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Mixture Toxicity of Three Toxicants with Similar and Dissimilar Modes of Action to *Daphnia magna*

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Abstract

Mixture toxicity of similar- and dissimilar-acting toxicants can be predicted by the models concentration addition (CA) and independent action (IA) using single substance toxicity data. Knowledge of the toxicants mode of action is thus required in order to use the models. In order to test the predictive capability of the models, we conducted *Daphnia magna* 48 h immobilization experiments with three toxicants with known modes of action (dimethoate, pirimicarb and linear alkyl benzene sulfonate) singly, and in binary and ternary mixtures. Our results indicate that CA and IA predict binary mixtures of similar- and dissimilar-acting toxicants equally well. CA and IA also equally predicted the ternary mixture consisting of both similar- and dissimilar-acting chemicals. The paper discusses the concept of mode of action and the implications the definition of mode of action has on the choice of reference model for mixture toxicity studies.

Keywords: *Daphnia magna,* Dimethoate, Pirimicarb, Linear alkyl benzene sulfonate, Mixture toxicity, Isobole method, Mode of action, Concentration addition, Independent action

1. Introduction

Risk assessment of anthropogenic toxicants is primarily derived from experiments conducted with single substances (ECB, 2003). However, toxicity in natural ecosystems typically does not result from single toxicant exposure, but is rather a result of exposure to mixtures of toxicants (Altenburger et al., 1996; Gardner et al., 1998) and therefore mixture toxicity has been a subject of ecotoxicological interest for several decades (Hermens et al., 1984; Altenburger et al., 1996; Silva et al., 2002; Backhaus et al., 2003).

Current understanding of ecological mixture toxicity is adopted from pharmacology (Berenbaum, 1989) and is based on two different theories, which are not mutually exclusive (Greco et al., 1995). These two theories are called concentration addition (CA) (Loewe and Muischnek, 1926) and independent action (IA) (Bliss, 1939) and describe mixture effects for similar- and dissimilar-acting toxicants, respectively. One common trait of the two models is that they assume non-interaction as the default. Deviation from the prediction is thus an indication of interaction (antagonistic (weaker) or synergistic (stronger) effects than predicted).

One way to determine whether the toxicity of a mixture deviates from such predictions is to conduct experiments based on the isobole method (Berenbaum, 1989). For a review on the use of isoboles in mixture toxicity studies see Kortenkamp and Altenburger (1998). A disadvantage with the standard isobole diagram described by Kortenkamp and Altenburger (1998) is that it is difficult to evaluate whether the isobole is statistically different from the additivity line. One way to overcome this problem is to apply an advanced isobole model to the data, which enables a statistical determination of whether the experimental isobole deviates from an additivity isobole (Sørensen et al., 2007). The model is based on Equation (1), in which the standard isobole equa-

tion (Kortenkamp and Altenburger, 1998) is extended with an extra parameter (λ).

$$\left(\frac{d_A}{D_A}\right)^{1/\lambda} + \left(\frac{d_B}{D_B}\right)^{1/\lambda} = 1 \quad (1)$$

where d_A and d_B represent the concentration of toxicant A and B applied in the mixture at a certain effect level and D_A and D_B represent the concentrations of toxicants A and B needed to obtain the same effect level when applied alone. Both toxicants are applied at isoeffective concentrations. The parameter λ describes the isobole's degree of concavity and thus reflects the degree of synergy/antagonism. If lo1 the effect is antagonistic, if $\lambda = 1$ the effect is additive and if $\lambda > 1$ the mixture effect is synergistic (Sørensen et al., 2007).

The isobole method is based on the theory of CA. However, the method is often used regardless of knowledge of mode of action (Merino-Garcia et al., 2003; Kortenkamp and Altenburger, 1998). In order to identify whether a binary mixture of toxicants with dissimilar modes of action is antagonistic, additive or synergistic an additional isobole must be applied to the conventional isobologram (Greco et al., 1995). This isobole is based on the theory of IA. If the toxicants A and B are applied jointly their mixture toxicity can be estimated by the following equation (Pöch, 1993; Faust et al., 2003):

$$(1 - E_A) = (1 - E_{A+B}) / (1 - E_B), \quad (2)$$

where E_A and E_B represent the fractional effects (ranging from 0 to 1) caused by the individual toxicants A and B and E_{A+B} is the total effect of the mixture.

For convenience the term "Loewe additivity" can be used to describe the additivity isobole of concentration addition (CA), and "Bliss independence" can be used to describe the additivity isobole of independent action (IA). Experimental deviation from these isoboles can be characterized as Loewe antagonism/Loewe synergism and Bliss antagonism/Bliss synergism, respectively (Greco et al., 1995).

In the current study the *Daphnia magna* 48 h immobilization test (OECD, 2000) was used to test three toxicants (dimethoate, pirimicarb and linear alkyl benzene sulfonate (LAS)) singly and in combination. The toxicants were chosen on the basis of their known modes of action, since the purpose of the study was to test whether CA and IA could predict the toxicity of mixtures with similar and dissimilar modes of action, respectively.

Dimethoate is a pesticide belonging to the group of organophosphate insecticides (OPs). These compounds inhibit the enzyme acetyl cholinesterase (AChE) and thus over-stimulate neurological activity in the organism (Pope, 1999). Many other insecticides also exhibit neurological activity. Pirimicarb belongs to another group of insecticides that also inhibit acetyl cholinesterase (AChE), the so-called carbamates (Levitin and Cohen, 1998). Dimethoate and pirimicarb were thus used to test whether CA could predict the combined action of similar-acting toxicants. The additivity of AChE inhibitors has been shown previously in experiments with cladocerans (Norbergking et al., 1991). LAS is one of the most frequently used anionic surfactants (Scott and Jones, 2000). It is known to be toxic to aquatic organisms (Lewis, 1991; Kusk and Petersen, 1997; Tanaka and Nakanishi, 2001). Bjerregaard (2001) showed that exposure to LAS resulted in a change in intracellular calcium due to an effect on ion transport over the cell membrane. Another study showed that LAS might influence important osmoregulatory functions in fish by interacting with cell membrane structures and thus inhibiting cell functions (Pareschi et al., 1997). Additionally, it has been shown that LAS influences the permeability of the plasma membrane in fibroblasts so that normally un-diffusible compounds could penetrate the membrane (Bianchi and Fortunati, 1990). Substances that interact with the plasma membrane are furthermore known to facilitate trans-membrane transport of hydrophobic substances with a potential increase in their toxicity and thus an overall synergistic effect of the mixture as a result (Jacobi et al., 1996). A binary combination of LAS and one of the two pesticides should be predictable by IA, except if LAS facilitates uptake and thereby increases the toxic effect of the hydrophobic pesticides resulting in a synergistic effect.

2. Materials and methods

2.1. Toxicants

Stock solutions of the three toxicants dimethoate (Pestinal®, Sigma-Aldrich, Denmark), pirimicarb (Pestinal®, Sigma-Aldrich, Denmark) and LAS (Chiron AS, Trondheim, Norway) were prepared by dissolving them in MilliQ water which was subsequently diluted in test medium to obtain test solutions. Pesticide stock solutions were treated with ultrasound to ensure that they were completely dissolved.

2.2. Test organisms

All experiments were performed with a cultured clone of the water flea *D. magna*, provided by DHI Water and Environment, following the OECD *Daphnia* sp. 48 h Immobilization Test (OECD, 2000). The culture was kept in Elendt M7 test medium at 19±1°C under a light (16 h): dark (8 h) photoperiod. The light intensity was 15 $\mu Em^{-2}s^{-1}$. Specimens were gradually acclimated to the test medium over a 1 month period, as recommended in the OECD guideline for *Daphnia* sp. reproduction test (OECD, 1998). The culture was maintained in six aquaria of 3 L each with approximately 30 specimens of *D. magna*. Test specimens were taken from cultures that were older than 14 days to ensure that no first broods were used, in accordance with the OECD Guideline (OECD, 2000). Furthermore tests with the reference chemical $K_2Cr_2O_7$ (MERCK, Darmstadt, Germany) were made periodically, to ensure that the test organisms were in a proper condition for the experiments.

The *Daphnia* cultures were fed with the green algae *Pseudokirchneriella subcapitata*. The algal cultures were kept in the laboratory at 19±1°C under an 8 h dark:16 h light photoperiod with 85 $\mu Em^{-2}s^{-1}$. The algal cultures were grown in the medium recommended in the OECD *Daphnia* sp. reproduction test guideline (OECD, 1998) with a modification of the amount of macronutrients (N and P). The concentrations of the two macronutrients were multiplied by five to optimize growth. Algal cultures were renewed every week, and the mature algal cultures were centrifuged (Centrikon T-42 K, Kontron instruments, Bletchley, UK) for 7 minutes at 450*g*. These algae were placed in a refrigerator at 5°C and kept cool and dark until

they were fed to the daphnids. The daphnid culture was fed every 12 h during acclimatization (0.3 mg C d^{-1}). *Daphnia* used in experiments were starved throughout the experimental period (48 h). The amount of algae added was calculated based on the relationship between algal carbon content (EAN 1110 CHNS elemental-analyzer, CE Elantech Inc., Lakewood, New Jersey) and light absorbance (Perkin-Elmer, Lambda II UV/VIS Spectrometer, Wellesley, Massachusetts) at 440 nm. This ensured that the cultures were fed consistent amounts of carbon. Algae were continuously stirred with a magnetic rotator and connected to the aquaria with a peristaltic pump (OLE DICH, Hvidover, Denmark).

2.3. Experimental design and data analyses

The toxicities of the three toxicants were tested both separately and in binary and ternary mixtures. All experiments were conducted in 100 mL glass containers with five specimens in each container. Each container was filled with 50 mL test medium. Ten toxicant concentrations were used in each dose-response experiment. Each experiment was conducted with five replicates at each concentration including the control group. Single toxicity experiments were performed twice in order to determine the reliability of the experimentally determined EC_{50} values. The EC_{50} values were obtained applying a three-parameter log-linear logistic regression model (Equation (3)):

$$f(x) = \frac{d}{1 + \exp\{(b \log(x) - \log(e))\}} \quad (3)$$

where d is the upper limit, b is proportional to the slope at e, which is the EC_{50} value of the doses-response curve. A comparison of the log-logistic fit and a probit fit showed that EC_{50} values did not deviate between the two fits (results not shown), indicating that the choice of the log-logistic model did not influence the isoeffective mixture ratio despite the fact that immobilization is a quantal response. The EC_{50}s values used for construction of the isoeffective binary and ternary mixtures were calculated as geometric means from the preliminary single toxicant experiments. The data from these preliminary experiments were not included in the mixture experiments, where new data for single substance toxicity were determined. This procedure was used since deviation in sensitivity of the test specimens between experiments might result in misinterpretation of the mixture effects.

Mixture experiments were conducted following a fixed ratio design (Greco et al., 1995; Sørensen et al., 2007). The compounds were mixed in ratios corresponding to five effect ratios (0:100, 25:75, 50:50, 75:25 and 100:0%) for binary mixtures and a 33:33:33% effect ratio for the ternary mixture. All ratios were based on the single toxicant EC_{50}-values (isoeffective concentrations) in accordance with the isobole method (Kortenkamp and Altenburger, 1998), giving a total of 260 glass containers with five specimens in each. The containers were marked and placed randomly under a light source, and exposed to the same light:dark photoperiod as the stock cultures.

Concentration addition- and experimental EC_{50} isoboles were fitted to all mixture toxicity data using the software package drc (http://www.bioassay.dk) and the statistical software R (R Development Core Team. 2004. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria). Deviations between CA-reference EC_{50} isoboles and experimental isoboles were analyzed according to Sørensen et al. (2007). This approach was chosen since it allows statistical comparison between the CA reference isobole and the isobole that fits the data best, and thus allows a determination of the deviation from CA. It is important to note, that since the CA reference isobole is a regression fitted to all data it is not a straight line connecting the EC-values of the single substances, as it appear in the classic isobologram. The EC_{10}-estimates and their standard errors were estimated with drc. The corresponding isoboles were estimated by weighted least squares, with the inverse variance estimates retrieved from drc as weights. Mixtures with dissimilar-acting chemicals were furthermore compared with an IA-isobole, in order to evaluate whether this reference isobole explained data better than the CA-isobole, as would be expected according to the theory (Greco et al., 1995). The IA-isobole was constructed in accordance with Pöch (1993). Deviations from the IA-isobole were determined by visual inspection of the isobolograms.

3. Results

3.1. Single substance toxicity

Clear sigmoid dose-response relationships were observed in all single toxicant experiments, with exposure concentrations spanning 0-100% effects. The dose- response parameters of two independent experiments are given in Table 1. The geometric means of the EC_{50} values from the two experiments were 19.02 µg/L for pirimicarb, 2290 µg/L for dimethoat and 3540 µg/L for LAS.

3.2. Binary and ternary mixtures

The dimethoate/pirimicarb isobologram with 50% effect level data showed no significant deviation from additivity ($p > 0.05$) (Figure 1 and Table 2). At the 10% effect level the isobole also appeared to be strictly Loewe additive ($p > 0.05$) (Figure 1 and Table 2).

The pirimicarb/LAS mixture did not deviate statistically from CA at the 50% effect level ($p > 0.05$) (Figure 2 and Table 2). However, the deviation from CA increased as exposure concentrations decreased, giving a deviation from Loewe additivity (strong Loewe antagonism) at lower concentrations (EC_{10})($p < 0.05$) (Figure 2 and Table 2). When comparing the observations with the IA-isobole, the effect seemed to be Bliss synergistic at EC_{50} while at EC_{10} the mixture approximated the IA-isobole and hence showed Bliss independence (Figure 2, Table 2 and Figure 4c).

Table 1. Parameter values from the three-parameter log logistic curves fitted from the preliminary single substance experiments (Equation (3) in text)

	Dimethoate		Pirimicarb		LAS	
	I	II	I	II	I	II
b	8.53 ±1.54	4.81 ±0.83	7.68 ±0.86	7.96 ±0.83	4.08 ±0.56	2.90 ±0.42
d	4.95 ±0.14	5.10 ±0.17	5.01 ±0.09	5.00 ±0.10	5.00 ±0.18	5.12 ±0.38
e	2410 ±50	2190 ±80	17.84 ±0.26	20.28 ±0.38	4050 ±170	3080 ±230

In the equation d gives the maximum response (number of mobile individuals) and b is the slope around e, the EC_{50} of the dose response curve. All values are given±standard error. The numbers I and II signifies two independent experiments.

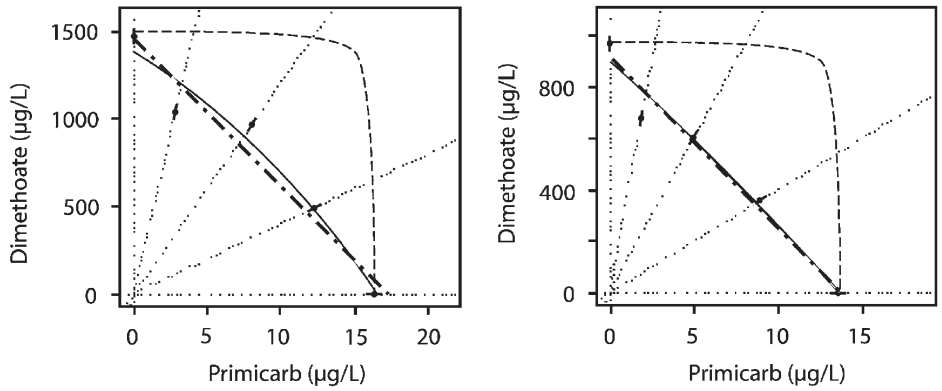

Figure 1. Isobolograms for dimethoate and pirimicarb mixtures at 50% (left) and 10% (right) effect concentrations. The solid line describes the fitted isobole and the straight line describes the fitted concentration addition reference isobole. The dashed line indicates the isobole according to independent action (Bliss independence). Data is given±standard error (plotted along the half lines which represent individual dose response curves). Half lines represent dose response curves from individual mixture experiments.

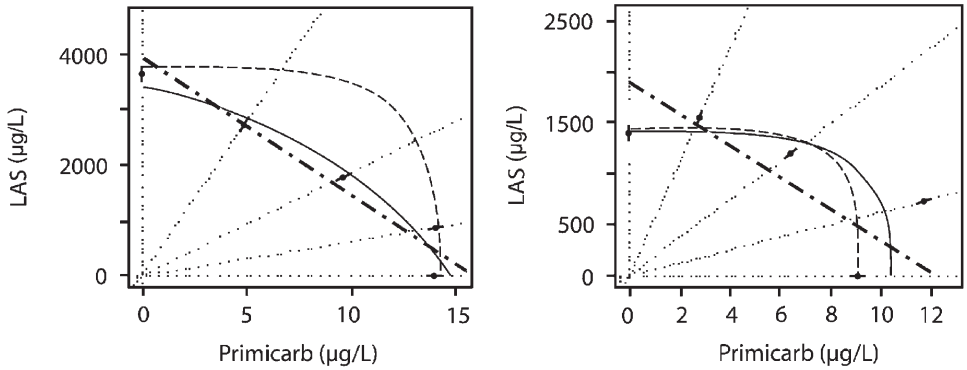

Figure 2. The diagrams show the isobologram for pirimicarb and LAS mixtures at 50% (left) and 10% (right) effect concentrations. The solid line describes the fitted isobole and the straight line describes the fitted concentration addition reference isobole. The dashed line indicates the isobole according to independent action (Bliss independence). Data are given±standard error and are plotted along the half lines which represent individual dose response curves.

The third binary mixture dimethoate/LAS showed no significant deviation from Loewe additivity at the EC_{50} effect level ($p > 0.05$) (Figure 3 and Table 2). Also the isobole at the EC_{25} effect level appeared to be Loewe additive ($p > 0.05$) (note that the EC_{25} concentration was used in this experiment, since a single outlier had such a great impact on the EC_{10} results that no proper regression could be made at this effect concentration). If the dimethoate/LAS results are compared with the IA-isobole instead of the CA-isobole, the mixture showed Bliss synergy at both effect concentrations (EC_{50} and EC_{25}).

The binary mixtures with 50:50% effect ratio and the ternary mixture with 33:33:33% effect ratio ratios were also evaluated in relation to CA and IA over their entire effect ranges. The results are shown in Figure 4 where experimental data and dose-response curves estimated by CA and IA are plotted. The curve for the pirimicarb/dimethoate mixture confirms the results of the isoboles showing that CA describes the data well over the entire effect range (Figure 4a). However, the data are also reasonably predictable with IA. Combinations of LAS and either of the two pesticides behaved in a similar way, being best described by IA at low effect concentrations, while being equally well described by both models at higher effect concentrations (Figure 4b and c). The ternary mixture was equally well described with both CA and IA (Figure 4d).

Dose-response parameters for all the dose-response curves included in the binary mixture experiments are given in Table 3. For the ternary mixture the maximal response (d) was 5.01±0.12 mobile *Daphnia* per beaker, the slope parameter (b) was 5.08±0.06 and the EC_{50} (e) was 1933.93±55.68 µg/L. The ternary mixture consisted of a mixture of 0.33% pirimicarb 39.21% dimethoate and 60.46% LAS when given quantitatively in µg/L, in total yielding effect ratios of approximately 33:33:33%.

4. Discussion

4.1. Chemicals with the same mode of action

As the results illustrate, definitions of synergism and antagonism critically depend upon the definition of "no interaction" (Greco et al., 1995). The mixture of the two acetylcholine esterase inhibiting insecticides clearly followed

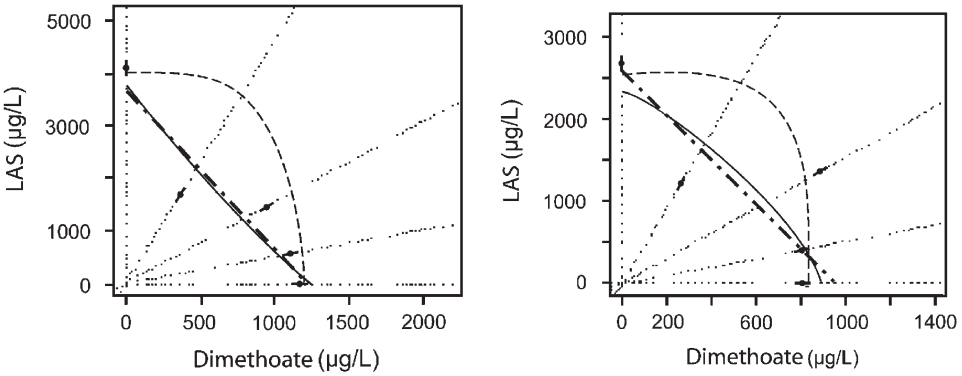

Figure 3. Isobolograms for dimethoate and LAS mixtures 50% (left) and 10% (right) effect concentrations. The dashed line indicates the isobole according to independent action (Bliss independence). The solid line describes the fitted isobole and the straight line describes the fitted concentration addition reference isobole. Data is given±standard error of the individually fitted dose response curves.

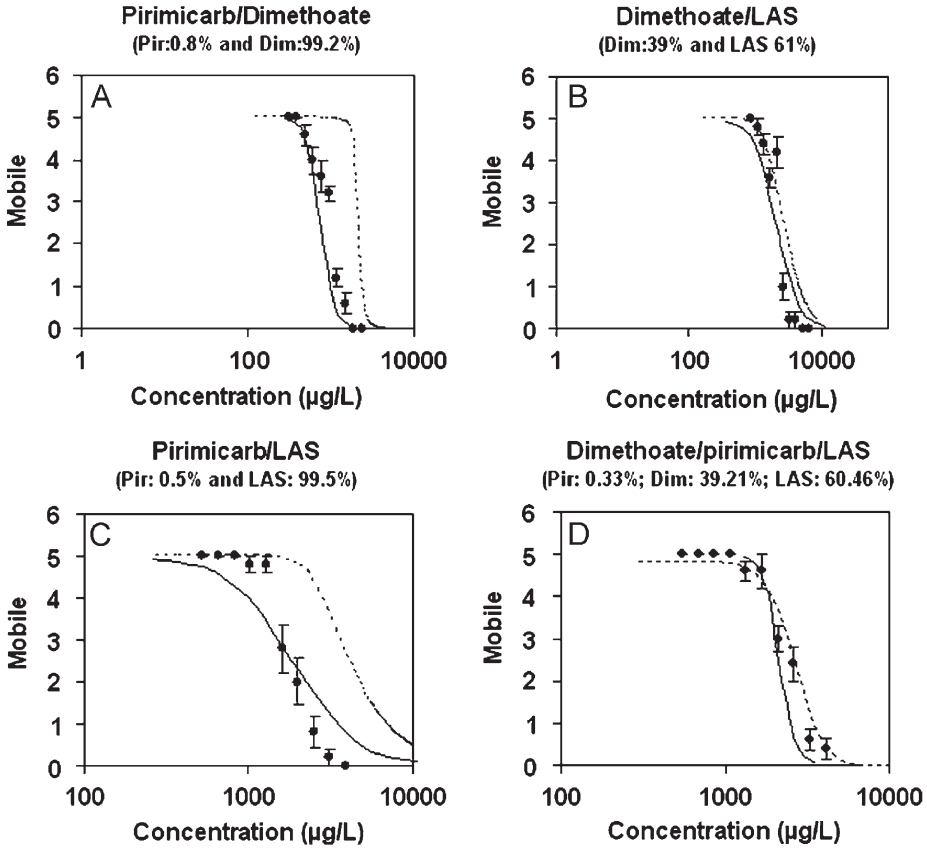

Figure 4. Experimentally determined toxicity of binary mixtures and ternary mixture. The toxicants were mixed to contribute with either 50:50% of the effect at EC_{50} (binary mixtures: A, B and C) or with 33:33:33% each of the effect at EC_{50} (ternary mixture: D). The actual concentration of each toxicant in the mixtures is given as % of the total mixture concentration. The dashed line indicates the predicted mixture toxicity according to independent action and the solid line indicates the predicted mixture toxicity according to concentration addition. Experimental observations are plotted with error bars indicating standard error.

Table 2. The parameter (λ) describing the curvature of the fitted isobole for the three binary mixture experiments

EC-value	Dimethoate/pirimicarb	Pirimicarb/LAS	Dimethoate/LAS
EC_{50}	$\lambda = 0.86\pm0.11$, $p > 0.05$	$\lambda = 0.71\pm0.1$, $p > 0.05$	$\lambda = 1.1\pm0.22$, $p > 0.05$
EC_{10}	$\lambda = 0.97\pm0.11$, $p > 0.05$	$\lambda = 0.31\pm0.19$, $p < 0.05$	$\lambda = 0.9\pm0.36$, $p < 0.05$

If $\lambda = 1$ the isobole follows CA, if $\lambda < 1$ the isobole indicates Loewe antagonism and if $\lambda > 1$ the isobole indicates Loewe synergism. p-values indicate whether the deviation of the estimated λ from one is significant.

Table 3. Parameter values from the three-parameter log logistic curves fitted from mixture toxicity experiments (Equation (3) in text)

Ratio	100:0	75:25	50:50	25:75	0:100
Dimethoate/pirimicarb					
b	5.29 ±0.83	5.19 ±0.85	4.58 ±0.53	6.89 ±1.02	17.37 ±2.99
d	4.92 ±0.15	5.00 ±0.19	4.92 ±0.11	4.90 ±0.11	4.83 ±0.07
e	1472.19 ±47.11	1040.80 ±41.78	978.67 ±29.04	506.10 ±11.71	16.62 ±0.18
	(dim 100%: pir 0%)	(dim 99.7%: pir 0.3%)	(dim 99.2%: 0.8%)	(dim 97.6% pir 2.4%)	(dim 0%: pir 100%)
Pirimicarb/LAS					
b	5.13 ±0.44	11.82 ±1.52	5.37 ±0.68	3.87 ±0.56	2.26 ±0.34
d	5.01 ±0.09	5.02 ±0.07	5.05 ±0.12	5.05 ±0.16	5.12 ±0.25
e	14.00 ±0.30	889.60 ±11.91	1793.63 ±53.53	2730.14 ±123.84	3637.66 ±306.80
	(pir 100%: LAS 0%)	(pir 1.6%: LAS 98.4%)	(pir 0.5%: LAS 99.5%)	(pir 0.2%: LAS 99.8%)	(pir 0%: LAS 100%)
Dimethoate/LAS					
b	4.99 ±0.86	3.47 ±0.42	15.03 ±3.82	3.37 ±0.46	2.62 ±0.43
d	4.88 ±0.20	5.00 ±0.19	4.57 ±0.11	5.03 ±0.22	4.84 ±0.22
e	1168.64 ±44.12	1671.50 ±80.90	2424.78 ±51.51	2069.68 ±116.45.	4092.82 ±312.71
	(dim 100%: LAS 0%)	(dim 66.1%: LAS 33.9%)	(dim 39.4%: LAS 60.6%)	(dim 17.8%: LAS 82.2%)	(dim 0%: LAS 100%)

In the equation d gives the maximum response (number of mobile individuals) and b is the slope around e, the EC_{50} of the dose response curve. All values are given±standard error. The EC_{50} values are given in µg/L total chemical and their individual quantitative contribution (as opposed to effect concentration/effect ratio) to the overall mixture is given in %.

concentration addition, just as expected from the theory of "same mode of action" (Bliss, 1939; Merino-Garcia et al., 2003; Faust and Scholze, 2004), though IA can explain the data reasonably well too. But does the AChE inhibiting ability necessarily mean that the two pesticides share the same mode of action? Earlier studies have shown that elevated cytochrome P450 mono-oxygenase may increase the toxic effect of dimethoate (Frasco and Guilhermino, 2002; Anderson and Zhu, 2004). The reason for this increased toxicity may result from the oxidative activation of dimethoate into the O-analog metabolite, omethoate, which is a more effective inhibitor of AChE (Frasco and Guilhermino, 2002; Anderson and Zhu, 2004). In a study exposing the insect *Aphis gossypii* to pirimicarb and omethoate, Benting and Nauen (2004) found that the same mutation in AChE (S431F) lead to resistance to both insecticides. These results indicate that the two compounds act within the same active site of the AChE molecule. However, even though the same mutation causes resistance to both insecticides, mutated *A. gossypii* was many fold less sensitive to pirimicarb than dimethoate (Benting and Nauen, 2004). It is therefore possible that different residues, besides the serine residue, may be involved in the inhibition mechanism of pirimicarb and omethoate. Is it then feasible to talk about the same mode of action, with regard to defining mixture effects, for pesticides that bind differently to target sites, and/or differ in potency. The theory of simple similar action, which has evolved into CA, was based on comparing mixtures with a "mixture" where a toxicant is mixed with a dilution of itself (Berenbaum, 1989). Toxicants that bind differently to the target sites do not mimic this kind of "mixture." Hence, if mixtures have to meet this criterion in order to be characterized as being similar in action, then CA is an exception rather than the rule. Another aspect that complicates the classification of chemicals into similar- or dissimilar-acting, is that even for those toxicants with a known mode of action (e.g. pesticides) it remains a question whether there are other modes of action, apart from the "designed" effect as well as a common narcotic effect (Pope, 1999). Dimethoate is one of the insecticides that is known to have several modes of action (Casida and Quistad, 2004). Studies with organophosphorus and carbamate insecticides have shown that these compounds can induce both necrotic cell death and apoptosis (Pena-Llopis et al., 2003; Caughlan et al., 2004; Kim et al., 2004), processes that are biochemically associated with release of cytochrome c from mitochondria into the cytoplasm (Skulachev, 1998). It has been suggested that apoptosis is a novel toxic mechanism of OPs that could be independent of AChE inhibition (Caughlan et al., 2004). Therefore, the Loewe additive effect of pirimicarb and dimethoate (see Figure 1) could as well be due to similar mode of action in relation to apoptosis or necrosis just as of inhibition of AChE.

In the case of the mixture of pirimicarb and dimethoate predictions, using independent action would not have made a big difference for the conclusion of the study, since predictions from the two models were very similar (Figure 4).

4.2. Chemicals with dissimilar mode of action

Turning to the mixtures of the insecticides with LAS, the quantification of the mixture effects depends on whether they are compared with Loewe additivity or Bliss independence as a reference model. In this case the IA reference

model might be the proper model to choose since both mixtures are made from toxicants with dissimilar modes of action. The insecticides inhibit an enzyme active in neural transmission while LAS disrupts cell membranes (Bjerregaard, 2001). The isobolograms showed Bliss synergy at EC_{50} levels, as was expected for combinations with LAS since its cell membrane disrupting mode of action potentially could facilitate the uptake of other xenobiotics (Jacobi et al., 1996). At lower effect levels the mixture approached Bliss independence, which could be due to the declining concentration of the individual toxicants in the mixture. With a decreasing number of LAS molecules that can react with the cell membranes, the facilitation of transport of insecticide molecules over the membranes is also likely to decrease.

The insecticides and LAS have different molecular target sites, but do they act independently at the level of a complex organism such as *Daphnia*? IA assumes that toxicants work independently and thus do not influence each others' effect at all (Bliss, 1939). As Greco et al. (1995) summarized, this is only realistic within very simple systems, such as 'in vitro' experiments with enzymatic processes. When applied to multicellular organisms it is likely that a toxicant does influence the toxicity of others. The application of a toxicant does at least provoke a reallocation of metabolic energy, which influences the capability to cope with another toxicant (Greco et al., 1995).

LAS disrupts plasma membranes resulting in a change in calcium (Ca^{2+}) homeostasis (Bjerregaard, 2001) and thus has a dissimilar mode of action compared to the two insecticides. However, it is well documented that an increase in the intracellular [Ca_{2+}] can lead to release of cytochrome c from mitochondria into the cytoplasm (Denecker et al., 2001). Dimethoate, pirimicarb and LAS may therefore exhibit the same "mode of action" in this regard. These observations could explain why CA provides such good estimation of both the binary and ternary mixtures in this study (Figure 4 and Table 2).

Yet another factor which complicates the use of mode of action for classifying different toxicants is that mode of action can shift with dose. Also, the selection of proper endpoint is important. Cedergreen (2005) found that quantification of mixture toxicity was highly dependent on choice of endpoint in plants. In this study it was found that herbicide mixture effects on *Lemna minor* appeared either antagonistic or additive depending on which of two different growth endpoints was selected (Cedergreen, 2005). With regard to assessing effects on ecosystems, which are important targets of ecological risk assessment of chemicals, the concept of mode of action breaks down, since the modes of action often deviates between organisms.

It is thus extremely complicated, and in some cases inappropriate, to define a toxicant's mode of action, even for toxicants with designed effects such as pesticides. This means that the choice of reference model is not straightforward, particularly when the mixtures are composed of a large array of compounds as are commonly found in the environment.

4.3. Choice of reference model for quantifying and predicting mixture effects

Several studies have applied both CA and IA in evaluating the predictability of mixture effects (Backhaus et al., 2000a, b, 2003; Faust et al., 2001, 2003; Junghans et al., 2003; Arrhenius et al., 2004). The results indicate that IA produces accurate predictions of the effects of multi-component mixtures of strictly dissimilarly acting toxicants tested on algae and bacteria. Under such conditions several authors found that CA generally overestimated the effects (Backhaus et al., 2000b; Faust et al., 2003). In experiments with similarly acting toxicants the observed effects were found to be in good agreement with CA predictions. IA predictions, however, underestimated the mixture effects by as much as a factor of four (Backhaus et al., 2000a; Junghans et al., 2003). The ternary mixture results of the present study also showed that CA and IA predicted the mixture effect equally well (Table 2). This mixture consisted of both similar-and dissimilar-acting chemicals, though the cause of the good prediction with CA could be due to the chemicals exhibiting a similar mode of action at a physiological level as discussed above. How should we choose between the two models when the basic assumptions are not met for either of them, but they both predict the mixture effect well? Irrespective of mode of action, CA will be the most conservative of the two models when the slopes of the individual toxicants' logistic dose-response curves are above 1.25, as described by Greco et al. (1995). Since this is common for many toxicants, CA will often predict the most conservative mixture effect. As environmentally realistic mixtures consist of both similar-and dissimilar-acting toxicants, concentration addition seems to be preferable as the general reference model in risk assessment. In studies investigating the physiological interactions between chemicals, both reference models might provide valuable information despite their specific limitations.

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All experiments were performed with a cultured clone of the water flea *Daphnia magna* following the OECD *Daphnia* sp. 48 h Immobilization Test (OECD, 2000).

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