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Multiple Regression Analysis of Toxic Interactions: Application to the Microtox Test and General Comments

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Interaction studies are now a challenging point of interest in environmental toxicology. Living organisms are more often exposed to multiple aggressions in a complex biotope than to a single toxicant in a pure medium. Since combined effects may result in additive, antagonistic or synergistic action - the last being the most harmful to organisms - it is important to determine the impact of mixed toxic substances in the environment.

We describe here the method currently utilized in our laboratory for determining the presence or absence of interactions between two or more compounds in mixture. First, a clear definition of interaction is necessary ; we propose the following one : interaction between two (or n) agents corresponds to the fact that the effect of each one depends on the dose of the other(s). The effect studied here is the response of a bacterial test (Microtox™ Test) to various concentrations of toxicants. We report results obtained with zinc (Zn) and pentachlorophenol (PCP) which are respectively among the most commonly found inorganic and organic pollutants in contaminated surface waters (USEPA,1980 ; CEE,1982).

The adopted approach consists in the multiple regression analysis of factorial experiments. Such an approach of interaction studies is now more and more utilized - see for example Kaitala et al. (1984). This method is powerful and well suited to toxicity screening provided that the expression of a test response obeys to a precise criterion - additivity. The last condition leads us to define a new expression of Microtox™ Test results. An other point discussed here concerns the isobolographic method, a classical analysis of interactions (Loewe, 1953), which is shown to be likely to lead to erroneous conclusions.

MATERIALS AND METHOD

Toxic effects of zinc sulfate ($ZnSO_4$, $7H_2O$, Prolabo 2915323) and pentachlorophenol (Merck 807042) were assessed with the Microtox™ test. This test consists in measuring the decrease of the bioluminescence of marine bacteria (Photobacterium phosphoreum) occuring when toxic substances are added to the bacterial suspen-

sion. This decrease is more important when the toxicant concentration is high : an EC50 can be defined as the effective concentration of a tested compound which inhibits 50 % of the bacterial luminescence. The luminescence intensity is recorded before (I_o) and a few minutes (I_t) after the introduction of toxicants in the test medium (Bulich, 1982).
A blank assay is performed simultaneously in order to correct from natural variations of luminescence ; the ratio $I_{t(b)}/I_{o(b)}$ for this assay is designated by BR (blank ratio). The standard procedure of the test has been followed (Beckman Instrument Inc. 1982). The test temperature was 20°C, the time of contact 30 min. The variations of light output are commonly expressed as Δ% :

$$\Delta\ \% = 100\ (I_{o(x)}.BR - I_{t(x)})/(I_{o(x)}.BR)$$

We also define log β which interest will be demonstrated in the next section and constitute a new expression of the results :

$$\log \beta = \log\ (I_{t(x)}/(I_{o(x)}.BR))$$

A set of response of the test to zinc and PCP alone or together has been obtained in a factorial experiment designed with four levels for each variable :

zinc concentration : 0 ; 0.2 ; 0.6 ; 1.4 mg/l

PCP concentration : 0 ; 0.25 ; 0.81 ; 2.08 mg/l

These concentrations range from 0 to twofold EC50 values. Each level of a variable is combined with the four levels of the other so that 4 x 4 = 16 experiments are needed. In addition triplicates were performed.

Multiple regression analysis was used to modellize the response y of the test by an equation of the form :

$$\hat{y} = b_o + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \ \cdots$$

where $\hat{y}$ stands for the calculated value from the model, x_1, x_2 for the controlled experimental factors (concentrations of Zn and PCP), and b for the regression coefficients. The term $b_{12}x_1x_2$ is characteristic of an interaction between x_1 and x_2 ; in fact if b_{12} is significant the above equation is equivalent to :

$$\hat{y} = b_o + b_1x_1 + (b_2 + b_{12}x_1)x_2 \ \cdots$$

where obviously the coefficient of x_2 depends on x_1, indicating that the effect of x_2 depends on the value of x_1 and conversely.
For computations, concentrations were transformed in standard units (by log transformation and centring). The independance of variables Zn and PCP, achieved with the factorial plan, sets their covariance to zero, making computations quick and easy with a microcomputer. For statistical assessment of the model the coeffi-

cients b are ranked by order of contribution to the regression. The less important terms are eliminated until the residual variance of the model becomes equal or just superior to the variance of triplicates (intra-group variance). By this mean the model has a precision adapted to the experimental reproducibility and does not reach an illusory accuracy. The coefficients of the remaining terms are also tested with a classical Student's test.

RESULTS AND DISCUSSION

Table 1 presents the results obtained for triplicate experiments. Results are expressed by $\Delta\,\%$ and $\log \beta$. Two models have been computed : $\Delta\,\% = f(Zn, PCP)$ and $\log \beta = f(Zn,PCP)$. The corresponding equations are :

$$\hat{\Delta}\% = 83 + 46X_1 + 50X_2 - 19X_2^2 - 84X_2^3 - 88X_1X_2 \qquad \text{(eq.1)}$$

and

$$-\log \hat{\beta} = 0.8 + 1.19X_1 + 0.62X_1^2 + 0.79X_2 + 0.645X_2^2 \qquad \text{(eq.2)}$$

where

$$X_1 = \log ((Zn^{++}) + 0.2) \qquad (Zn^{++}) : \text{mg/l}$$

$$X_2 = \log ((PCP) + 0.2) \qquad (PCP) : \text{mg/l}$$

These two models are respectively represented on figures 1 and 2 ; although they derive from the same observations they differ greatly. This will be the first point of our discussion. The model $\hat{\Delta}\%$ indicates an antagonism between zinc and PCP (a significant negative coefficient is assigned to the term X_1X_2) whereas the model $\log \hat{\beta}$ does not. It would be vain to attribute to the term $-88X_1X_2$ in eq. 1 the meaning of an actual antagonism. This false interaction comes from the fact that the expression $\Delta\,\%$ (which cannot exceed 100 %) does not suit mixture studies. This can be illustrated as follows : if we take a concentration of toxicant 1 which produces alone 80 % of inhibition of luminescence and a concentration of toxicant 2 which produces 70 % of inhibition, of course, a mixture of both at previous concentrations will not achieve 150 % of inhibition.

Therefore the mathematical process of multiple regression introduces artificially the term X_1X_2 with minus sign only for keeping the computed values under the limit of 100 %. It is necessary that the mathematical composition law of the observed variable be additive i.e. in the case of non-interaction between compounds 1 and 2, the expression accounting for the effect of their mixture have to be theoretically the sum of the expressions describing their effects when alone. This applies also when n compounds are studied.

We propose the new expression of results, i.e. $\log \beta$ which fulfills the above requirement. We can consider that the ratio

Table 1. The responses of Microtox™ Test to zinc (Zn) and Pentachlorophenol (PCP) alone or in combination, expressed by Δ% and log β (triplicate experiments)

N°	Zn mg/l	PCP mg/l	Δ %			- log β		
1	0	0	- 4.9	- 0.1	-10.8	-0.021	-0.004	-0.045
2	0	0.25	15.0	18.9	13.7	0.071	0.091	0.064
3	0	0.81	51.9	48.1	52.7	0.318	0.285	0.325
4	0	2.08	83.5	83.5	81.7	0.784	0.780	0.740
5	0.2	0	22.9	26.5	26.5	0.113	0.130	0.130
6	0.2	0.25	27.8	33.9	32.4	0.142	0.181	0.170
7	0.2	0.81	66.3	62.0	61.2	0.472	0.420	0.410
8	0.2	2.08	87.2	86.1	85.4	0.892	0.856	0.839
9	0.6	0	58.8	60.0	62.9	0.385	0.410	0.430
10	0.6	0.25	60.3	62.5	62.8	0.400	0.429	0.430
11	0.6	0.81	82.5	80.7	76.1	0.756	0.710	0.620
12	0.6	2.08	91.2	92.1	91.8	1.06	1.10	1.10
13	1.4	0	90.6	87.1	87.0	1.03	0.890	0.870
14	1.4	0.25	92.6	84.1	83.4	1.13	0.800	0.780
15	1.4	0.8	94.6	90.0	89.8	1.30	1.00	0.990
16	1.4	2.08	96.7	97.3	95.0	1.49	1.57	1.30

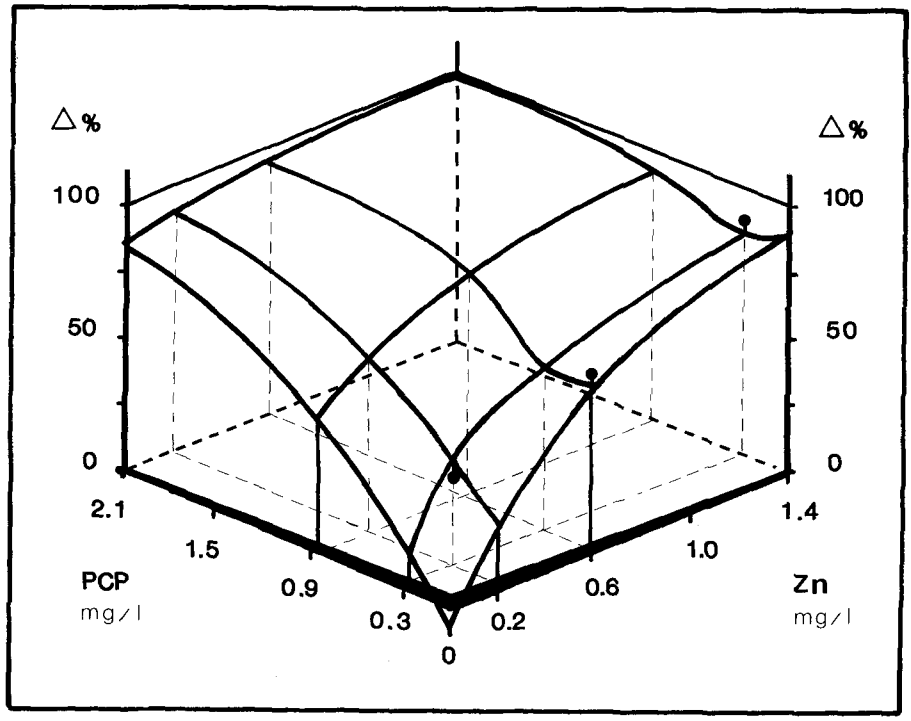

Figure 1. Model representation of bacterial luminescence variations (expressed as $\Delta\%$) as a function of zinc (Zn) and Pentachlorophenol (PCP) concentrations reported in linear scale
(↕ indicates deviations from experimental values)

$I_{t(i)}/I_{o(i)}BR$ represents the fraction of an inhibited light-producing unit (enzyme or enzymatic complex...) after action of a compound i during time t. Clearly when two compounds (1) and (2) act independently the effect of their mixture is :

$$\log (\beta_{1+2}) = \log (\beta_1 \times \beta_2)$$

so that : $$\log (\beta_{1+2}) = \log (\beta_1) + \log (\beta_2)$$

That is exactly what occurs in the case of zinc and PCP : each point of the surface in figure 2 has an elevation equal to the sum of marginal elevations obtained for zinc and PCP alone at the same concentrations. In the equation 2 there is no term as X_1X_2 : we can affirm, owing to the use of $\log \beta$, that there is no antagonism or synergism between zinc and PCP towards Microtox™ Test. Note that in eq.2 responses of toxicant alone are not linear (a linear response was not expected over a so large range of concentrations).

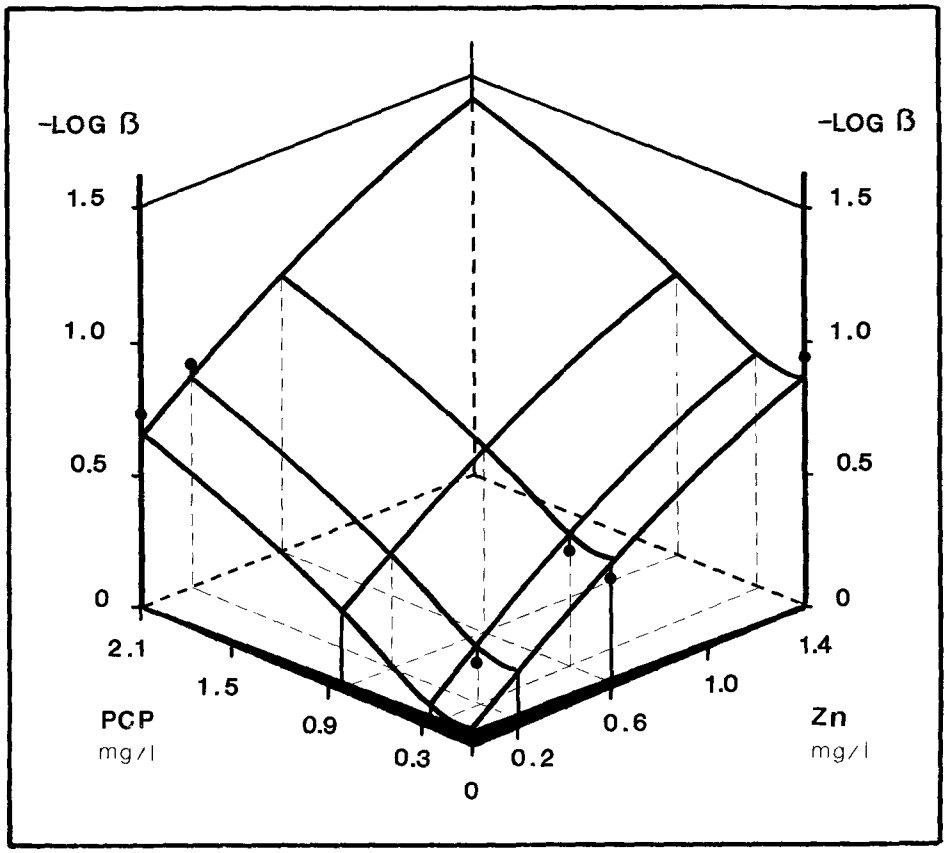

Figure 2. Model representation of bacterial luminescence variations (expressed as -log ß) as a function of zinc (Zn) and Pentachlorophenol (PCP) concentrations reported in linear scale
(↑ indicates deviations from experimental values)

The second point to be discussed is that a partial analysis of data, as performed by isobolograms, would have been misleading even when using log ß as observed variable. Isobolograms are obtained by plotting various doses of two substances which, if combined, produce a certain constant effect (Ariens, 1972 ; Loewe, 1953 ; Zipf and Hamacher, 1966). It is easy to see that from our data an isobologram is obtained by taking an horizontal cross-section of the surface in figure 2. For example, with a median effect (- log ß = 0.30) the corresponding curve is presented in figure 3. Obviously, this is not a linear isobologram although we have previously shown that Zn and PCP act independently. This effect is due to the fact that dose-response curves of compounds alone are not linear, inducing a deformation of the constant effect curve. According to the classical interpretation of isobolograms linearity is related to additivity of effects : in fact, this is only true if dose-response curves of compounds alone are linear over the entire range of concentrations tested. So linearity of isobolograms is an irrelevant criterion of

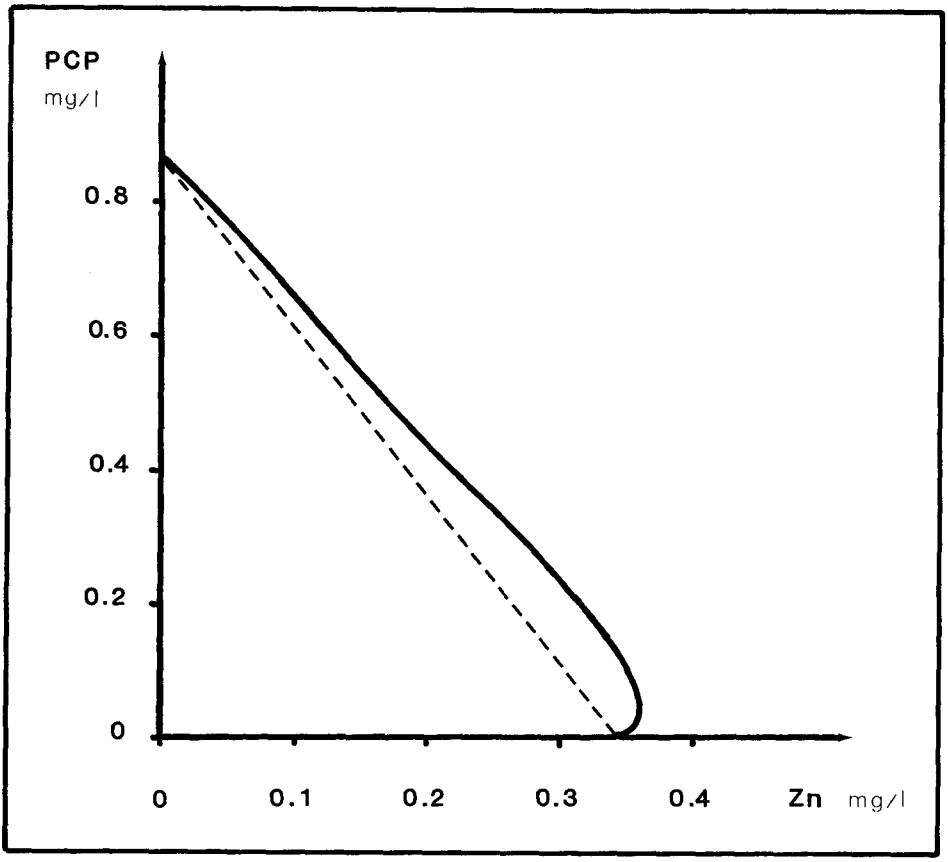

Figure 3. Horizontal cross-section of the model -log β = f(Zn;PCP) corresponding to 50 % of inhibition of bacterial luminescence (- log β = 0.30)

non-interaction. In our model representation, the absence of interaction can be indicated geometrically by the series of curves $\hat{y} = f[Zn,(PCP \text{ constant})]$ for various concentrations of pentachlorophenol, obtained by the vertical cross sections of the surface in figure 2 for a given dose of PCP. These curves presenting a similar shape can be deduced from each other by translation without any deformation ; this property is characteristic of non-interaction. The same is true for the curves $\hat{y} = f[PCP,(Zn \text{ constant})]$ for various concentrations of Zinc. For these reasons, the use of isobolograms when analyzing their linearity only, is not suited for interaction studies.

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