A DYNAMIC ANALYSIS OF TOXIC MIXTURE EFFECTS

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1. Introduction

A major hurdle in clarifying underlying mechanisms of toxic effects of mixtures is obtaining detailed information about the interaction between the individual substances during exposure, uptake, physiological processes and processes at the target receptors. Within many applications it is not feasible to investigate the modes of action of the total range of possible toxic chemicals in the broad variety of biological systems. However, in order to enable the analysis of mixture toxicity data it is required to derive a hypothesized combined effect from the toxicity of the individual compounds. For that purpose the principle of additive action and the principle of independent action are available. Both principles are embedded in a mechanistic context. Additive action (or concentration addition (Loewe and Muischnek, 1926)) is generally related to a similar mode of action and target sides of the individual components in the mixture. Consequently, each compound acts as a dilution of the other. On the other hand, independent action (Bliss, 1939), it is related to a dissimilar mode of action and different target sides of the individual compounds. Hence, it is assumed that the chemicals in the mixture do not interfere with each other during exposure, uptake and toxic action.

In order to capture the different modes of action of the individual chemicals in a conceptual framework, Hewlett and Plackett (1959) defined four possible combination mechanisms (table1). Ideally, for drawing solid conclusions from a mixture experiment, it is required to identify these combination mechanisms in the data set. However, at present sound criteria are still lacking and the way to use additive and independent action is still subject to discussion (Greco *et al*., 1995). Nevertheless both principles, especially the additive action principle, are generally applied in many fields of research (e.g. Cassee *et al*., 1998, Hömme *et al*., 2000, Nielsen *et al*., 2000, Backhaus *et al*., 2000, Altenburger *et al*., 2000). Usually the major interest is to assess the mixture effect relative to the toxicity of the individual toxicants, beyond the experimental conditions (e.g. cancer research and ecological risk assessment). Thus, within this context the possibilities and shortcomings of these theorems should be defined accurately.

Table 1: Combination mechanisms defined by Hewlett and Plackett (1959) to characterize combined effects of toxicants.

	Similar action	**Dissimilar action**
No Interaction	Simple similar	Independent
Interaction	Complex similar	Dependent

I feel, after thorough evaluation of conceptual and statistical discussions in literature (Calabrese, 1995, Chou and Hayball, 1996, Greco *et al*., 1990, 1995, Gessner, 1995, Haas *et al*., 1996, 1997, Könemann and Pieters, 1996) that both the additive and the independent principle should not be interpreted mechanistically (Jonker *et al*., *in prep.*). As a result they do not constitute real alternatives. They should be considered as reference models, relative to which the data can be analyzed. The reference can be chosen more or less arbitrarily, dependent on the research question. The models enable a description of the combined effect without revealing physiological or chemical interactions. Predicting toxic effects beyond the

experimental conditions should therefore be performed with caution. In order to enable this kind of extrapolations, mechanistic/dynamic insight in the combined effects is inevitable. It has been reported that deviations from additive action show time dependence (Singh *et al*., 1998), which illustrates the necessity of a dynamic approach. In this way time independent mixture effects can be characterized and quantified. It would be interesting to evaluate whether DEB provides tools to accomplish this task. Hence:

Research question:
To what extent is the prediction of toxic mixture effects feasible, when DEB theory is used for data interpretation?

In this assay an experiment is proposed to investigate this question.

2. Toxicology in DEB

2.1. Background

The DEB (Dynamic Energy Budgets) theory provides a framework to analyze toxicity data mechanistically. The interpretation of single toxicity data has been worked out in detail (Kooijman, 2000, Kooijman and Bedaux, 1996). The quantification procedures have the following attractive properties:

- Uptake/elimination kinetics is included, whereas it is not necessary to actually measure toxicant fluxes.
- The organism can handle low concentrations of toxicants, which is a realistic feature.
- The dynamic properties allow the calculation of a time independent (ultimate) N.E.C. (No Effect Concentration).
- Sublethal toxicity is quantified by analyzing to what extent the toxicant changes the energy allocation in the organism. This concept is backed up with strong, generally applicable theoretical support.
- The DEB theory also provides a framework to extrapolate toxic effects at the individual level to higher trophic levels.

The analysis of single toxicity data is robust, and can easily be implemented in regular toxicity testing. Thus, it is worthwhile to investigate whether these quantification methods can also be applied to analyze the effects of chemicals administered jointly.

2.2. Problems of combined toxic effects

Even within a very simple binary mixture the amount of combinations is endless. Therefore it is interesting to comprehend to what extent this variability hampers the predictability of the toxic effect. After all, also single compounds are actually mixtures of different ionic forms and/or metabolites, which is generally not accounted for in toxicity testing.

In general dynamic models allow predictions as long as parameter values “in the organism” are relatively constant. Also in DEB theory it is assumed that toxicokinetic parameter do not change during exposure time. Only certain physiological target parameters are changed (which regulate the energy allocation in the organism) due to the harmful action of the internal toxic chemical concentration. However, the toxic action can also comprise inhibition of

defense mechanisms and detoxifying enzymes, and an increased permeability of membranes. Combination toxicity complicates these effects due to (possible) interactions. Hence, the question raises to what extent it is reasonable to assume that toxicokinetic parameters stay constant.

The predictability of the (combined) toxic effect might improve if toxicokinetics is linked to the physiological state of the organism. For instance, prolonged exposure time might change elimination rates of toxicants, due to irreversible damage to defense mechanisms. Then it might be important to tie a decrease in elimination rate to a change in maximum specific assimilation rate, specific maintenance rate or costs of growth, which represent the "health state" of the organism. Maybe it is necessary to link the value of interaction parameters to internal toxicant concentrations: the more chemicals are taken up, the higher the chance of interaction. The objective is to obtain a very first indication of the importance of this kind of "confounding factors".

2.3. Implementing combination toxicity

This is the "technical" part of this assay. It is added in order to illustrate the current possibilities of implementing combination toxicity in the DEB theory. Background information, interpretation of the symbols and dimensions of the quantities are omitted for brevity. They are well described in the references. The inclusion of mixture effects has been discussed (Houte and Bedaux, 1997, Kooijman, 2000, Kooijman and Bedaux, 1996), but it is still in a developmental stage. At present, two possibilities can be distinguished: 1) extending the toxicokinetic functions that are described for single toxicants, and 2) extending the number of reserves in the DEB model.

Toxicokinetics in DEB is delineated from one compartments kinetics. The toxic effect is induced by an internal concentration of the chemical, which is quantified by integrating the chemical flux. Assuming that the environmental concentration (c_i) of the chemical is constant, and that the initial concentration in the organism is negligibly small, yields:

$$c_{V_i}(t) = c_i(1 - e^{-k_a t}) \tag{1}$$

In this equation c_V is the scaled concentration of the chemical in the organism. The actual concentration in the organism ([Q]) can be quantified by $[Q](t) = k_u / k_a c_V$, where k_u and k_a are uptake and elimination rates.

When data is analyzed, it is required to distinguish "time to event effects" (e.g. survival, duration of the juvenile period, etc.) from the other toxicological endpoints (e.g. growth, amount of eggs, etc.). If "time to event effects" are of interest, then the hazard function can be expanded. For a single toxicant the hazard rate is quantified by:

$$h(t;c) = k_\dagger \left(c(1 - e^{-k_a t}) - c_0 \right)_+ \tag{2}$$

where $(x)_+$ means the maximum of x and 0, and c_0 denotes the environmental (ultimate) no-effect concentration. If we define $c_i^*(t) = c_i(1 - e^{-k_{a_i} t}) - c_{0_i}$, then the hazard rate of toxicants administered simultaneously can be calculated by:

$$h(t;c_1,c_2,...,c_n) = \sum_{i=1}^{n} k_{\dagger_i} c_i^*(t) + k_{\dagger_{1,2,...,n}} \prod_{i=1}^{n} c_i^*(t) \tag{3}$$

for every compound 1,2,…,*n*. The combination killing rate $k_{1,2,...,n}$ can be considered as an interaction term. In this model, specific no-effect concentrations (c_0) are tied to each individual toxicant. The model can also be defined in such a way that all individual toxicants have a joint no-effect concentration, which might be more realistic.

$$h(t; c_1, c_2, ..., c_n) = \left(\sum_{i=1}^{n} k_i c_{V_i}(t) + k_{1,2,...,n} \prod_{i=1}^{n} c_{V_i}(t) - k_t \right)_+ \tag{4}$$

for *n* compounds, and The parameter k_t can be considered as the combined hazard toleration. The survival probability (for eq (2): if $c_i > c_{0,i}$, and $t > t_0$) is given by:

$$q(t; c_1, c_2, ..., c_n) = \exp\left\{ -\int_0^t h(\tau; c_1, c_2, ..., c_n) d\tau \right\} \tag{5}$$

for every compound 1,2,…,*n*.

Effects, other then "time to event" effects, can be quantified by a dimensionless stress value *s*. If the individual compounds in the mixture have the same physiological target parameter, *s* is defined by:

$$s = \left(\sum_{i=1}^{n} \frac{c_{V_i}(t)}{c_{Ti}} + \frac{\prod_{i=1}^{n} c_{V_i}(t)}{C_T} - s_0 \right)_+ \tag{6}$$

for every compound 1,2,…,*n*. In this formula, c_{Ti} is the tolerance concentration, and C_T is inversely proportional to an interaction term. The stress value can be used to calculate the effect of the mixture on a certain target parameter, e.g. maximum specific assimilation rate, specific maintenance rate, costs of growth, costs of reproduction or hazard of the ovum (Kooijman, 2000).

Note that in eq 3, 4 and 6 it is difficult to interpret the interaction term. The terms are not backed up mechanistically, and are actually incorporated to enable the quantification of effects that cannot be explained by simply the addition of c_V's. Interaction should be quantified more elegantly. At this stage, I do not have a sound solution, but regarding the descriptive nature of the term it might be more appropriate to define a dimensionless term. For instance, we can calculate:

$$x_i = c_{V\,i} \left(\sum_{i=1}^{n} c_{V\,i} \right)^{-1} \tag{7}$$

and define an interaction term as:

$$I = k_I \prod_{i=1}^{n} x_i \tag{8}$$

and use this term instead of the product term in eq 3, 4 and 6. In this equation the interaction is quantified by k_I. Note that this term does not have any mechanistic basis, but is only a method to obtain a dimensionless interaction quantity. Its appropriateness has to be evaluated critically, if implementation is of interest.

The second method to include mixture toxicity in DEB is to extend the number of reserves. Theoretically, this should be a suitable approach for quantifying effects of toxicants that can be regulated physiologically. The balance equation for reserves M_{Ei} is defined as:

$$\frac{d}{dt} M_{Ei} = \dot{J}_{Ei,A} - \dot{J}_{Ei,C} + \kappa_{Ei} \dot{J}_{Ei,R} \qquad (9)$$

Thus, the dynamics of reserves is determined by the difference between the anabolic flux and the catabolic flux. The third term on the right hand site denotes the fraction of the rejected flux (by the synthesizing units involved), that returns to the reserves. This approach has not been tried yet to analyze (mixture) toxicity endpoints. When its applicability is evaluated the following aspects have to be considered:

- Is it a solid method that allows the translation of mass fluxes to toxic effects?
- In the toxicokinetic approach toxicant fluxes are incorporated without the necessity of actually measuring them, which is of practical interest. Is this also possible with the multiple reserves method?
- How to quantify interactions?

The preliminary character of this approach does not allow any analysis of experimental data yet, therefore in the next session only the toxicokinetic models are considered.

2.4. Proposed experiment

Experimental approach:

As indicated above, the main interest is to analyze the predictive possibilities when the DEB theory is adopted for interpreting mixture toxicity. Therefore it is required to comprehend to what extent toxicokinetic parameter values change due to “confounding factors”. The following experimental set up might reveal some of the possible interactions.

It is required to investigate a dose response relationship: organisms (e.g. *Daphnia*, *C. elegans*) are exposed to different relevant combinations of a certain mixture with *n* compounds, and the toxic effect is determined. It is important to test enough combinations to cover the relevant concentrations on the *n* dimensional combination plane. The experimental set-up should allow for a certain amount of observations of the toxic effect in time. See for an example: Houte and Bedaux (1997). Either a sublethal or a lethal toxicological endpoint can be of interest, but can be chosen arbitrarily. Note that it is very likely that different endpoints yield different results. When the toxic effect of the mixture is determined in time, this data can be used to analyze the possibilities of predicting combination effects.

Modeling approach

The basic idea behind the modeling approach is to use only a part of the data set to estimate the parameters in the model. In this way it can be evaluated to what extent the other part of the data set is predicted satisfactorily. With regard to mixture toxicity, the behavior of the interaction parameter ($\dot{k}_{\dagger 1,2,\ldots n}$ or C_T) is of special interest. The following questions can be addressed:

- *To what extent do parameter values change over time?*

This can be evaluated by using only data from the first time points to estimate model parameters and to analyze whether the effects at the last time points are predicted accurately.

Alternatively it can also be evaluated by using only data from the last time points to estimate model parameters and to analyze whether the effects at the first time points are predicted accurately.

- *To what extent do parameter values change due to toxic strength of the mixture?*

This can be evaluated by using only data related to low toxicity to estimate model parameters and to analyze whether the effects at high toxicity are predicted accurately. Alternatively it can also be evaluated by using only data related to high toxicity to estimate model parameters and to analyze whether the effects at low toxicity are predicted accurately.

- *To what extent do parameter values change due to differences in toxicant ratios?*

This can be evaluated by using only data related to a certain combination ratio to estimate model parameters and to analyze whether the effects related to other combinations are predicted accurately.

Of course, also the comparison of parameter values, obtained from each calculation run, is of interest. Insight in these questions can help developing tools for predicting toxic mixture effects beyond experimental conditions. Note however that it is probably extremely difficult to identify the exact cause of the variability in the parameter values. This observational study can be used to determine how serious the variability is, and to what extent it should be taken into account when predictions are performed.

3. References

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