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Can the joint effect of ternary mixtures be predicted from binary mixture toxicity results?

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ABSTRACT

The joint effect of the majority of chemical mixtures can be predicted using the reference model of Concentration Addition (CA). It becomes a challenge, however, when the mixtures include chemicals that synergise or antagonise the effect of each other. In this study we examine if the deviation from CA of seven ternary mixtures of interacting chemicals can be predicted from knowledge of the binary mixture responses involved. We hypothesise that the strongest interactions will take place in the binary mixtures and that the size of the ternary mixture response can be predicted from knowledge of the binary interactions. The hypotheses were tested using a stepwise modelling approach of incorporating the information held in binary mixtures into a ternary mixture model, and comparing the model predictions with observed ternary mixture toxicity data derived from studies of interacting chemical mixtures on the floating plant *Lemna minor* and the bacteria *Vibrio fischeri*. The results showed that for both the antagonistic and the synergistic ternary mixtures the ternary model predictions were superior to the conventional CA reference model and provided robust estimations of the size of the experimentally derived ternary mixture toxicity effects.

1. Introduction

In the environment pollutants occur in mixtures, both because sources contain multiple pollutants and because the pollutants from different sources mix. During the recent decades models have been developed to predict the joint effect of chemical mixtures on organisms and processes in the environment (Altenburger et al., 2003; George et al., 2003; Greco et al., 1995). These models are based on two concepts: Concentration Addition (CA), also called Loewe additivity, additive action, additive dose model etc., and independent action (IA), also called Bliss independence, response multiplication, etc. (Altenburger et al., 2003; Greco et al., 1992; Streibig et al., 1998). Both CA and IA predictions have proved to closely reflect the observed joint effects in studies with >10 components (Altenburger et al., 2000; Arrhenius et al., 2004; Backhaus et al., 2000; Faust et al., 2001, 2003), and there is evidence that the compliance of observations with predictions increases as the number of components increases (Warne and Hawker, 1995). This so-called funnel hypothesis seems to hold, not only for non-polar narcotics which the hypothesis was based upon, but also for chemicals with specific site of action (McCarty and Borgert, 2006), at least as long as a large number of chemicals each contribute with a similar proportion to the joint effect. Hence, biologically significant deviations from a reference model are most likely to occur when toxicity from a few components dominates a multi component mixture (McCarty and Borgert, 2006). For environmental samples this is often the case. Measurements of pesticides in surface waters in USA and Denmark find more than 5 and 7 pesticides in 50% of the water samples tested positive (Gilliom et al., 1999; Jensen et al., 2000), but looking at the concentrations of the individual pesticides in the samples and relating them to the EC50 for e.g. algae or daphnia often show 2–3 pesticides or pollutants to dominate the total sample toxicity (Baas et al., 2009; Junghans et al., 2006).

In terms of risk assessment, addressing the joint effect of known mixtures behaving according to a reference model is trivial, but it becomes a challenge when mixtures include chemicals that induce deviations from the reference model. Though both synergists and antagonists are known (Belden et al., 2007; Cedergreen et al., 2008), it is not straight-forward to predict which mixtures will give rise to deviations from the reference model or to predict the magnitude and direction of the deviations. According to the funnel hypothesis the maximal deviation from a reference model should be found in mixtures of two chemicals. But even though literature studies seem to confirm the funnel hypothesis (McCarty and Borgert, 2006), few

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experiments have been designed to systematically test this hypothesis. And not many attempts have been made to extend models describing deviations from reference models to include mixtures of more than two chemicals (Charles et al., 2002; Jonker et al., 2005; Ren et al., 2004). Binary mixture toxicity experiments can be set up in different ways depending on the hypothesis to be tested and the resources available. The most complete picture of the interactions between two chemicals is achieved by creating concentration–response-surfaces describing the joint effect at all effect levels and mixture ratios (Greco et al., 1995; Jonker et al., 2005; Sørensen et al., 2007). These concentration–response-surfaces can be modelled and the significance and size of the deviation from a chosen reference model can be quantified (Jonker et al., 2005; Sørensen et al., 2007). The question therefore becomes: Can the detailed information gathered from binary concentration–response-surfaces be used to more accurately predict the joint effects of more complex mixtures? From a risk assessor's point of view the major question is: What happens if a potential synergist enters a multiple mixture where it might potentiate more than one compound? Some chemicals are known to induce synergy, for example by affecting the rate at which the organism metabolises other chemicals (Thompson, 1996; Walker, 2009). It has been proposed that safety factors should be increased, if such compounds are present in a mixture (Thompson, 1996). However, it is unknown if and how the maximal level of potentiation can be predicted in order to assess the needed increase in such specific safety factors.

In this study we examine if the deviation from the reference model of seven ternary mixtures of interacting chemicals can be predicted from knowledge of the binary mixture responses involved, assuming that the maximal deviation from the reference model occurs at the binary mixture level. That is, we hypothesise that no stronger interactions will take place in the ternary mixture. The hypothesis will be investigated using a stepwise modelling approach of incorporating the information held in binary mixtures to improve the prediction of ternary mixture effects. First, the binary concentration response surface model of Jonker et al. (2005) will be applied to the three binary sub-mixtures of a ternary mixture. In that way synergistic or antagonistic deviations from the reference model are quantified by three deviation parameters (one per binary sub-mixture). We will use CA as the reference model, as it is considered the most conservative in most cases and often is the model proposed for risk assessment (Cedergreen et al., 2008; Grimme and Backhaus, 2003; Syberg et al., 2009). For the used datasets, previous analyses have shown no differences between CA and IA (Fig. 3 in Cedergreen et al., 2008). This information will then be used to create a joint effect prediction model for the ternary mixture based on the binary deviations only. This ternary model will be constructed based on inclusion of the observed binary deviations proportionately to how much each binary mixture dominates at any given point of the ternary part of the response surface. In a final step, a ternary element will be included to allow the ternary mixtures to deviate from the model based on the binary data. The joint effect predictions of both the CA reference model (based solely on the single chemical concentration response relationships), the binary model (where we add information about the binary deviation patterns), and the truly ternary model (fitted from ternary mixtures) are then compared in terms of the sum of toxic units.

The models were tested on *Lemna minor* and *Vibrio fischeri* exposed to ternary mixtures of chemicals known to interact either antagonistically or synergistically, when tested in binary mixtures. The model of Jonker et al. (2005) was used as a starting point. Only overall antagony/synergy for the binary mixtures was addressed, using only one parameter of Jonker et al., 2005 for describing the deviation from CA, thus ignoring possible concentration level and concentration ratio deviations. This is done as such more detailed deviation patterns have proven difficult to reproduce (Cedergreen et al., 2007a) and to limit the model parameters needed in this exercise.

2. Materials and methods

2.1. Test-systems

The aquatic macrophytes *L. minor* and the bacterial test using the luminescent bacteria *Vibrio fischeri* were chosen because of their relatively small size and short test durations making large experimental setups possible. The test conditions and design for the binary mixtures are published in Cedergreen et al. (2006). For this study, in addition to the three binary mixtures where the ratios 25:75, 50:50 and 75:25% effect concentration were tested, four ternary mixtures were included. The fixed ternary mixture ratios were chosen to give 20:20:60, 20:60:20, 60:20:20 and 33:33:33% effect concentrations based on EC50 estimates from previous experiments. Full concentration response curves were tested at these mixture ratios using six concentrations, three replicates and 12 control treatments for *L. minor*, and eight concentrations, two replicates and four control treatments for *V. fischeri*. The single chemicals, binary and ternary mixtures were always tested simultaneously. A schematic presentation of the treatment design is shown in Fig. 1. For the *L. minor* test, the complete experiments were run twice at separate times for all four ternary mixtures to test the reproducibility of the results.

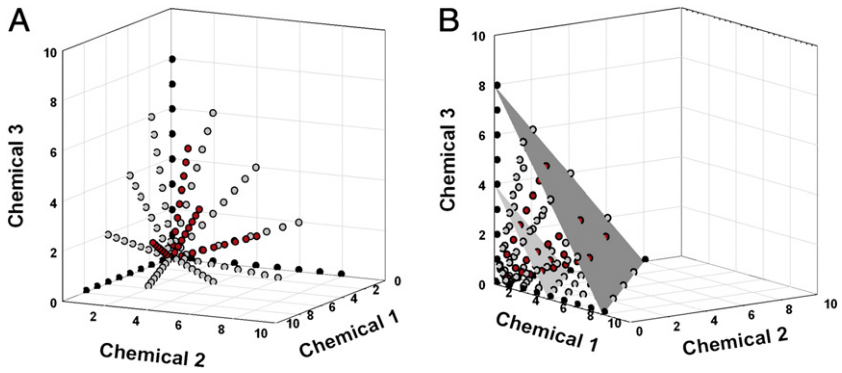

Fig. 1. The experiments were designed to test series of concentration of the three single chemicals (black symbols), three mixture ratios of each of the three binary mixtures (grey symbols) and four mixture ratios of ternary mixtures (red symbols). The binary mixture ratios were chosen to give 25:75, 50:50 and 75:25% effect concentrations while the ternary mixture ratios were chosen to give 20:20:60, 20:60:20, 60:20:20 and 33:33:33% effect concentrations based on EC50 estimates from previous experiments. Panel A shows the raw test design. In panel B we have inserted the ideal Concentration Addition (CA) isobole planes, describing the concentrations of the individual chemicals and their mixtures giving a certain effect, for example a 50% effect.

2.2. Chemicals

Previous experiments with binary combinations of pesticides on *L. minor* did not show any synergistic interactions (Cedergreen et al., 2006, 2007b; Munkegaard et al., 2008). The combinations of pesticides were therefore based on mixtures of chemicals responding in accordance to CA and exhibiting different degrees of antagonism. Six herbicides with different modes of action were used: acifluorfen (a protoporphyrinogen oxidase inhibitor), diquat (a photosystem I inhibitor), glyphosate (an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase), mecoprop (a synthetic auxin), mesotrione (an inhibitor of carotenoid synthesis), and terbuthylazine (a photosystem II inhibitor) (Tomlin, 2002). In the *V. fischeri* test system, previous studies on binary mixtures showed synergy both when the imidazole fungicide prochloraz (an ergosterol biosynthesis inhibitor), the organophosphorous insecticide chlorfenvinphos (acetylcholinesterase inhibitor) and, at times, the herbicide acifluorfen were present in the mixture (Cedergreen et al., 2006). Mixtures of pesticides for *V. fischeri* were therefore designed to include one or two of these pesticides. In addition diquat and dimethoate (acetylcholinesterase inhibitor) were included, as they had been observed to be synergised by the above-mentioned pesticides.

2.3. Model formulation

For binary mixtures the deviation patterns from the CA model were described in the model framework of Jonker et al. (2005). For a full appreciation of this framework please see the original paper. In the following a simplified description of the model elements used in this paper is presented.

2.3.1. Single concentration response relationship

The log–logistic function is used to describe how the biological response relates to exposure concentrations for the individual chemicals (Ritz, 2010). This model was chosen for its simplicity and adequacy of describing data, as discussed in Jonker et al. (2005) and Sørensen et al. (2007). Hence, for chemical i, the concentration c of the chemical and the expected biological response Y are assumed to be related as follows:

$$Y = f_i(c) = \frac{Y_0}{1 + (c/\mathrm{EC50}_i)^{b_i}}. \quad (1)$$

Here, Y_0 is the average control response, and $\mathrm{EC50}_i$ and b_i denote the 50% effect concentration and the slope parameter for chemical i, respectively. The relationship is illustrated in the top of Fig. 2. The relationship can be rearranged to express concentration on the basis of observed effect:

$$c = f_i^{-1}(Y) = \mathrm{EC50}_i \left(\frac{Y_0 - Y}{Y}\right)^{1/b_i}. \quad (2)$$

For a given biological response Y, Eq. (2) gives the concentration c of chemical i that on average would result in response Y if the chemical is applied on its own.

2.3.2. Concentration Addition

A mixture of n chemicals that results in an x% joint effect compared to the control response is said to be additive according to

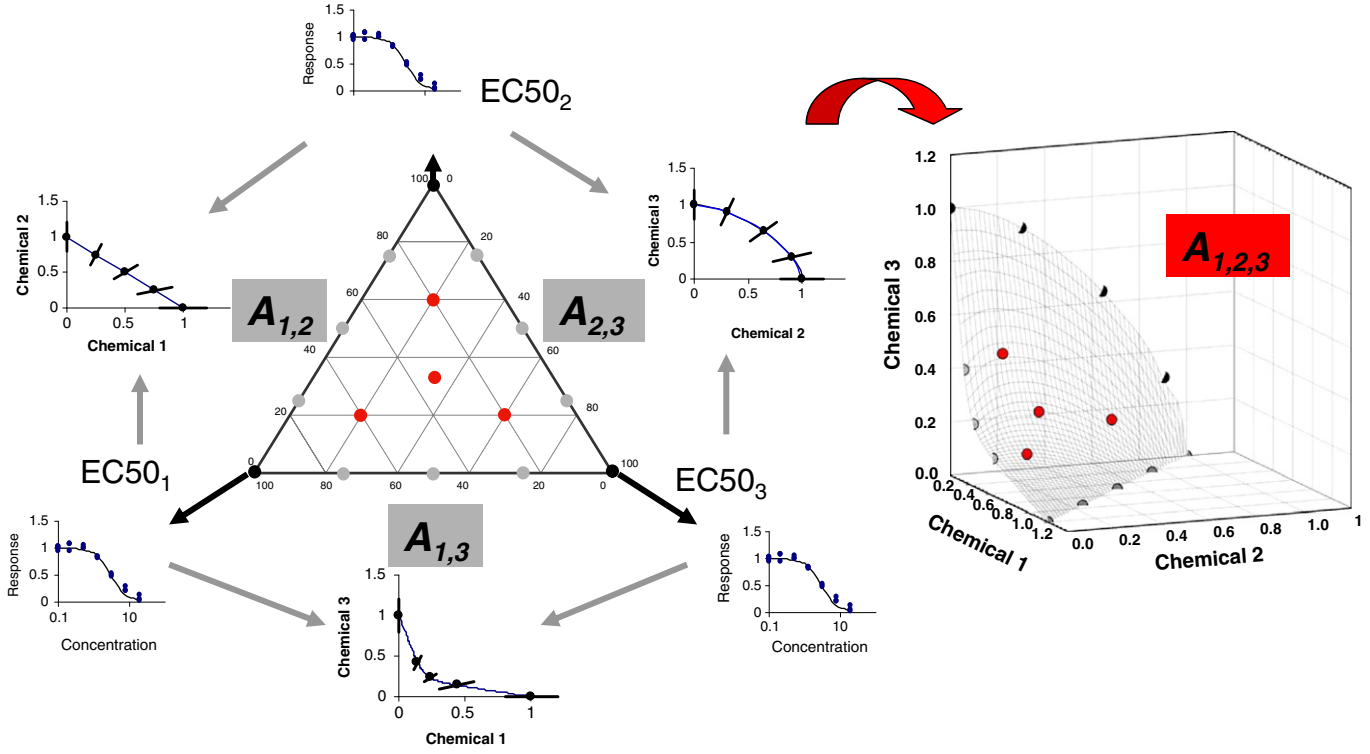

Fig. 2. The figure shows the mixture ratios of the experimental setup (central triangle) as illustrated as an isobole plane (Fig. 1B). The basic steps of the data analysis are illustrated around it. First the single compound data were described by a concentration–response model including an EC50, see Eq. (1) (black arrows). Then all binary mixtures were described by a concentration–response surface model including an interaction parameter that allows the response surface to deviate from CA (Jonker et al., 2005). The EC50 isoboles of response surfaces with interaction parameters of −3 ($a_{1,3}$), 0 ($a_{1,2}$) and 2 ($a_{2,3}$) are shown to illustrate examples of synergy ($a<0$), CA ($a=0$) and antagony ($a>0$). Finally, ternary isobole planes were predicted based on the three binary isoboles alone (Eq. (7), red arrow), or including the ternary data and a fourth deviation parameter, $a_{1,2,3}$ (see text for details). Introducing deviations from CA on the binary isoboles gives a curved isobole plane rather than the flat isobole plane shown in Fig. 1B. Introducing $a_{1,2,3}$ into the model can make the isobole plane curve more outwards ($a_{1,2,3}>0$), indicating antagony of the ternary mixture compared to predicted, or more inwards ($a_{1,2,3}<0$), indicating synergy compared to the predicted.

the reference model of CA, if the following relationship holds (Sprague, 1970):

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1. \quad (3)$$

Here, c_i denotes the concentration of chemical i in the mixture, and $\mathrm{EC}x_i$ is the effect concentration of chemical i alone that results in the same effect (x%) as the mixture (i.e. $\mathrm{EC50}_i$ in case of a 50% mixture effect). The quotient $c_i/\mathrm{EC}x_i$ is the dimensionless toxic unit ($\mathrm{TU}x_i$) that quantifies the relative contribution to toxicity of the individual chemical i in the mixture. This can be calculated at various effect levels indicated by x (for example, $\mathrm{TU}_{10i} = c_i/\mathrm{EC}_{10i}$ when the mixture with concentrations $c_1,\ldots,c_n$ has a 10% effect). If a mixture follows CA, the binary isobole at any effect level will be a straight line, while the isobole plane of the ternary mixture will form a plane surface (Fig. 1B). Notice that the CA model only involves parameters from the single chemical concentration response curves.

2.3.3. Incorporation of deviations from CA

To enable quantification of antagonistic or synergistic deviations from CA, Eq. (3) is rewritten in terms of the biological response, Y, and extended with a deviation function G as follows (Jonker et al., 2005):

$$\sum_{i=1}^{n} \frac{c_i}{f_i^{-1}(Y)} = \exp(G). \quad (4)$$

The degree of deviations from CA is given by the quantity G. When $G = 0$, the right hand side becomes 1 corresponding to CA (Eq. (3)). In combination, Eqs. (2) and (4) give the relationship between the concentrations of the chemicals in a mixture and the combined biological response. For a given value of G and concentrations $c_1,\ldots,c_n$ the equations can be solved by numerical methods.

The toxicity of each of the individual chemicals in a mixture can differ substantially, hence, the deviation function G should depend on each chemical's relative contribution to toxicity (i.e. the toxic units: TUx, see above) rather than on their actual concentrations. The relative proportion of toxic units of each chemical i in a mixture can be calculated as follows:

$$z_i = \frac{\mathrm{TU}x_i}{\sum_{j=1}^{n} \mathrm{TU}x_j} \quad \text{where } \mathrm{TU}x_i = \frac{c_i}{\mathrm{EC}x_i}. \quad (5)$$

While effect concentration (ECx) upon which the TUx's are based, can obviously be chosen arbitrarily, then the toxic units referred to in the remainder of this paper will be EC50 based. We choose EC50, as it is the response level that can be determined with the greatest precision.

2.3.4. Deviation function for binary mixtures

In the analysis of binary mixtures the deviation function which describes synergism or antagonism and is substituted into Eq. (4) is as follows:

$$G(z_1, z_2) = a \cdot z_1 \cdot z_2. \quad (6)$$

This deviation function describes antagonism when the parameter a is positive, and synergism when a is negative. As we have three separate binary mixtures within each ternary mixture, each data analysis will consequently include three separate a parameters, namely $a_{1,2}$ for the interaction between chemicals 1 and 2, $a_{1,3}$ for the interaction between chemicals 1 and 3, and $a_{2,3}$ for the interaction between chemicals 2 and 3 (Fig. 2). The model given by Eqs. (4) and (6) (for each mixture) will be referred to as the binary model.

2.3.5. Incorporation of binary deviations into ternary mixture effect predictions

The model for binary mixtures can be used to construct predictions of ternary mixtures if a deviation function for ternary mixtures is defined using the parameters from the binary model. The ternary deviation function combines the three binary deviations as follows:

$$G(z_1, z_2, z_3) = a_{1,2} \cdot z_1 \cdot z_2 + a_{1,3} \cdot z_1 \cdot z_3 + a_{2,3} \cdot z_2 \cdot z_3. \quad (7)$$

When Eq. (7) is substituted into Eq. (4), it defines a ternary response surface.

At the edges of the ternary response surface where the mixture consists solely of two chemicals, e.g. chemical 1 and chemical 2, the predicted joint effect will match the deviation from the corresponding binary model since the two other terms are zero ($z_3 = 0$). Once a slight amount of the third chemical is added and the mixture becomes truly ternary, the ternary deviation will be made up of a large fraction of the deviation from the binary mixture of chemicals 1 and 2 plus small amounts of the deviations from the binary mixtures of chemicals 1 and 3, and chemicals 2 and 3, respectively. The model given by Eq. (4) combined with Eq. (7) will be referred to as the ternary model.

2.3.6. Including additional ternary deviations

In order to examine if the ternary model given by Eq. (7) provides full description of the ternary mixture data, an extension with a separate ternary deviation parameter $a_{1,2,3}$ has been introduced. The deviation function is now

$$G(z_1, z_2, z_3) = a_{1,2} \cdot z_1 \cdot z_2 + a_{1,3} \cdot z_1 \cdot z_3 + a_{2,3} \cdot z_2 \cdot z_3 + a_{1,2,3} \cdot z_1 \cdot z_2 \cdot z_3. \quad (8)$$

Inclusion of the truly ternary parameter allows the ternary response surface to be above or below the one predicted from combination of the binary deviations (Eq. (7)). When $a_{1,2,3}$ is positive it describes antagonism compared to the isobole plane predicted by Eq. (7), and when $a_{1,2,3}$ is negative, it describes synergism compared to Eq. (7) (Fig. 2). The model given by Eq. (4) combined with Eq. (8) will be referred to as the Ternary-Plus model.

2.3.7. Error model and model fitting

Eq. (1) defines expected values for the biological response for single chemicals. The unknown parameters consist of the average control response Y_0, the 50% effect level concentrations $\mathrm{EC50}_1$, $\mathrm{EC50}_2$, $\mathrm{EC50}_3$ and the slopes b_1, b_2 and b_3. The expected values for the binary model (Eq. (4) combined with Eq. (6)) have extra parameters $a_{1,2}$, $a_{1,3}$, and $a_{2,3}$, and finally the Ternary-Plus model (Eq. (4) combined with Eq. (8)) involves an extra parameter $a_{1,2,3}$. Notice that the model equations cannot be solved in closed form. Hence, the expected value of Y cannot be written in closed form as a function of parameters and concentrations, but should be computed with numerical algorithms. Fig. 2 gives a schematic illustration of the model. As is common for continuous data, it is assumed that the random variation around the expected values is normally distributed with the same variance for all exposure combinations. Parameters are estimated by least squares, i.e. by minimising $SS = \Sigma(y_h - Y_h)^2$ where y denotes observed responses, $\hat{Y}$ denotes predicted values, and the sum is over all observations used for estimation. The objective function was minimised in a spreadsheet environment (Microsoft Excel) using the built-in Solver tool that uses a Newton algorithm. Notice that least squares estimation is equivalent to maximum likelihood estimation under the model assumptions (Neter et al., 1996).

2.4. The data analysis process

The data was analysed in the following steps: 1) The single chemical concentration response curves were fitted in one operation and with a common maximum/control value by minimising the sum of squared residuals for the single chemical data only. If the curve parameters of the single substances compared well with the observed data, then their parameters were held constant for the remaining analyses. The fitted model for single chemicals and the assumption of CA give rise to a predicted ternary response surface, denoted the "CA surface". 2) The model for binary mixtures, obtained by substituting the binary deviation function given in Eq. (6) into Eq. (4), was fitted. This was done for the three binary mixtures in one go, minimising the sum of squared residuals for the binary mixture data with the estimates from step 1 held fixed. The resulting estimates of $a_{1,2}$, $a_{1,3}$ and $a_{2,3}$ were then held constant in the remaining analyses. The estimated parameters together with Eq. (7) define a predicted ternary response surface, denoted the ternary surface. 3) The truly ternary model, obtained by substituting Eq. (8) into Eq. (4), was fitted by optimising the sum of squared residuals for data from ternary mixtures with all other parameters than $a_{1,2,3}$ held fixed. The fitted model defines a "fitted" response surface describing pure ternary deviation from the "ternary surface" using $a_{1,2,3}$, the Ternary-Plus surface. 4) The predicted and "fitted" ternary surfaces are compared as described below.

2.5. Quantification of deviations between the models

One aim of the study was to examine whether the difference between the ternary prediction models is likely to be of biological/ecological significance in the context it is used. We therefore wished to quantify the size of possible deviations between the models. The maximum difference between CA and the two ternary models will be located on the transect between the point of maximal binary deviation (the 50:50% mixture effect ratio of the binary combination with the largest numerical a-value) and the pure third chemical as shown in Fig. 3. The maximum deviations from CA of the ternary prediction model as well as the Ternary-Plus model were therefore quantified in terms of sums of toxic units for all three transect lines of each mixture as a function of the relative contribution of the third chemical for each of the ternary model transect lines. The CA prediction corresponds to a sum of toxic units of 1.

2.6. Comparing observed data and predicted models

The compliance between the observed data and the predicted models was evaluated graphically at the EC50 level. This was done by evaluating the EC50 values of all binary and ternary mixtures together with the EC50 isobole plane, as shown in Figs. 4 and 5 and the supplementary information. Moreover, the EC50 value of each of the ternary mixtures and their 95% confidence intervals were compared with the model predictions of the isobole plane transects giving the largest deviation from CA. The transects are illustrated in Fig. 3. These comparisons allow for individual assessments of the predictive power of the models for the EC50 of each of the ternary mixtures. To illustrate how the models predicted the entire concentration–response relationship for selected ternary mixture, the observed *L. minor* growth rates for the four ternary mixtures of glyphosate, mesotrione and mecoprop presented in Fig. 4A and C were plotted together with the model predictions of Concentration Addition, the ternary model based on the binary mixture deviations (with parameters $a_{1,2}$, $a_{1,3}$ and $a_{2,3}$) and on the Ternary-Plus model (including the ternary deviation parameter $a_{1,2,3}$) in Fig. 6. Bootstrap methods would make it possible to take into account the estimation error on the model predictions, but this is a topic for future research. All figures were made using SigmaPlot 11.0.

2.7. Limitations and assumptions of the analysis

As stated in the Introduction the main question of this paper is to address if inclusion of knowledge of deviations from additivity in the three binary mixtures will help provide better prediction of the ternary response surface compared to the CA reference model. Fixing the parameters for the single concentration response curves (during steps 2 and 3) and the binary deviations (in step 3) makes it possible to examine the predictive power of extending the CA model with information about binary and ternary mixtures. In step 2, for example, we want the full deviation from CA to be accounted for in the binary deviation parameters. The single concentration response curves were fitted to the single chemical observations only and kept fixed when fitting the binary mixture data. The reason for this is that if parameters for these curves were left free to vary when fitting the binary model to data which genuinely deviate from CA, then the deviation would "drag" the single curves away from where they are known to be, in particular if there are many data points from mixtures compared to single chemicals. The estimates for the binary deviations would therefore be smaller as a consequence of having "twisted" the single chemical concentration response parameters to match the CA prediction best possible. The reasoning for fixing the binary parameters (as well as the single chemical concentration response parameters) when fitting the ternary data is similar. We want to examine if it is possible to model the ternary mixtures given what is known about the binary deviations.

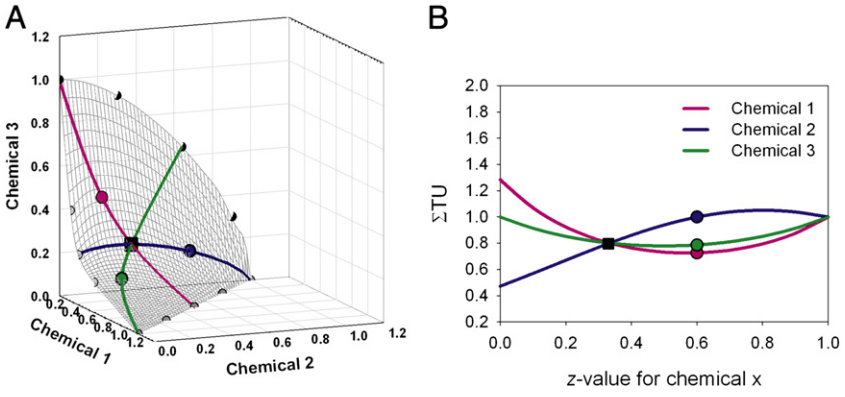

Fig. 3. Panel A shows the three transects of the isobole plane where the deviation from additivity is the largest. To illustrate the size of deviation between the observed ternary EC50 and the predicted isobole plane, these transects are shown as the sum of TU for the mixture as a function of the fraction (z) for each of the three chemicals (panel B).

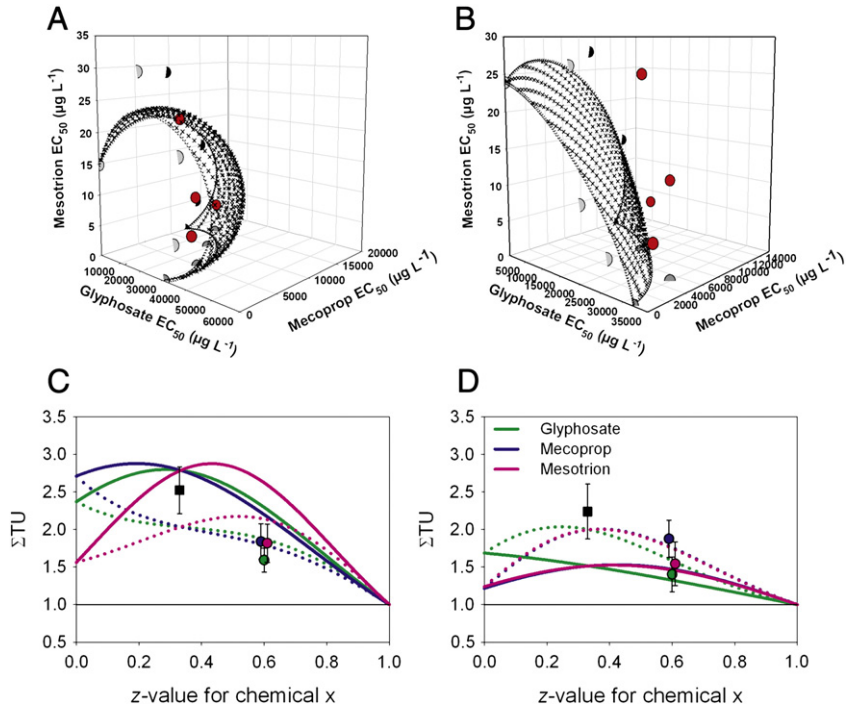

Fig. 4. The EC50 isobole plane of two experiments mixing glyphosate, mecoprop and mesotrione and testing their joint effect of the growth of *Lemna minor* are illustrated in panels A and B together with the EC50 values of the individually fitted concentration response curves of each mixture ratio. Binary mixtures between glyphosate and mecoprop are given with dark grey half-circles, glyphosate and mesotrione with light grey half-moons, mecoprop and mesotrione with black half-moons and the four ternary mixtures with red circles. In panels C and D the transects of the isobole plane are shown in bold lines (see Fig. 3 for explanation) together with the four ternary mixture EC50 values given in sums of toxic units (ΣTU) ± 95% confidence intervals. The confidence intervals are computed from the individual response curves. The ternary mixtures containing 60% effect concentration of glyphosate, mecoprop and mesotrione are given in green, blue and pink circle respectively, while the 33:33:33% mixture is given with a black square. As the theoretical 20:20:60 and 33:33:33% mixtures will always be situated in a slightly different place on the response surface in a real experiment (A and B), where obtained effects do not correspond 100% with theoretical predictions, their location on the two dimensional plots (C and D) cannot be determined precisely. While the bold lines give the transects for the ternary model including the deviation parameters $a_{1,2}$, $a_{1,3}$ and $a_{2,3}$ (Eq. (7)), the dotted line gives the transects for the model Ternary-Plus including the deviation parameter $a_{1,2,3}$ (Eq. (8)). CA is given with a thin straight line at ΣTU = 1. The EC50 values of the single compounds, the size of the deviation parameters and the maximal deviation from CA for binary mixtures and the two ternary models are given in Table 1. Figures for the remaining six mixtures conducted on *L. minor* are given in Appendix I and II.

Notice that we cannot carry out likelihood ratio tests when we fix parameter values from step to step even though the CA model, the ternary model and the Ternary-Plus models are nested. For the likelihood ratio test all data (single chemicals, binary and ternary mixtures) should be used to fit all models and all parameters should be re-estimated for each model. The above-mentioned reasons, however, led us not to do so.

3. Results and discussion

The study showed that the ternary model based on the binary mixture toxicity data was far superior to the CA reference model in predicting joint effects of interacting ternary mixtures. This was illustrated in Figs. 4, 5 and 6, and in the Supplementary material S1 and S2. The improvement from the ternary to the Ternary-Plus model was not substantial, and model predictions were not changed more than they were by repeating the experiment. This is illustrated by the example given in Fig. 4. For this ternary mixture the estimate of $a_{1,2,3}$ was negative in the first experiment, indicating synergy compared to the ternary prediction, while it was positive in the next (Table 1). The difference between the two predictions was mainly caused by the EC50 for the single compound mesotrione shifting from 14.8 to 23.3 µg L^{-1} between experiment one and two (Table 1, Fig. 4). A less than two-fold shift in EC50 estimates between experiments is not uncommon and must be regarded as part of the natural variance occurring in many test systems (Cedergreen et al., 2007a).

For the synergistic mixtures tested on *V. fischeri* the maximal deviation from CA, was not any larger for the Ternary-Plus model than it was in any of the binary mixtures (Table 1, Fig. 5). For the mixture of acifluorfen, diquat and prochloraz the ternary model predicted 4% stronger synergy compared to Ternary-Plus and the binary mixtures. This small difference is, nonetheless, not likely to be of any major biological significance. Hence, we conclude that for both the antagonistic and the synergistic ternary mixtures the ternary model including the binary deviation information provides robust estimations of the experimentally derived ternary mixture toxicity data.

For the synergistic mixtures the estimations of worst case ternary synergy can be simplified even further, as it is described by the strongest synergy of the involved binary mixtures. This pattern of the severest synergy occurring in the binary mixture is confirmed by other studies mixing three or four chemicals, which in binary mixtures interact synergistically (Cooper et al., 2009; Lin et al., 2005; Rosal et al., 2010; Wang et al., 2011; Woods et al., 2002). For studies where the mechanisms behind the synergism involve transformation of chemicals outside the organisms, rather than inside, which is the hypothesis behind some of the above-mentioned interactions, the patterns might be different. This was discussed by Koutsaftis and Aoyama (2007) in a study of mixtures of antifouling biocides where the proportions of chelated and non-chelated metal ions were known to be able to change when mixed, thereby changing the toxicity of the individual metals. The results presented in the study did, nevertheless, still show the most severe synergy to occur in the binary mixtures (Koutsaftis and Aoyama, 2007).

Other models have been proposed to evaluate interacting ternary mixtures, but most have been descriptive simply testing the significance of deviation from additivity (Charles et al., 2002). Lin et al. (2005), for example, propose a Climax Hypothesis stating that the

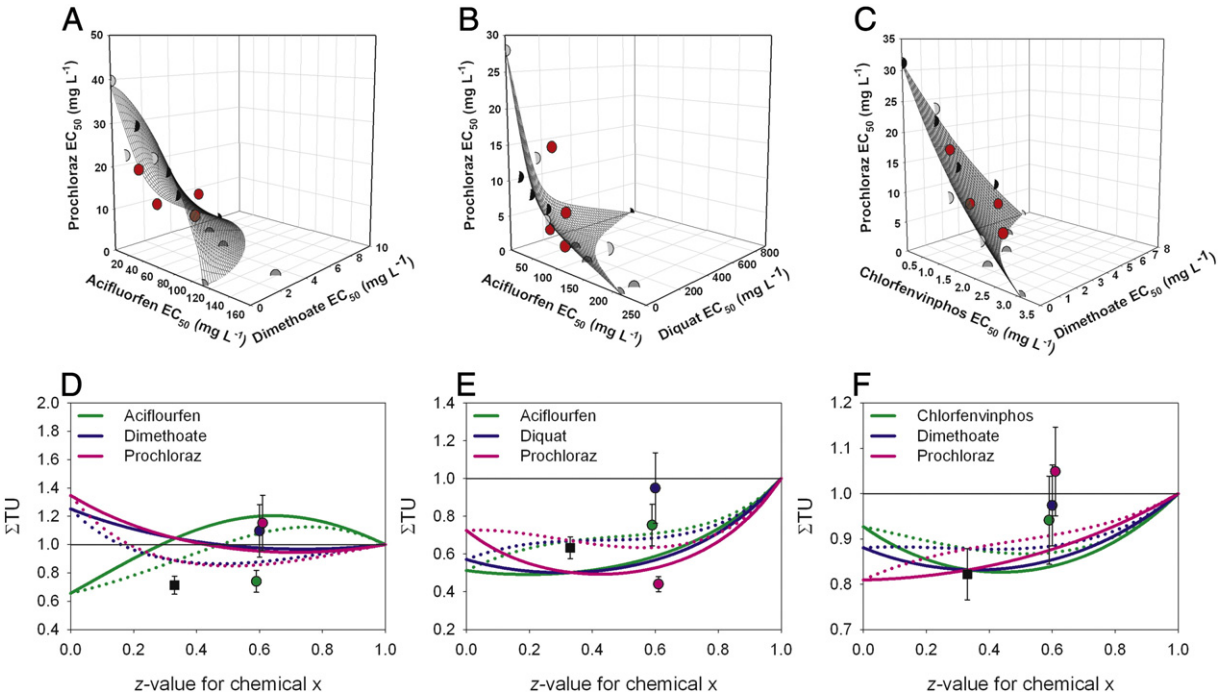

Fig. 5. The isobole planes and corresponding transects of the three mixture experiments performed on *V. fischeri*. All symbols are as explained in Fig. 4.

maximal deviation from additivity will occur at equitoxic ratios. They verified this for both binary and ternary mixtures of interacting chemicals using the *V. fischeri* test. They did not, however, try to quantify the size of the deviations in the ternary and quaternary mixtures based on the binary deviations. Also Lin et al. found the largest synergistic effects in the binary mixtures (Lin et al., 2005). Ren et al. (2004) wished to predict the joint effect of ternary mixtures of interacting chemicals. They did this by constructing a model for binary and ternary interacting metal mixtures on a luminescent bacterium (Shk1) using both binary and ternary data to construct the model. Thereafter they verified the model using other binary and ternary data at other mixture ratios (Ren et al., 2004). Hence, contrary to the present

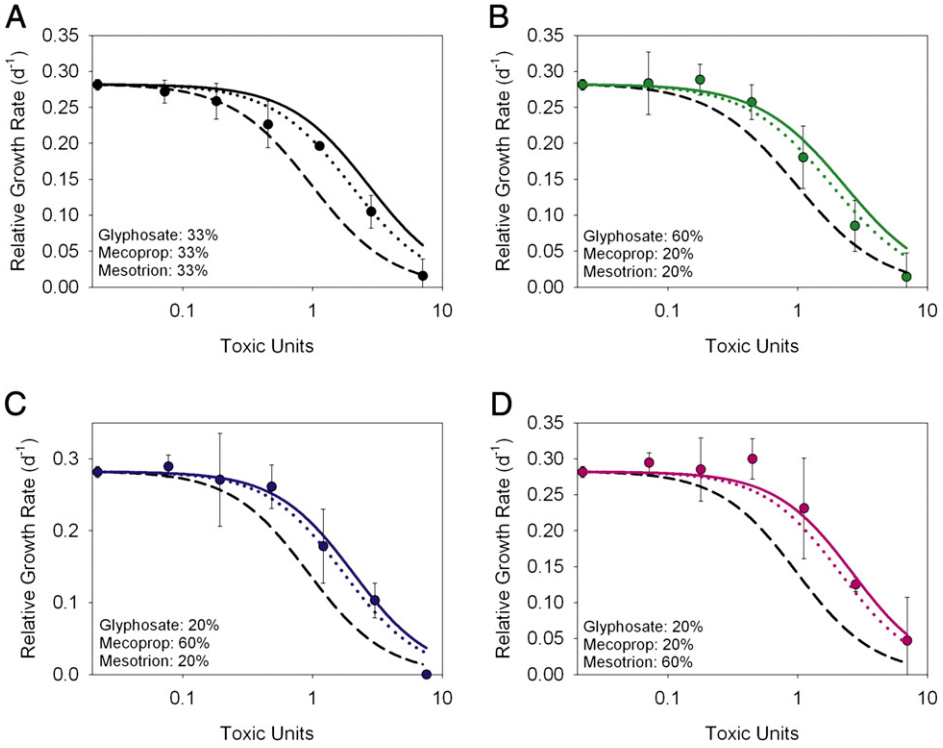

Fig. 6. To illustrate the predictive power of the models for the entire concentration–response curve, the observed data for the four ternary mixtures of Fig. 4A,C are pictured together with the model predictions of Concentration Addition (broken curve), the ternary model based on the binary mixture deviations (with parameters $a_{1,2}$, $a_{1,3}$ and $a_{2,3}$) (solid curve) and on the Ternary-Plus model (including the ternary deviation parameter $a_{1,2,3}$) (dotted line). The observed data are given as mean ± 95% confidence intervals ($n = 3$). The mixture ratios of the three herbicides are given in the legends at each panel A–D.

Table 1
Results of the model fits to the 11 sets of experimental data. EC50 values of each of the three single chemicals (in μg L^{-1} for the *L. minor* tests and in mg L^{-1} for the *V. fischeri* tests) are given together with the deviation parameters described in Eqs. (7) and (8). The maximal deviation from CA at the EC50 isobole plane is given in sums of toxic units of chemical needed to obtain EC50 both for the binary mixture with the largest deviation parameter (Bin), the ternary model based on the binary mixture deviations (with parameters $a_{1,2}$, $a_{1,3}$ and $a_{2,3}$) and on the Ternary-Plus model (including the ternary deviation parameter $a_{1,2,3}$). The CA model predicts a ΣTU of 1.

Chemicals			EC50			Deviation parameters (a)				ΣTU at max dev.		
Chemical 1	Chemical 2	Chemical 3	1	2	3	a_{12}	a_{13}	a_{23}	a_{123}	Bin	Ter	Ter+
Lemna minor												
Glyphosate	Mecoprop	Mesotrione	30,611	12,284	14.8	1.78	3.99	3.45	−8.51	2.71	2.88	2.71
Glyphosate	Mecoprop	Mesotrione	31,905	12,315	23.9	0.86	0.78	2.09	7.47	1.69	1.69	2.04
Glyphosate	Mecoprop	Terbuthylazine	28,406	13,235	244	−0.03	0.86	1.94	0.67	1.63	1.63	1.63
Glyphosate	Mecoprop	Terbuthylazine	34,213	12,289	276	0.75	1.10	2.83	5.07	2.03	2.03	2.15
Terbuthylazine	Mesotrione	Mecoprop	142	15.1	9486	0.91	3.07	2.95	−0.91	2.16	2.26	2.19
Terbuthylazine	Mesotrione	Mecoprop	117	25.0	14,000	−0.29	0.90	0.88	10.91	1.25	1.25	1.79
Diquat	Acifluorfen	Mesotrione	25.7	220	20.5	1.44	0.93	1.13	−0.49	1.43	1.49	1.46
Diquat	Acifluorfen	Mesotrione	27.4	399	20.9	2.00	1.90	1.44	−0.75	1.64	1.82	1.77
Vibrio fischeri												
Acifluorfen	Dimethoate	Prochloraz	112	8.04	38.7	1.19	0.90	−1.69	−4.45	0.66	0.66	0.66
Acifluorfen	Diquat	Prochloraz	214	856	28.1	−1.29	−2.24	−2.67	7.78	0.51	0.49	0.51
Chlorfenvinphos	Dimethoate	Prochloraz	3.08	7.44	31.2	−0.84	−0.51	−0.30	1.46	0.81	0.81	0.81

study, ternary data was needed to make predictions on ternary mixtures of interacting chemicals. This limits the extent of the model to those areas of the ternary mixture response surface that have already been tested, while the ternary model presented in the present study can be applied to all ternary combinations based solely on established binary interaction terms.

The funnel hypothesis, which we referred to in the Introduction, states that as the number of components in a mixture increases, the range of deviation from toxic additivity decreases (Warne and Hawker, 1995). This was true for all the synergistic mixtures of this study as the largest deviation from CA occurred in the binary and not the ternary mixtures, also for mixtures of up to three synergistically interacting binary mixtures (Table 1, Fig. 5). However, for the antagonistic mixtures, three of the eight ternary predictions were larger than any of the binary deviations included in the mixture (Table 1, Fig. 4, Supplementary material S2), which is not strictly in compliance with the funnel hypothesis. It is uncertain what the maximal deviation would be, if a fourth antagonistic herbicide was added. But there is no scientific argument saying that the joint antagony would decrease. We therefore propose a reformulation of the funnel hypothesis to: As the number of possible interactions in a mixture increases, the likelihood of large joint deviation from toxic additivity decreases. Shifting the focus from number of components to number of possible interactions, makes it plausible that mixing an increasing number of antagonistically interacting chemicals might not necessarily lead to CA, just as mixing an increasing number of synergistically interacting chemicals most likely will not. Only if the chemicals are chosen at random from a sample of substances that, in binary combinations, will lead to an equal amount of synergy as well as antagony, the Warne definition of the funnel hypothesis is likely to apply.

Implementing the results of the present study in a risk assessment context, the synergistic mixtures are the most interesting. The key observation that the deviation from the reference model in the ternary mixture did not increase above that of the binary mixture showing the highest degree of synergy, was confirmed by other studies (Cooper et al., 2009; Lin et al., 2005; Rosal et al., 2010; Wang et al., 2011; Woods et al., 2002). It indicates that knowledge of the strongest degree of synergy of a binary mixture can be used to determine the size of safety factors used for complex mixtures including potential synergists, such as suggested by Thompson (1996). In the present study, for example, the strongest synergy was found between diquat and prochloraz. In this mixture 50% effect was observed at only 51% of the mixture concentrations expected from CA (Table 1). This would mean that for a multiple mixture containing these two chemicals an extra safety factor of >2 would be necessary. The examples of synergy presented in this study are not very severe. There are examples of 6–16 fold synergies in other organisms than *V. fischeri* in studies including P450 inhibitors such as piperonyl butoxide and azole fungicides tested on crustaceans and insects (El-Merhibi et al., 2004; Nørgaard and Cedergreen, 2010; Pilling and Jepson, 1993) and a study on antifouling mixtures on sea urchin embryos showed similar strong synergies ranging from 2 to 7-fold for the ten tested binary mixtures, while the range was 2 to 30-fold for the resulting ten ternary and 3 to 10-fold for the five quaternary mixtures tested (Wang et al., 2011). Though the synergy in this study was higher in the ternary and quaternary mixtures compared to the binary mixtures, the differences were not statistically significant (Wang et al., 2011). Hence, for those types of mixtures considerable extra safety factors should be included to account for synergy if they are evaluated to occur at realistic concentrations.

Regulatory risk assessments of mixtures are, for various reasons, not yet very common. But when they are implemented, as for example for product preparations in the European legislation of chemicals (REACH), CA is used as a concept for mixture assessment (European Council, 1999; Syberg, 2009). In that context, determining the chemicals likely to induce synergy in different organisms is important. Interactions with the activity of metabolic enzymes are, as mentioned, a well known mechanism behind much of the synergy observed (Thompson, 1996; Varsano et al., 1992; Walker, 2009). Other mechanisms behind synergistic interactions, such as the promotion of chemical uptake or chemical interactions outside the organism are less well studied (Chalvet-Monfray et al., 1996; Koutsaftis and Aoyama, 2007). A future challenge therefore seems to be to develop methods that can identify and distinguish different kinds of potential synergists, and evaluate their environmental relevance.

Supplementary data related to this article can be found online at doi:10.1016/j.scitotenv.2012.03.086.

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