# USING CA AND IA CONCEPTS TO ASSESS MIXTURE TOXICITY OF DISSIMILARLY ACTING TOXICANTS TO CLADOCERANS

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***ABSTRACT:*** *The effect of salinity on the toxicity of diazinon, methyl parathion or mercury was assessed with the test organisms – Daphnia magna and Ceriodaphnia cornuta. The results obtained for the mixture toxicity of salinity (NaCl) and the different toxicants were evaluated by the concept of Concentration Addition or Independent Action. The results for the mixture toxicity of NaCl and diazinon, methyl parathion or mercury showed independent action.*

***Keywords:*** *Daphnia magna*; *Ceriodaphnia cornuta*; mixture toxicity; combined effects; Concentration addition; Independent action

## INTRODUCTION

Aquatic organisms are typically not exposed to single toxicants but rather simultaneously to multiple mixtures of chemicals. Evaluation of the mixture toxicity and direct measurement through toxicity testing is an arduous task due to the temporal and spatial variability of chemical composition, a direct effect assessment is, although desirable, not feasible in most case. The combined effects pose many problems to the researchers due in part to the differences in terminology, e.g. in the use of the same term for phenomena as well as for mechanisms, like "additivity", "synergism" and "antagonism". Also, different methods are used for describing and explaining experimental results, from traditional graphics of "isobolograms" (Loewe and Muischnek, 1926), Multiple Toxicity Indices (Kưnemann, 1981) to modern mathematical models of "Concentration Addition" and "Independent Action" (Pưch et al., 1993; Altenburger et al., 2000).

Study on toxicity of mixtures to *C. cornuta*, *D. magna* will be presented in this paper in dealing with the evaluation of toxicity effects in mixtures of chosen substances and sodium chloride.

Much of the literature about assessing combination effects is concerned with establishing the theoretical basis for defining the expected effects combinations of agents (Berenbaum, 1989; Greco et al., 1995). An analysis of the relationship between dose and response provides such a basis. The goal is to analyze dose-response relationships of single compounds in a way that reveals something about the effects of combinations. Two main reference models for defining the expected effects of mixtures of agents have emerged, the models of *concentration addition* (CA) or Loewe additivity (Loewe and Muischnek, 1926) and *response addition* or *independent action* (IA), or Bliss independence or effect multiplication (Bliss, 1939).

The model of *concentration addition* is based on the assumption that chemicals act in a similar way, such that effects can be produced by replacing one compound totally or in part with the other. Each individual component of a multiple mixture is assumed to contribute to the observed overall effect by acting in proportion to its concentration, regardless of any effect thresholds.

The concept of *response addition* was developed on the basis of stochastic considerations and later evolved to assume that compounds act on different sub-systems in organisms, with different sites of action i.e. the combination effect $E_{A,B}$ of a mixture is the product of the individual activities of its components, $E_A * E_B$.

The two concepts were developed independently to suit different experimental contexts, and there are no rational criteria for choosing between them. Nevertheless, both models are currently regarded as equally valid reference points for predicting the effects of mixtures of chemicals (Greco et al., 1992, 1995). Now we return to the suspended point of additivity from the beginning, as defined by

both concepts, it implies that the combined effect of chemicals is greater than that of the single most potent constituent of the mixture.

The concentration addition (CA) is mathematically expressed as

$$\sum_{i=1}^{n}\frac{c_i}{ECx_i}=1 \qquad (1)$$

where n is the number of mixture components, $ECx_i$ is the concentration of the $i^{th}$ mixture component that provokes $x\%$ effect when applied singularly, and $c_i$ is the concentration of the respective component in the mixture. Each fraction $c_i/ECx_i$ represents the concentration of a mixture component scaled for its relative toxicity and is generally termed the toxic unit of that component. Consequently, each compound in the mixture can be replaced by another without changing the overall toxicity as long as the sum of toxic units remains unchanged. If, at a total concentration of the mixture provoking $x\%$ effect, the sum of toxic units equal one, concentration addition holds. For that reason, the sum of toxic units has been frequently used as a measure for comparing the observed toxicity with the prediction made by concentration addition. There is a general consensus that concentration addition is suitable for the prediction of the toxicity of mixtures of similarly acting substances.

In contrast to concentration addition, the concept of independent action (also known as response addition, Bliss independence, or effect multiplication) is based on the assumption that the compounds of a given mixture act on different physiological systems within the exposed organisms. The mathematical formulation of independent action (IA) is as follows.

$$E(c_{mix})=E(c_1+...+c_n)=1-\prod_{i=1}^{n}\left[1-E(c_i)\right] \qquad (2)$$

where $E(c_{mix})$ denotes the predicted effect (scale from 0-1) of an $n$-compound mixture, $c_i$ is again the concentration of the $i^{th}$ compound, and $E(c_i)$ is the effect of that concentration if the compound is applied singly.

Both concepts predict that the overall effect of a mixture is enhanced by adding more compounds. Both require knowledge about the concentration-response relationship of each mixture component which is statistically estimated on the basis of experimental data. A comparative evaluation of the validity of these approaches require an adequate experimental design. The following questions have to be considered:

**How to calculate expected mixture toxicity?**

How to perform concentration-response analysis of mixtures?

To answer these questions, in this work we examine toxicity mixtures of chosen substances and different sodium chloride concentrations by using both concepts, Concentration Addition and Independent Action. The predictive power of both concepts then is compared with another tool, Multiple Toxicity Index which is an accommodated tool to both CA and IA.

## MATERIAL AND METHODS

**Test chemicals**: all chemicals selected including sodium chloride, diazinon, methyl parathion and mercury chloride as mixture components were purchased in the available highest purity. Stock solutions were prepared of all chemicals and stored at 4°C. The same set of stock solutions was used throughout the study.

*Diazinon* (CA: O,O-diethyl O-(2- isopropyl-6-methyl-4 -pyrimidinyl) phosphorothioate; IUPAC: O,O-diethyl,O-2-isopropyl-6-methylpyrimidinyl-4-yl phosphorothioate), formula $C_{12}H_{21}N_2O_3PS$, molecular weight: 304.35, purity 95.0 %, lot number 80723, stored at 4°C was obtained from the laboratory of Dr. Ehrenstorfer-Schäfers, Augsburg, Germany. Separate stock solutions of the chemical were prepared by dissolving weighed amounts into DMSO to achieve a nominal concentration of 100mg/l. Stock solutions were stored at 4°C in parafin film-wrapped glass containers.

*Methyl parathion* (CA: O,O-dimethyl O-(4-nitrophenyl) phosphorothioate; IUPAC: O,O-diethyl,O-4-nitrophenyl phosphorothioate), formula $C_8H_{10}NO_5PS$, molecular weight: 263.21, purity 98.5 %, lot number 90318, stored at -18°C was obtained from the laboratory, Dr. Ehrenstorfer-Schfers, Augsburg, Germany. Separate stock solutions of the chemical were prepared by dissolving weighed amounts into DMSO to achieve a nominal concentration of 100mg/l. Stock solutions were stored at 4°C in parafin film-wrapped glass containers. The first stock solutions (100 mg/l) of diazinon and methyl parathion were diluted in DMSO. These solutions were then diluted in ultra pure water at lower concentrations to obtain the test preparations.

*Mercuric chloride, formula* $HgCl_2$, molecular weight 271.52, was obtained from Merck. Separate stock solutions of the chemical were prepared by dissolving weighed amounts into bi-distilled water to achieve a concentration of 100mg/l. Stock solutions were stored at 4°C in parafin film-wrapped glass containers. The results related to this substance will be expressed as mercury ion and not $HgCl_2$.

**Toxicity tests**: Acute toxicity tests were applied to *C. cornuta* and *D. magna*. The results of EC50 were determined after an expose time of 24h and 48h to the tested toxicants.

**Experimental concentration range:** an identical experiment design was carried out for the toxicity determination of the single substances and the mixtures. At least 8 different concentrations were tested for each substance, each test was done in 4 replicates. The concentrations were chosen to allow for a valid description of the complete range from 1 to at least 80% effect in the case of single substance and from 5 to at least 90% in the case of mixtures. Untreated samples served as the blank and were conducted simultaneously with the tests. A preliminary toxicity assessment of single substances was already reported in chapter three and the mixture toxicity analyses will be presented in this section. First, to plot isobolograms, one substance was tested at various concentration levels (fixed dilution factor) while the other is kept constant. Consequently, the second substance is tested at various concentration levels (same dilution factor) while the first one is kept constant. Next, to calculate CA and IA, we kept a fixed ratio between the two substances identical for every mixture concentration. This ratio corresponded to the ratio between the two EC50s of the single toxicity experiments.

**Data analysis**: It is important to express the experimental results according to the models of interaction. This may be observed by the dose-response curves (DRCs). However, this finding has first to be seen as a phenomenon that theoretically could be brought about by competitive interaction but by other mechanisms as well. DRCs are the graphical expressions of dose-response relationships, whose inherent information we can directly exploit. A DRC is also characterized by the doses necessary to produce certain effects, e.g. by the 50% effective dose, the EC50, by the 10% effective dose, the EC10. EC50 is the dose or concentration at which 50% effect or response is attained. Increasing doses exert increasing effects up to a maximum effect, 100%. Furthermore, the relationship between increases in effects with increasing doses varies with different steepness of DRCs, mathematically expressed, as "slope".

**Prediction of mixture toxicities**: We worked with a mixture of two substances, A and B, the **concentration** addition (CA) therefore developed from equation 1 (Grimme et al., 1998) and expresses as

$$\frac{c_{s1}}{ECx_{(A)}}+\frac{c_{s2}}{ECx_{(B)}}=1 \qquad (3)$$

as the ratio of the mixture components is know, the concentration of each component $c_i$ can be expressed as a fraction of the total concentration in the mixture with $p_i$ being the fraction of component i present in the mixture. Consequently, for the calculation of the effect concentration predicted by CA, equation 2 can be written:

$$ECx_{mix}=\left(\sum_{i=1}^{n}\frac{p_i}{ECx_i}\right)^{-1} \qquad (4)$$

where $ECx_{mix}$ is the total concentration of the mixture provoking $x\%$ effect and $p_i$ denotes the fraction of component $i$ in the mixture. The fixed-ratio design allows to calculate the complete concentration-response curves for both CA and IA by using the following procedures:

1. Calculate for an effect x the corresponding mixture concentration $ECx_{mix}$ according to CA by using the rewritten formula 4.

   *Step i:* Assume effect concentration values is a log normal distribution, calculate Probit corresponding to fixed value of percent of inhibition ray from 5 to 95%,

   *Step ii:* Using results of linear regression developed for substance A, calculate the concentration of A corresponding to a fixed percentage of effect.

   *Step iii:* Repeat step i and ii for substance B

   *Step iv:* Using CA equation (3), develop it as

$$CA=\frac{1}{\frac{1}{EC_A}+\frac{1}{EC_B}\times(ratioA/B)} \quad (5)$$

   and calculate the corresponding mixture concentration that has the fixed percentage of inhibition. From here we calculate concentrations of A and B in mixtures that give a definite fixed inhibition.

2. Use the effect concentration $ECx_{mix}$ of 1. as a starting point to calculate the mixture effect according to IA by developing equation 2, the Independent Action (IA) of two substances is mathematically expressed as

$$E(c_{s1+s2})=E(c_{s1})+E(c_{s2})-E(c_{s1})*E(c_{s2}) \quad (6)$$

3. Repeat steps 1 and 2 for all experimental data.
4. Plot graph with resulting concentration/mixture effect pairs for CA and IA to have a preliminary visualization of experimental concentration-response curves.

These procedures give a preliminary idea showing if the experimental data are close to CA or IA based on mainly linear regression. To be more precise, we conducted curve-fitting approaches.

**Models and curve-fitting**: After having a preliminary visualization of expected concentration-response curves in comparing to CA and IA by prediction step, we applied a mathematical model to improve the goodness of the fit and to provide a more robust model for achieving homogeneity and normality of the residuals. Various models are used to describe and to fit experimental data points to curves. Fitting curves to data using nonlinear regression was recently considered superior to linear regression of transformed data (Puch, 1993). In this study, we present a best fit approach for our DRCs by fitting data points to nonlinear curves using the computer program "THETA.exe". This is a computer program written in Fortran language by Nguyen (2001) to provide a preliminary estimate of the initial values for theta parameters of the survival S-shaped, non-linear parametric regression models. This preliminary estimation of initial values of model parameters is necessary for determining the accurate parameters of the models using SPSS package.

A set of 10 different two- or three-parametric regression models are chosen and described in the following to calculate the concentration-response relationship of the single substances as well as of the mixtures.

For all these models, however, the response is required to be from 0 to 1. Even if the response is the percentage (e.g., y ranging from 0 to 100), then the simple transformation of dividing by 100 should be used. For parameters of models 1 to 4, after simple transformations, the non-linear models become linear and can be easily solved by many available softwares, e.g. MS Excel, SPSS. In the case of model numbers 5 to 10, the determination of the parameters can be performed by using SPSS after the input of the initial parameter values calculated with the THETA software.

## RESULTS and DISCUSSIONS

We found that the dose-response relationships of mixtures of sodium chloride and diazinon, methyl parathion or mercuric ion tested with *C. cornuta* and *D. magna* show a better fit using the models of three-parameter as Logit generalized, Aranda, and the three Box-Cox transformations including Logit, Weibull and Probit than with the two-parameter model as Probit, Logit, Morgan and Weibul. The best fitted models with the highest *R* square coefficients are written in bold in the table 1 and 2.

Using CA and IA concepts to interpret experimental data show that our six experimental mixtures are closer to IA than CA, as presented in Fig. 1 to 6. In IA, the action of one component of the mixture not affects the action of the other component, for example in the case of sodium chloride and diazinon as a mixture, sodium chloride does not affect the action of diazinon, and diazinon does not change the relative effects of sodium chloride. This can be explained by the fact that sodium chloride and the other pollutants have a different mode of action on a cellular level.

The experimental mixtures that we used have a quite high concentration ratio, about $10^6 - 10^7$, this causes some difficulty to evaluate the toxicity of mixtures by observing the figure in which the dose-response curves of single substances and mixtures were plotted simultaneously with the real concentrations. We hypothesize that such substances having an effect in two very different concentrations ranges always have a different mode of action and that the effect of their mixture would therefore always follow the prediction of IA, but this hypothesis would need to be verified.

Tab. 1 Single substance toxicity of mixture components and their mixture toxicities to *C. cornuta*, the $\theta_i$ denotes the parameter of the concentration-response function, the 95% confidence intervals are given in brackets

| Toxicity of the mixture | Fit | $\theta_1$ | $\theta_2$ | $\theta_3$ | $R^2$ | EC1 (mMole/l) | EC50 (mMole/l) |
|---|---|---|---|---|---|---|---|
| NaCl+diazinon | Probit | 1.478 | 3.137 | | 0.870 | 5.2 (4.3-6.4) | 13.3 (10.9-16.2) |
| | Logit | -6.416 | 5.737 | | 0.866 | | |
| | Morgan-Mercier | 0.076 | 2.492 | | 0.866 | | |
| | Weibull/Gompertz | -4.449 | 3.402 | | 0.914 | | |
| | Generalized Logit 1 | -15.78 | 12.70 | 0.70 | 0.990 | | |
| | Generalized Logit 2 | -8.57 | 6.89 | 1.29 | 0.990 | | |
| | Aranda-Ordaz | -7.05 | 5.33 | -14865 | 0.988 | | |
| | Logit with Box-Cox | -3.98 | 0.38 | 0.76 | **0.991** | | |
| | Weibull w Box-Cox | -38.39 | 38.36 | -0.93 | **0.991** | | |
| | Probit w Box-Cox | -2.34 | 0.22 | 0.77 | 0.989 | | |
| NaCl+methyl parathion | Probit | -0.377 | 4.029 | | 0.965 | 5.7 (4.4-7.3) | 21.6 (16.8-27.8) |
| | Logit | -9.654 | 7.286 | | 0.960 | | |
| | Morgan-Mercier | 0.047 | 3.164 | | 0.960 | | |
| | Weibull/Gompertz | -6.579 | 4.450 | | 0.977 | | |
| | Generalized Logit 1 | -16.33 | 11.90 | 0.62 | 0.989 | | |
| | Generalized Logit 2 | -3.38 | 2.47 | 3.01 | 0.989 | | |
| | Aranda-Ordaz | -7.58 | 5.20 | 4935 | 0.997 | | |
| | Logit with Box-Cox | -3.78 | 0.23 | 0.84 | **0.998** | | |
| | Weibull w Box-Cox | -5.67 | 1.15 | 0.22 | 0.997 | | |
| | Probit w Box-Cox | -2.18 | 0.12 | 0.88 | **0.998** | | |
| NaCl+mercury | Probit | -1.002 | 4.332 | | 0.965 | 7.1 (5.2-9.8) | 24.3 (17.6-33.5) |
| | Logit | -10.92 | 7.919 | | 0.945 | | |
| | Morgan-Mercier | 0.042 | 3.439 | | 0.945 | | |
| | Weibull/Gompertz | -6.530 | 4.359 | | 0.981 | | |
| | Generalized Logit 1 | -12.77 | 9.09 | 0.76 | 0.977 | | |
| | Generalized Logit 2 | -6.31 | 4.49 | 1.53 | 0.977 | | |
| | Aranda-Ordaz | -7.50 | 5.05 | 19215 | 0.989 | | |
| | Logit with Box-Cox | -2.39 | 0.04 | 1.39 | **0.996** | | |
| | Weibull w Box-Cox | -3.15 | 0.19 | 0.77 | **0.995** | | |
| | Probit w Box-Cox | -1.43 | 0.02 | 1.42 | **0.995** | | |

Tab. 2 Single substance toxicity of mixture components and their mixture toxicities to *D. magna*, the $\theta_i$ denotes the parameter of the concentration-response function, the 95% confidence intervals are given in brackets

| Toxicity of the mixture | Fit | $\theta_1$ | $\theta_2$ | $\theta_3$ | $R^2$ | EC1 (mMole/l) | EC50 (mMole/l) |
|---|---|---|---|---|---|---|---|
| NaCl+diazinon | Probit | -2.781 | 4.463 | | 0.880 | 16.5 (10.4-26.2) | 55.5 (34.9-76.1) |
| | Logit | -14.678 | 8.448 | | 0.885 | | |
| | Morgan-Mercier | 0.018 | 3.668 | | 0.885 | | |
| | Weibull/Gompertz | -11.476 | 6.188 | | 0.987 | | |
| | Generalized Logit 1 | -25.14 | 13.99 | 0.66 | 0.973 | | |
| | Generalized Logit 2 | -9.66 | 5.37 | 1.72 | 0.973 | | |
| | Aranda-Ordaz | -12.05 | 6.51 | 6215 | 0.998 | | |
| | Logit with Box-Cox | -3.59 | 0.03 | 1.17 | **0.998** | | |
| | Weibull w Box-Cox | -5.13 | 0.29 | 0.55 | 0.991 | | |
| | Probit w Box-Cox | -2.06 | 0.01 | 1.24 | 0.990 | | |
| NaCl+methyl parathion | Probit | -2.005 | 4.151 | | 0.849 | 13.5 (8.7-21.0) | 49.0 (31.5-76.1) |
| | Logit | -12.609 | 7.498 | | 0.835 | | |
| | Morgan-Mercier | 0.021 | 3.256 | | 0.835 | | |
| | Weibull/Gompertz | -9.217 | 5.069 | | 0.921 | | |
| | Generalized Logit 1 | -25.66 | 14.74 | 0.67 | 0.991 | | |
| | Generalized Logit 2 | -16.70 | 9.59 | 1.03 | 0.991 | | |
| | Aranda-Ordaz | -14.74 | 8.35 | 2 | **0.992** | | |
| | Logit with Box-Cox | -7.32 | 0.57 | 0.49 | **0.992** | | |
| | Weibull w Box-Cox | -48.89 | 31.73 | -0.59 | 0.991 | | |
| | Probit w Box-Cox | -5.31 | 0.58 | 0.37 | 0.991 | | |
| NaCl+mercury | Probit | -0.185 | 3.010 | | 0.951 | 9.0 (6.4-12.7) | 53.0 (37.7-74.6) |
| | Logit | -8.873 | 5.143 | | 0.963 | | |
| | Morgan-Mercier | 0.019 | 2.234 | | 0.963 | | |
| | Weibull/Gompertz | -7.307 | 3.947 | | 0.981 | | |
| | Generalized Logit 1 | -4.60 | 2.64 | 2.41 | 0.992 | | |
| | Generalized Logit 2 | -11.58 | 6.65 | 0.96 | 0.992 | | |
| | Aranda-Ordaz | -10.21 | 5.80 | 1.36 | 0.992 | | |
| | Logit with Box-Cox | -6.91 | 0.98 | 0.26 | **0.993** | | |
| | Weibull w Box-Cox | -17.66 | 8.18 | -0.36 | 0.990 | | |
| | Probit w Box-Cox | -3.91 | 0.51 | 0.29 | 0.990 | | |

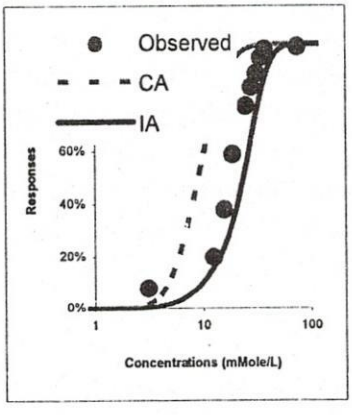


Fig. 1 Predicted and observed mixture toxicity of sodium chloride and diazinon to *C. cornuta*

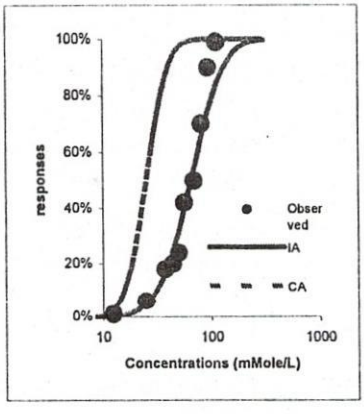


Fig. 2 Predicted and observed mixture toxicity of sodium chloride and diazinon to *D. magna*

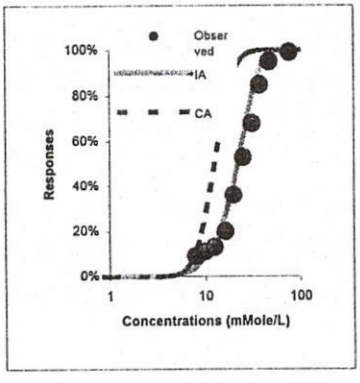


Fig. 3 Predicted and observed mixture toxicity of sodium chloride and methyl parathion to *C. cornuta*

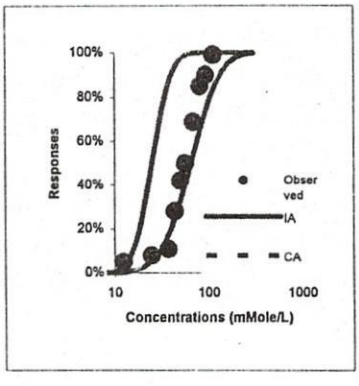


Fig. 4 Predicted and observed mixture toxicity of sodium chloride and methyl parathion to *D. magna*

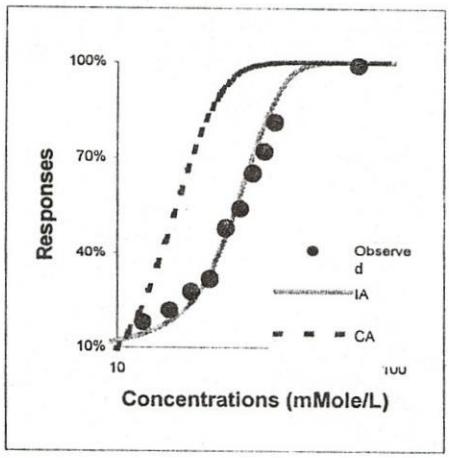


Fig. 5 Predicted and observed mixture toxicity of sodium chloride and mercury to *C. cornuta*

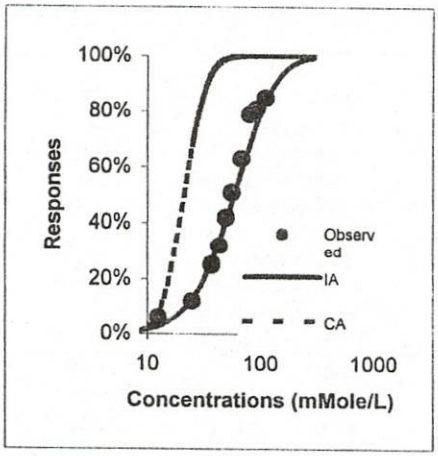


Fig. 6 Predicted and observed mixture toxicity of sodium chloride and mercury to *D. magna*

The graphical indication of effects in DRCs show equi-effective doses of the mixture 's components. From here we can interpret mixture actions as independent or concentration addition. Independent actions can be additive, under additive or antagonistic, over additive or synergistic in DRCs studies (Unkelbach, 1988) as well as in isobolograms (Christensen and Chen, 1985). The CA-IA concept takes the biological system as black boxes. Without implicating any biological premises, CA-IA concept just accounts for the concentrations as input and the observed effects as output. With input/output models the assessment of mixture effects is based either on concentration addition or on Independen Action.

The choice of the right concept for the assessment of mixture toxicity depends on which situation is found. In most experimental studies there will be little or no knowledge about the specific sites and mode of actions of toxicants. If sufficient data is available, we therefore recommend the choice of a concept with reference to the concept CA-IA by taking into account the concentration responses curves. However, the application of the mentioned concept has to follow an explicit decision on the shape of the curves. This can be extremely difficult, especially with poor data. Making use of isobolographics or algebraic equivalent (MTI) instead of the apparently convenient CA-IA concept might bypass this problem. The evaluation of mixture toxicity could therefore be better achieved by not only one method but also by using CA-IA and also the other methods including isobolographics, DRCs of and Mixture Toxicity Index (Kunemann, 1981, Svenson, 2001).

## CONCLUSIONS

The dose-response relationships of tested mixtures with tested organisms showed a better fit in using the three-parameter models than with the two-parameter models. Consequently, they contributed as a great predicted power of Concentration Addtion or Independent Action in evaluating mixture toxicity.

Interactions of sodium chloride with tested substances consist of diazinon or methyl parathion or mercury to *C. cornuta* and *D. magna* were neither antagonism nor synergism. These mixtures of sodium chloride and the tested substances can be considered as "independent actions" and "additivity" at the same time.

The toxicity of the salt present in the Sai Gon–Dong Nai rivers at high tide will add to the toxicity of the other substances. This mixture toxicity is expected probably from the effects of the individual substances, maybe "concentration addition" or "independent action" depends on mode of action of toxicants.

# SỬ DỤNG NGUYÊN LÝ CA VÀ IA ĐỂ ĐÁNH GIÁ ĐỘC TÍNH HỖN HỢP CÁC CHẤT Ô NHIỄM LÊN VI GIÁP XÁC

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***TÓM TẮT:*** *Ảnh hưởng của độ mặn lên độc tính của diazinon, methyl parathion hoặc thủy ngân lên sinh vật thí nghiệm - **Daphnia magna** and **Ceriodaphnia cornuta** qua nghiên cứu này. Độc tính hỗn hợp của muối và các chất ô nhiễm khác nhau được đánh giá thông qua nguyên lý "nồng độ bổ sung" và "hoạt động độc lập". Độc tính của hỗn hợp muối và diazinon, methyl parathion hoặc thủy ngân tuan theo kiểu "hoạt động độc lập" là kết quả thu được của nghiên cứu này.*

***Từ khóa:*** *Daphnia magna; Ceriodaphnia cornuta; độc tính hỗn hợp, ảnh hưởng phối hợp, "Nồng độ Bổ sung", "Hoạt động Độc lập"*

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