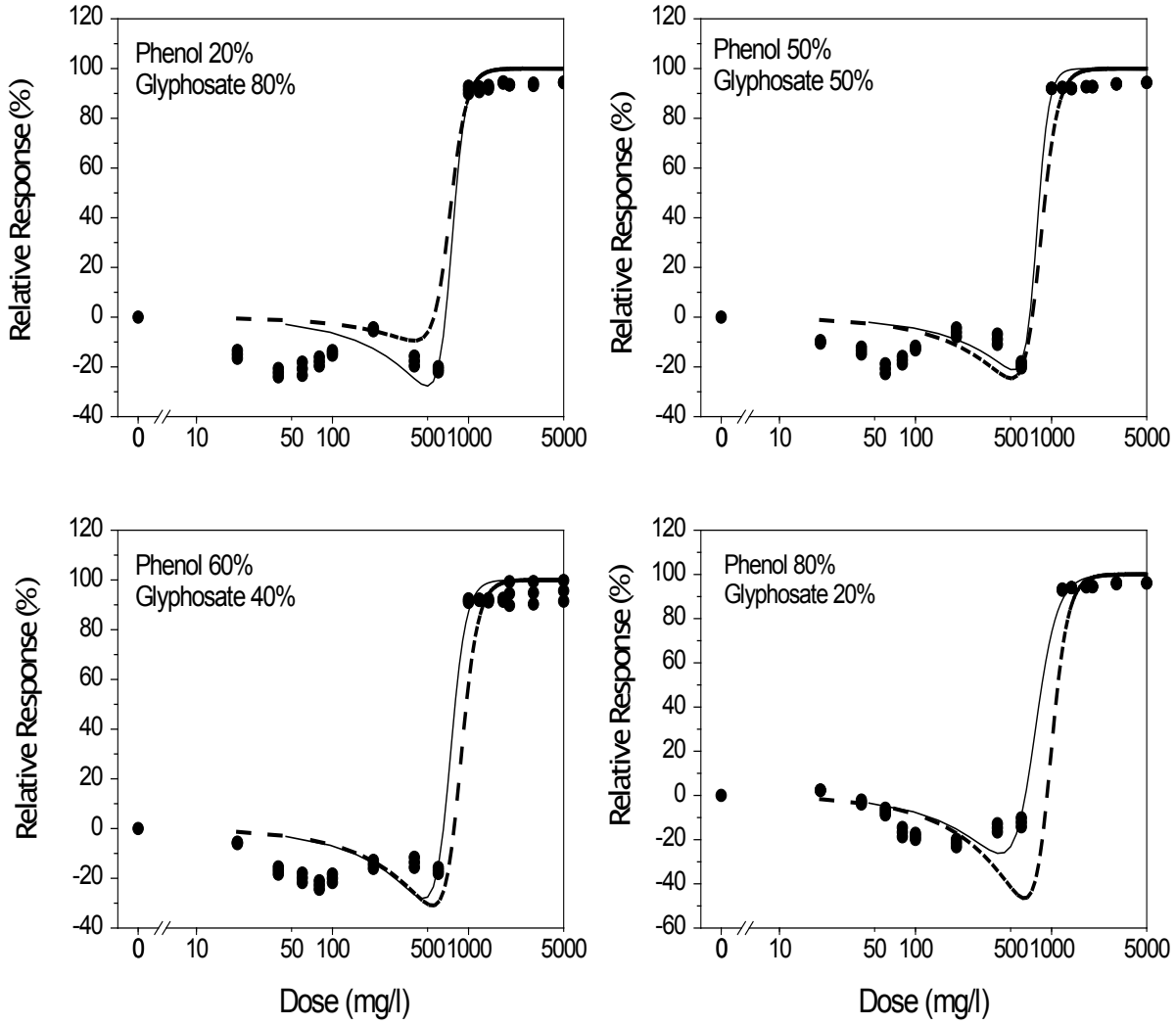

Figure 4. Experimental (data points) and model-predicted toxicities (lines) of binary mixtures of glyphosate and phenol to the dehydrogenase activity of *Rhizobium* species. The solid lines are the toxicities predicted from the hormesis model (Eq. 6). The dashed lines represent values predicted from the CA model (Eq. 10)

The CA model predicted significantly lower toxicities than the experimental data would suggest for 20% 2,4-DCP/80% glyphosate (Fig. 2). Based on the monotonic model, there were no significant difference ($p > 0.05$) between CA-predicted and experimentally derived IC_{50} values for 50% 2,4-DCP/50% glyphosate and 80% 2,4-DCP/20% glyphosate mixtures. Considering hormesis, all the predicted IC_{50} values were significantly different from the experimental values except for 50% 2,4-DCP/50% glyphosate mixture. In the case of 4-CP/glyphosate mixtures, with or without hormesis, CA model also underestimated the toxicity of 20% 4-CP/80% glyphosate mixture (Fig. 3). Although the CA model underestimated the toxicity of 50% 4-CP/50% glyphosate mixture, the IC_{50} values obtained was close to the experimentally-derived value. On the other hand, the toxicity of 80% 4-CP/20% glyphosate was slightly overestimated by CA model. The CA model predicted IC_{50} that was not significantly different from the experimental values for 60% 4-CP/40% glyphosate with or without hormesis. Based on the hormesis model, for 20% phenol/80% glyphosate and 50% phenol/50% glyphosate mixtures, the CA model predictions were similar to the experimentally derived toxicities, although the size of hormetic effect was underestimated with 20% phenol/80% glyphosate mixtures. CA model slightly underestimated the toxicities of 60% phenol/40% glyphosate and 80% phenol/20% glyphosate mixtures. However, the predicted IC_{50} and experimental IC_{50} were only significantly different with 60% phenol/40% glyphosate and 80% phenol/20% glyphosate mixtures. The size of hormetic effects of 80% phenol/20% glyphosate was overestimated by the CA model (Fig. 4). Similarly, on the basis of the monotonic logistic model, the IA model showed similar pattern of predictions with CA model for 2,4-DCP/glyphosate and 4-CP/glyphosate mixtures. However, IA model predicted lower toxicities of 20% 2,4-DCP/80% glyphosate and 20% 4-CP/80% glyphosate mixtures than CA model (Figs. 2 and 3).

The parameters of the dose-response functions for the individual compounds and mixtures and the statistical associations of the IC_{50} values are shown in Table 1. In all

the mixtures and individual chemicals, the IC_{50} values obtained from the monotonic logistic models were not significantly different from the values derived from the hormesis model. With the exception of 80% 4-CP/20% glyphosate mixture and phenol as an individual chemical, the IC_{50} values derived from hormesis model (Eq. 6) by restricting minimum and maximum response to 0 and 100% respectively were not significantly different from the IC_{50} values derived from hormesis model (Eq. 5) considering the saturation effect at inhibitions below 100%.

Table 1. Parameters of the dose-response functions for the individual chemicals and mixtures

Mixture/Ratio	Monotonic Logistic Model (Eq. 4)			Hormesis model (Eq. 6)			
(%)	IC_{50} (mg/l) †	*b*	R^2	IC_{50} (mg/l)‡,†	*f*	*b*	R^2
2,4-DCP/GLY							
100:0	49.236 ± 2.585a	6.686 ± 1.441	0.979	48.776 ± 2.136a*	0.312 ± 0.375	5.874 ± 1.229	0.981
80:20	64.742 ± 1.125a,b	13.390 ± 2.529	0.995	64.737 ± 1.168b*	0.00013 ± 0.087	13,248 ± 2.588	0.995
60:40	85.489 ± 1.702b	15.342 ± 3.621	0.988	84.599 ± 1.773c*	7.92E-6 ± 0.087	14.336 ± 3.960	0.985
50:50	87.009 ± 1.825b	20.500 ± 4.594	0.992	86.894 ± 1.915c*	3.58E-6 ± 0.068	20.122 ± 4.779	0.991
20:80	141.670 ± 12.737c	7.167 ± 1.819	0.983	133,106 ± 5.649d*	0.163 ± 0.056	5.503 ± 0.738	0.994
0:100	689.511 ± 32.021d	12.502 ± 4.102	0.998	687.240 ± 19.100e*	0.008 ± 0.003	11.473 ± 2.311	0.999
4-CP/GLY							
100:0	167.741 ± 6.784a	3.434 ± 0.363	0.995	165.045 ± 6.876a*	0.105 ± 0.079	2.929 ± 0.292	0.996
80:20	245.105 ± 14.846c	5.687 ± 1.413	0.990	253.201 ±16.742c*	1.97E-5 ± 0.049	4.046 ± 0.834	0.988
60:40	226.985 ± 9.710c	4.893 ± 1.066	0.993	234.140 ± 11.375b,c*	5.50E-6 ± 0.039	4.108 ± 0.770	0.992
50:50	216.217 ± 6.786b,c	4.896 ± 1.088	0.996	216.030 ± 6.498b*	0.029 ± 0.037	4.644 ± 0.935	0.996
20:80	188.373 ± 15.308a,b	3.851 ± 0.436	0.997	180.907 ± 20.330a*	4.66E-6 ± 0.046	4.004 ± 0.672	0.997
0:100	689.511 ± 32.021d	12.502 ± 4.102	0.998	687.240 ± 19.100d*	0.008 ± 0.003	11.473 ± 2.311	0.999
Phenol/GLY							
100:0	1411.555 ± 99.184	4.035 ± 1.203	0.969	1315.416 ± 66.439a*	0.053 ± 0.020	2.714 ± 0.286	0.987
80:20	-	-	-	848.218 ± 55.413b	0.077 ± 0.020	5.532 ± 0.966	0.985
60:40	-	-	-	796.537 ± 63.012b,c	0.070 ± 0.027	7.760 ± 2.208	0.968
50:50	-	-	-	818.378 ± 82.205b	0.045 ± 0.023	10.112 ± 4.204	0.971
20:80	-	-	-	808.321 ± 71.487b	0.063 ± 0.026	8.710 ± 2.836	0.966
0:100	689.511 ± 32.021	12.502 ± 4.102	0.998	687.240 ± 19.100c*	0.008 ± 0.003	11.473 ± 2.311	0.999

‡ In each mixture ratio, values with asterisk indicates that IC50 derived from the hormesis model is not significantly ($p > 0.05$) different from the IC50 values derived from monotonic logistic model.

† In each mixture type and IC_{50} derived from a particular model, values with same letters are not significantly ($p > 0.05$) different

Table 2. Model Deviation Ratios (MDR) for comparison of predicted and experimentally-observed toxicity

Mixture/Ratio	Monotonic Logistic Model (Eq. 4)		Hormesis model (Eq. 6)
(%)	CA	IA	CA
2,4-DCP/GLY			
80:20	0.933	0.923	0.925
60:40	0.916	0.936	0.918
50:50	1.056	1.140	1.048
20:80	1.352	1.723	1.427
4-CP/GLY			
80:20	0.806	0.881	0.769
60:40	1.060	1.239	1.013
50:50	1.248	1.335	1.232
20:80	2.257	3.992	2.327
Phenol/GLY			
80:20	-	-	1.311
60:40	-	-	1.209
50:50	-	-	1.103
20:80	-	-	0.940

The comparisons of the experimental IC_{50} values derived from the monotonic logistic model (Eq. 4) and hormesis model (Eq. 6) fits with the IC_{50} values predicted from CA and IA models are shown in Fig. 5. The IC_{50} values predicted from the CA and IA models were close to the experimental IC_{50} values except for 20% 2,4-DCP or 4-CP and 80% glyphosate. The slight differences between the observed and the predicted IC_{50s} could be attributed to marginal synergistic or antagonistic effects observed in these mixtures. There were strong positive correlations between the proportion of the components and the observed IC_{50} for the 2,4-DCP/glyphosate ($R^2 = 0.96$) and 4-CP/glyphosate ($R^2 = 0.82$) mixtures. On the contrary, there was a very weak correlation ($R^2 = 0.04$) between the proportion of phenol and the IC_{50} for phenol/glyphosate mixtures. The model deviation ratios (MDR) for comparison of predicted and experimentally observed toxicity of the mixtures are shown in Table 2. With the exception of 20% 4-CP/80% glyphosate, all MDR values lie between 0.5 and 2.0.

4. Discussion

The monotonic and hormesis models gave good descriptions of the experimental data with R^2 values ranging from 0.966 to 0.999 and greater than 0.99 in most cases. The effects of using monotonic logistic model to determine the toxicity of hormetic mixtures were marginal. Changes in IC_{50} values of the mixtures were insignificant when monotonic logistic model was used instead of hormesis model. This indicates, as was observed by Calabrese [20], that above the toxic threshold, the shape of the dose-response curve is similar for monotonic and hormesis model. Similar observation was made by Belz et al. [17] on the growth of *Lactuca sativa* and *Lemna minor* exposed to binary mixtures of herbicides. Hormesis in microorganisms have been reported to be associated with microbial exposure to phenols and glyphosate for a number of microbial responses. This phenomenon has been widely reported among bioluminescent bacteria [21-23]. Activities of dehydrogenase enzymes in bacterial cells have also been reported to be stimulated by phenols at low doses [24-26]. Above the hormetic doses, hormetic substances become toxic. This was also observed with the phenolic compounds and formulated glyphosate used in this study. At high concentrations, the phenols inhibited the dehydrogenase activity of the *Rhizobium* species in a manner proportional to their octanol-water partition coefficient. Similarly, the formulated glyphosate exerted toxic effects on the activity of dehydrogenases in the test organism. These substances are known to be toxic to microorganisms. Phenols for instance are known membrane-damaging substances [27-29]. Glyphosate is reportedly toxic to microorganisms [30, 31]. In Roundup®, its toxicity may further be increased by the surfactant component, polyoxyethyleneamine [30].

In mixtures, these substances could interact to modulate the toxicity of each other. The presence of glyphosate modulated the toxicity of the phenolic compounds. This modulation was more prominent with 20% 2,4-DCP/80% glyphosate and 20% 4-CP/80% glyphosate mixtures.

According to our previous analyses, synergistic, additive and antagonistic actions depending on the relative ratios of the individual components were possible [13]. The isobolographic analysis indicated that 20% 2,4-DCP/80% glyphosate and 20% 4-CP/80% glyphosate mixtures were synergistic [13]. This observed deviation from additivity was supported in the present study by the predictions of CA models based on the monotonic logistic model and the hormesis model. These variations in the predictions of the CA model could be attributed to synergistic and antagonistic effects of some mixtures [32-34], as were suggested by the isobolographic analysis of the mixtures [13]. The model deviation ratios (MDR) between predicted and experimentally observed effect concentrations lie between 0.5 and 2.0, suggesting that the deviations are marginal and within the expected inter-laboratory/inter-experiment deviation for most species [18, 19]. These ratios and their effects corroborated the results of the previous study done with isobolographic analysis of the experimental data [13].

The size of hormetic effects were either overestimated or underestimated by the CA model, which might be related to non-linear relationship of the size of hormesis with the mixture ratio. This observation could be attributed to marginal hormetic responses in the mixtures. Size of hormesis was suggested to change linearly with mixture ratio if there are no interactions between the chemicals showing large and reproducible hormesis [17].

The results indicated that the toxicities of the mixtures could be predicted by CA and IA models. These models were intended for the prediction of toxicity of chemical mixture on the basis of dose-response curve of the individual components in the mixture. While the CA model assumes that the mixture components have similar mode of action, IA model assumes that the components have dissimilar mode of action. Although the phenolic compounds and the glyphosate or its adjuvant, POEA may have different modes of action against bacteria, significant difference did not exist between the predicted values of mixture toxicity on the basis of CA and IA for most mixtures of glyphosate with 2,4-DCP or 4-CP. Similar insignificant differences between mixture toxicity predicted on the basis of CA and IA for phenolic compounds with similar and dissimilar action mechanisms was reported by Huang et al. [32]. Likewise, in a similar study, Barata et al [35] reported accurate prediction of mixture toxicity of dissimilarly acting chemicals by the CA and IA models. In addition, virtually identical toxicities predicted from CA and IA model for mixtures of similarly-acting phenylurea derivatives were reported [36]. This showed that both concepts, concentration addition and independent action, may serve as valuable tools for predicting toxicity of chemical mixtures [37].

5. Conclusions

The toxicities of the binary mixtures of formulated glyphosate and phenols (2,4-DCP, 4-CP and phenol) were analyzed using concentration addition (CA) and independent action (IA) models. The CA and IA models predicted the toxicity of the mixtures with variable degree of accuracy in relation to experimental values, indicating possibility of synergistic, additive and antagonistic actions depending on the relative ratios of the individual components. However, most model deviation ratios (MDR) between predicted and experimentally observed effect concentrations lie between 0.5 and 2.0. These values are within the expected inter-laboratory/inter-experiment deviation for most species. We therefore concluded that the joint action of the mixtures on *Rhizobium* species dehydrogenase activity were additive.

This information constitutes an essential contribution towards assessing the environmental risk of glyphosate and some intermediates of 2,4-D degradation. Biodegradation processes and the prevailing environmental conditions that determine the residual amounts of these herbicides and their metabolic intermediates in the environment may constitute important factors affecting toxicity of these chemicals. This study needs to be extended to microbial communities of soil or aquatic ecosystems, since microbes exist as a consortium rather than pure cultures in natural environments.

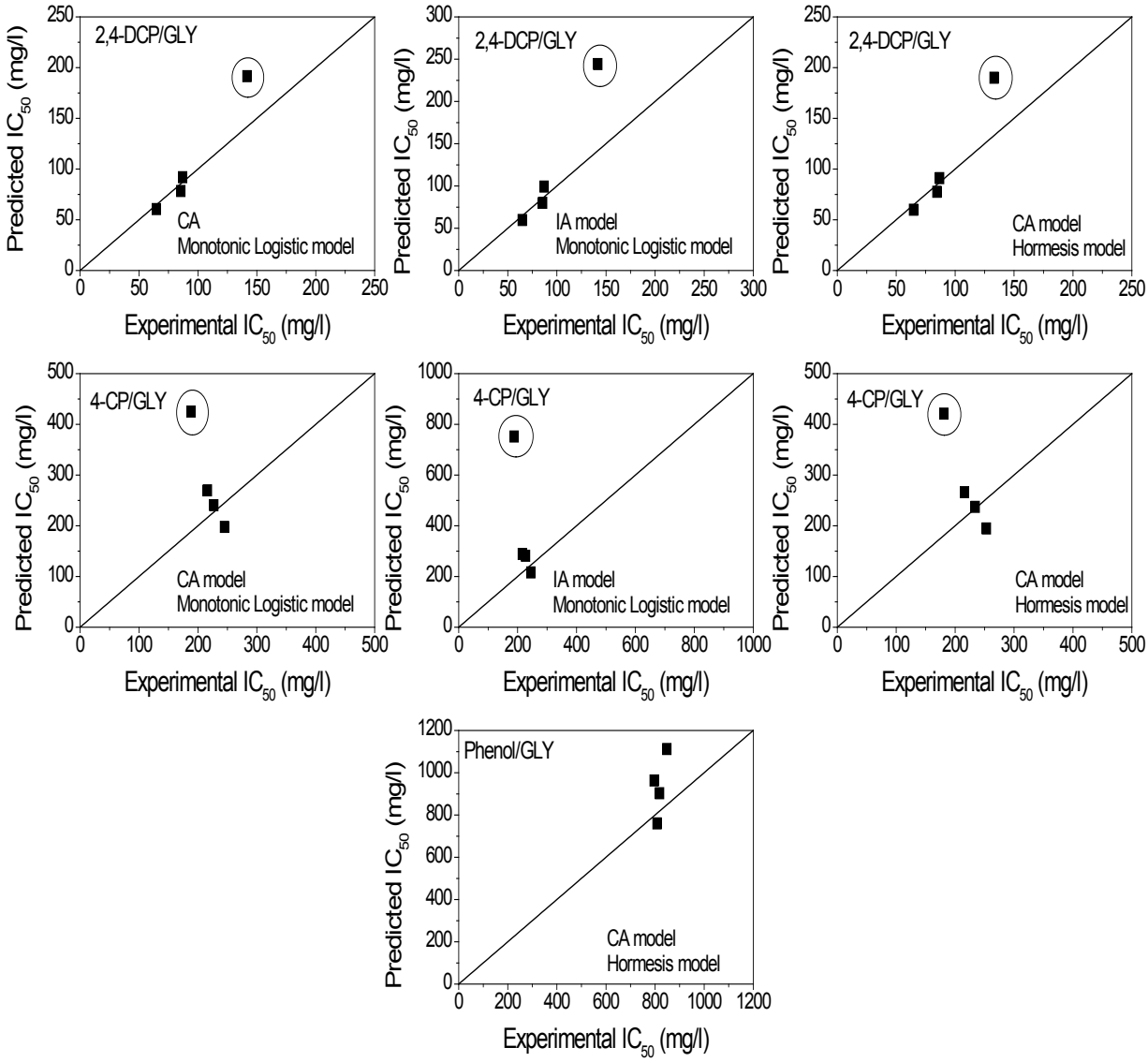

Figure 5. Comparison of the experimental IC_{50} derived from the monotonic logistic model (Eq. 4) and hormesis model (Eq. 6) with the predicted IC_{50} according to CA and IA models. The encircled data point is that of 20% 2,4-dichlorophenol (2,4-DCP) or 4-chlorophenol (4-CP) and 80% glyphosate (GLY); solid lines represent $y = x$ plots

REFERENCES

[1] O'Sullivan, P. A., and O'Donovan, J. T., 1980, Influence of various herbicides and Tween 20 on the effectiveness of glyphosate. Can. J. Plant Sci. 60, 939 – 945.

[2] Lym, R. G., 2000, Leafy spurge (*Euphorbia esula*) control with glyphosate plus 2,4-D. J. Range Manage. 53, 66 – 72.

[3] Sharma, S. D., and Singh, M., 2001, Surfactants increase toxicity of glyphosate and 2,4-D to Brazil Pulsey. HortSci. 36(4), 726 – 728.

[4] U.S.D.A., Forest Service, 1984. Pesticide background statements. p. G1-G72. In Agriculture Handbook No. 633. Vol. 1. Herbicides. Part 2.

[5] WHO, 1988. Environmental Health Criteria 159, Toxicological Evaluations - Glyphosate; International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland.

[6] U.S. EPA, 1990. Pesticide Fact Handbook. Vol. 2. p. 301-312. Noyes Data Corporation. Park Ridge, New Jersey.

[7] Giesey, J. P., Dobson, S., and Solomon, K. R., 2000, Ecotoxicological risk assessment for Roundup herbicide. Rev. Environ. Contam. Toxicol. 167, 35 – 120.

[8] U.S. EPA, 2005, Reregistration Eligibility Decision (RED) 2,4-D; EPA 738-R-05-002; U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, U.S. Government Printing Office: Washington, DC, 2005.

[9] Daugherty, D., and Karel, S., 1994, Degradation of 2,4-dichlorophenoxyacetic acid by *Pseudomonas cepacia* DBO1 (pRO101) in a dual-substrate chemostat. Appl. Environ. Microbiol. 60, 3261 – 3267.

[10] Kohring, G-W., Zhang, X., and Wiegel J., 1989, Anaerobic dechlorination of 2,4-dichlorophenol in freshwater sediments in the presence of sulphate. Appl. Environ. Microbiol. 55(10), 2735 – 2737.

[11] Zhang, X., and Wiegel, J., 1990, Sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments. Appl. Environ. Microbiol. 56(4), 1119 – 1127.

[12] Fukumori, F., and Hausinger, R., 1993, *Alcaligenes eutrophus* JMP134 "2,4- dichlorophenoxyacetatemonooxyge nase" is an α-ketoglutarate-dependent dioxygenase. J. Bacteriol. 175, 2083 – 2086.

[13] Nweke, C. O., Ahumibe N. C.,and Orji J. C., 2014, Toxicity of binary mixtures of formulated Glyphosate and phenols to *Rhizobium* species dehydrogenase activity. J. Microbiol. Res. 2014, 4(4): 161–169.

[14] Brain, P., and Cousens, R., 1989, An equation to describe dose responses where there is stimulation of growth at low doses. Weed Res. 29, 93 – 96.

[15] Schabenberger, O., Tharp, B. E., Kells, J. J., and Penner, D., 1999, Statistical test for hormesis and effective dosages in herbicide dose–response. Agron J. 91, 713–721.

[16] Cedergreen, N., Ritz, C. and Streibig, J. C., 2005, Improved empirical models describing hormesis. Environ. Toxicol. Chem. 24(12), 3166 – 3172.

[17] Belz R. G., Cedergreen N., and Sørensen H. 2008, Hormesis in mixtures — Can it be predicted? Science of the Total Environ. 404, 77 – 87.

[18] Petersen, K., and Tollefsen, K.E. 2011, Assessing combined toxicity of estrogen receptor agonists in a primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat. Toxicol. 101, 186 – 195.

[19] Li,Y., Zhang B., He X., Cheng W-H., Xu W., Luo Y., Liang R., Luo H.and Huang K., 2014, Analysis of individual and combined effects of ochratoxin A and zearalenone on HepG2 and KK-1 cells with mathematical models. Toxins *6*, 1177 – 1192.

[20] Calabrese, E. J., 2008, Hormesis: why it is important to toxicology and toxicologists. Environ. Toxicol. Chem. 27, 1451– 1474.

[21] Brack, A., Strube J., Stolz P., and Decker H., 2003, Effects of ultrahigh dilutions of 3,5-dichlorophenol on the luminescence of the bacterium *Vibrio fischeri*. Biochimica et Biophysica Acta 1621: 253 – 260.

[22] Zhang, J., Liu, S-S, Yu, Z-Y., and Zhang, J., 2013, Time -dependent hormetic effects of 1-alkyl-3-methyllimidazolium bromide on *Vibrio qinghaiensis* sp.-Q67: Luminescence, redox reactants and antioxidases. Chemosphere 91, 462 – 467.

[23] Zou, X., Lin, Z., Deng, Z., and Yin, D., 2013, Novel approach to predicting hormetic effects of antibiotic mixtures on *Vibrio fischeri*. Chemosphere 90, 2070 – 2076.

[24] Okolo, J.C., Nweke, C.O., Nwabueze, R.N., Dik,e C.U., and Nwanyanwu, C.E., 2007, Toxicity of phenolic compounds to oxidoreductases of *Acinetobacter* species isolated from a tropical soil. Sci. Res. Essay 2(7), 244 – 250.

[25] Nweke, C.O. and Okpokwasili G.C., 2010a, Influence of exposure time on phenol toxicity to refinery wastewater bacteria. J Environ. Chem. Ecotoxicol. 2(2), 20 – 27.

[26] Nweke C.O. and Okpokwasili G. C., 2010b, Inhibition of dehydrogenase activity in petroleum refinery wastewater bacteria by phenolic compounds. Ambi-Água 5(1), 6 – 16.

[27] Keweloh, H., Weyrauch, G., and Rehm, H. J., 1990, Phenol induced membrane changes in free and immobilized *Escherichia coli*. Appl. Microbiol. Biotechnol. 33 65 – 71.

[28] Heipieper, H. J., Keweloh, H., Rehm, H. J., 1991, Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. Appl. Environ. Microbiol. 57, 1213 –1217

[29] Heipieper, H. J., Diefenbach, R., and Keweloh, H., 1992, Conversion of cis unsaturated fatty acids to trans, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Appl. Environ. Microbiol. 58, 1847 – 1852.

[30] Tsui, M. T. K., and Chu, L. M., 2003, Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189 – 1197.

[31] Bonnet, J. L., Bonnemoy, F., Dusser, M., and Bohatier, J.,

2007, Assessment of the potential toxicity of herbicides and their degradation products to non target cells using two microorganisms, the bacteria *Vibriof ischeri* and the ciliate *Tetrahymena pyriformis*. Environ. Toxicol. 22: 78 – 91.

[32] Huang, W. Y., Liu, F., Liu, S. S.. Ge, H. L., and Chen, H. H., 2011, Predicting mixture toxicity of seven phenolic compounds with similar and dissimilar actionmechanisms to *Vibrio qinghaiensis* sp.nov.Q67. Ecotoxicol. Environ. Saf. 74, 1600 – 1606.

[33] Mo, L. Y., Liu, S. S., Zhu, Y. N., Liu, H. L., Liu, H. Y., and Yi, Z. S., 2011, Combined toxicity of the mixtures of phenol and aniline derivatives to *Vibrio qinghaiensis* sp.-Q67, Bull. Environ. Contam. Toxicol. 87 (4), 473 – 479.

[34] Mo, L., Zhu, Z., Zhu, Y. Zeng, H., and Li Y., 2014, Prediction and evaluation of the mixture toxicity of twelve phenols and ten anilines to the freshwater photobacterium *Vibrio qinghaiensis* sp.- Q67. J. Chem. 2014, 1 – 9.

[35] Barata, C., Baird, D.J., Nogueira, A.J.A., Soares, A., and Riva, M.C., 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to Daphnia magna Straus. Implications for multi-substance risks assessment. Aquat. Toxicol. 78, 1 – 14.

[36] Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M., and Grimme, L.H., 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. Environ. Toxicol. Chem. 23, 258 – 264.

[37] Faust, M., Altenburger, R., Backhaus, T., Bödeker, W., Scholze, M., and Grimme, L.H., 2000, Predictive assessment of the aquatic toxicity of multiple chemical mixtures. J. Environ. Qual. 29, 1063 – 1068.