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ANALYSIS OF BIOASSAYS WITH TIME-VARYING CONCENTRATIONS

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Abstract—In the analysis of ecotoxicological bioassays the concentration of the test compounds is assumed to be constant. In many situations this assumption is questionable, as various processes may cause a substantial decline in the concentration during exposure. This leads to difficulties in the estimation of parameters that characterise the toxicity of the test compound. As a solution to this problem, time-varying concentrations are often replaced by their mean values for the estimation of toxicity parameters. However, Monte-Carlo simulations show that this approach results in biased estimates of the toxicity parameters. As an alternative approach, we propose models to estimate one important toxicity parameter, the no effect concentration, in situations where the concentration of the compound varies in time. These models are extensions of the DEBtox model (Kooijman and Bedaux, 1996) which is based on biological assumptions about toxicokinetics and toxic effects. We also propose a new approach for the estimation of toxicity parameters for strongly accumulating non-metabolisable compounds. This approach does not require any kinetics assumption. Computer simulation and experimental data confirm the relevance of the different proposals. © 2001 Elsevier Science Ltd. All rights reserved

Key words—aquatic bioassays, temporal variation, biological model, bioconcentration, no effect concentration

INTRODUCTION

In environmental risk assessment one always has to extrapolate findings from laboratory experiments to field situations. Two major problems bedevil this extrapolation. First, while in the field exposure is rarely if ever constant in time, standard toxicity tests prescribe just that. In line with this prescription, methods to analyse data from laboratory studies mostly assume a constant exposure. The second problem is that this assumption of a constant exposure during the test is rarely satisfied. This hampers a proper analysis of the experiments, so that the extrapolation starts from dubious information.

Although standard tests prescribe exposure to be constant, in aquatic bioassays nearly all test compounds tend to disappear from the solution. This disappearance may result from evaporation, adsorption to the vessels or to food particles, formation of inaccessible complexes, degradation, etc. For instance, heavy metals can adsorb to the walls of the vessel (Van Hattum *et al*., 1989, Widianarko *et al*., 1997). Phenanthrene, a polycyclic aromatic hydrocarbon disappears during experiments, possibly due to microbial degradation (Landrum *et al*., 1991, 1994).

To approach the ideal of a constant exposure, one often regularly replaces the solution by freshly prepared medium. If, despite such efforts, the concentration is not approximately constant, the data will be difficult to analyse. For instance, Van Hattum *et al*., (1989) had to express concentration factors for Cadmium by the freshwater isopod *Asellus aquaticus* as large ranges, due to disappearance of the compound in the solution (loss of about 60% of the compound between two renewals of the solution).

A substantial decline of the concentration of the test compound to which the animal is exposed leads to an underestimate of the toxicity of the compound if the concentration is assumed to be constant. In other words, it leads to an overestimate of the concentration associated with a specified level of adverse response. Many attempts have been undertaken to find methods that increase the renewal rate of the solution, but these methods are costly and produce a large amount of chemical compounds.

As an alternative to high renewal rates, Kooijman (1981) proposed to estimate simultaneously the parameters of toxicity and the rate of disappearance

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of the compound from survival data only. Widianarko *et al.* (1997) measured the time-varying Zn exposure and estimated its effects on the survival of *Poecilia reticulata* with a model for kinetics and lethal effects. Nevertheless, these methods involve a large number of parameters and can only be applied to an exponential decrease of the concentration in the bioassay.

It is not obvious how to incorporate time-varying concentrations into the analysis of bio-assays. Most methods used do not account for time of exposure. They are based on dose-response models which look at some toxicological endpoint after a fixed exposure time. A drawback of such methods is that the estimated toxicity measures tend to depend on the exposure time. The only way to account for this time dependency is to formulate models that explicitly deal with exposure time. One such model is the DEBtox model put forward by Kooijman and Bedaux (1996). It explicitly addresses the toxicokinetics and thus in principle allows for time-varying concentrations.

In this study, we focus on an important toxicity parameter, the no effect concentration (NEC) as implemented in the DEBtox model. This NEC is the highest environmental concentration having no effect on the organism after prolonged exposure. This parameter can for instance be used to set safety limits or discharge of chemicals in the environment. Current implementation in DEBtox assumes that the environmental concentration is constant, an assumption that is highly contentious for many chemicals. The aim of this paper is to present a general approach to account for time-varying concentrations of tests compounds.

We introduce the DEBtox approach in the next section. Subsequently, we present three extensions of the DEBtox model that deal with time-varying concentrations in three different ways. We then study these extensions with computer-generated data. Finally, we illustrate the methods by a reanalysis of some experimental data.

THE DEBTOX APPROACH: FROM EXPOSURE TO EFFECTS

The basic idea of the DEBtox approach is that a compound is taken up from the environment by an organism, and that its internal concentration enhances the organism's risk to die if this concentration exceeds a certain threshold level. The internal concentration of the chemical compound is deduced from its concentration in the solution (i.e. the external concentration) according to a simple model for the kinetics. The current implementation of DEBtox assumes the external concentration to be constant. Within this context, the NEC is the constant external concentration that after an infinite exposure makes the internal concentration just to reach the threshold. Next, toxic effects are related to the internal concentration of the compound. This yields an expression for the survival function. Experimental data on survival together with knowledge of the compound's external concentration can then be used to estimate the parameters in the model.

The kinetics of the compound is a simple linear one-compartment kinetics. The uptake of the compound is assumed to be proportional to its concentration in the solution, and the elimination proportional to its concentration in the body. This leads to the following equation:

$$\frac{\mathrm{d}C_\mathrm{i}}{\mathrm{d}t} = k_\mathrm{u} c_\mathrm{e}(t) - k_\mathrm{e} C_\mathrm{i}(t) \tag{1}$$

where k_u and k_e are, respectively, the uptake and the elimination rate, c_e is the external concentration of the compound and C_i its internal concentration, i.e. the ratio of the amount of compound in the body and the body volume.

A well known parameter in toxicity studies is the bio-concentration factor (BCF). The BCF is defined as the ultimate ratio between concentration in the body of an organism and concentration in the solution when the latter is kept constant. Within the context of a one-compartment kinetics the BCF equals the ratio of the uptake and elimination rate, so $\mathrm{BCF} = k_\mathrm{u}/k_\mathrm{e}$.

Because data on the internal concentration are generally lacking, we express it in terms of the external concentration. To this end we use the BCF. The scaled internal concentration relates to the original one according to $c_\mathrm{i} = C_\mathrm{i}/\mathrm{BCF}$. The kinetics of the internal concentration thus becomes:

$$\frac{\mathrm{d}c_\mathrm{i}}{\mathrm{d}t} = k_\mathrm{e}(c_\mathrm{e}(t) - c_\mathrm{i}(t)) \tag{2}$$

The probability $q(t)$ to survive till time t is defined as

$$q(t) = \exp\left\{ -\int_0^t h(\tau)\,\mathrm{d}\tau \right\}$$

where $h(\tau)$ is the hazard rate at time τ. For a small time interval $\mathrm{d}\tau, h(\tau)\,\mathrm{d}\tau$ represents the probability of dying between τ and $\tau + \mathrm{d}\tau$ for an organism which has survived until time τ. The description of the death process is thus not deterministic but stochastic. In the DEBtox approach, the effects of exposure to a chemical is modelled as an increase in the hazard rate, which depends on the mean concentration of the compound in the body, $C_\mathrm{i}(t)$. This assumption does not imply that the concentration of the compound is the same in all organs, but that the ratio of concentrations in different organs is fixed.

The model assumes the hazard rate to be proportional to the difference between $C_\mathrm{i}(t)$ and an internal threshold value. By scaling via BCF, like in equation (2), we can say that the hazard rate is proportional to the difference between $c_\mathrm{i}(t)$ and an external threshold

concentration, the No Effect concentration (NEC). The NEC can be interpreted as the concentration in the environment that will not have any adverse effect, even after prolonged exposure.

The hazard due to the toxicant comes on top of the background hazard $d_\dagger$. We assume the latter to be constant, which implies that the animals are not subject to aging. This relates to the duration of the experiments, which often is short relative to the organism's life span. (The assumption of a constant background hazard is not essential to the approach. Ageing may be easily accounted for by a different baseline hazard when appropriate.) The hazard rate is thus given by

$$h(t) = \begin{cases} b_\dagger(c_i(t) - NEC) + d_\dagger & \text{if } c_i(t) > NEC \\ d_\dagger & \text{otherwise} \end{cases}$$

where $b_\dagger$ is the killing rate.

EXTENSIONS OF THE BASIC DEBTOX MODEL

The original DEBtox approach assumes that the external concentration of the test compound is constant; moreover, its value must be known. The extensions we present all differ from DEBtox in that the requirement of a constant concentration is relaxed. The extensions require additional information, namely the time course of the external concentration. For the so-called deduction model, one also needs an estimate for the BCF.

The spline model

In this approach, the decrease of the external concentration is described by a spline function, which is a function that interpolates between the measurements (e.g. Lancaster and Salkauskas, 1986). This purely descriptive approach makes no assumption regarding the disappearance of the compound. Splines have some attractive properties as an interpolating device although one should pay attention to the degree of accuracy of the interpolation. The latter problem is related to the fact that a spline is a piecewise polynomial function. To interpolate between data, one chooses a number of values on the horizontal axes. For these so-called knots, one has to find y-values. Between any pair of adjacent (t, y) values, a piece of a cubic polynomial is used to interpolate. The pieces are chosen such that two pieces always join in their common knots values; also their derivatives as well as the second derivatives in this point are taken to be identical. In this way, a smooth interpolation is achieved.

To apply a spline interpolation, one has to choose the knots, the number of which should not exceed the number of observations. In our approach, we take the knots equal to the time points of the observations on the external concentration of the compound. To find the accompanying $y_s(t_i)$-values, we minimise the following sum:

$$\sum_i^n \{(y(t_i) - y_s(t_i))^2 + \lambda(y_s''(t_i))^2\}$$

where $y(t_i)$ are the observations at time t_i and $y_s(t_i)$ the values of the spline at time t_i. The left hand side is the sum of the squared deviations between data and spline. The right hand side concerns the second derivative of the spline in the knots. If these have high values, the spline fluctuates wildly. The resulting interpolations are then likely to be inaccurate. The fluctuations can be reduced by increasing the value of the weight coefficient λ. The higher the value of the weight coefficient, the more gradual the spline function will be; the price to be paid for this is that the spline will less closely follows the data.

Once the data on the external concentration are described, the parameters can be estimated. For any set of parameters k_e, $b_\dagger$, NEC and $d_\dagger$, the DEBtox model provides the scaled internal concentration of the compound (see equation (2) and the survival function. To estimate the parameters from the survival data (an example of such data is given in Table 3), we use the maximum likelihood method for binary data (McCullagh and Nelder, 1992, p. 114) as explained in Bedaux and Kooijman (1994).

The function model

In general, the decrease in the concentration of the compound in the environment can be described by

$$\frac{dc_e(t)}{dt} = f(t, c_e(t), c_i(t)) \tag{3}$$

where f can be any function of time t, internal concentration c_i and external concentration c_e. The function model assumes that the general profile of f is known. The description of the external concentration data (with a least-squares method) yields estimates of the parameters needed to determine f. For instance, one might have:

$$\frac{dc_e(t)}{dt} = -\alpha c_e(t) \tag{4}$$

where α is the disappearance rate constant of the compound. This behaviour can be expected for evaporation or adsorption to the vessel (Widianarko *et al.*, 1997).

A special situation arises when the decrease in the external concentration is due to uptake by the organisms. Two cases can be distinguished. First, if each molecule taken up by the organisms is metabolised, we can express the disappearance rate (which is no longer constant because the number of animals decreases) as

$$\alpha = k_u \frac{V_o}{V_s} N(t) \tag{5}$$

where k_u is the individual uptake rate, defined in equation (1), $N(t)$ the number of surviving organisms

at time t, V_o the volume of an organism and V_s the volume of the solution. Here, the description of the external concentration data provides an estimate for the uptake rate. In the second case, the compound is not metabolised. If the BCF is large, the decrease of the external concentration can be explained by the fact that most of the compound initially present in the solution transfers to the tissue of the organisms. The following equation applies in this situation:

$$\frac{dc_e(t)}{dt} = -N(t)a\frac{dc_i(t)}{dt} - f(t, c_e(t)) \qquad (6)$$

where $a = BCF(V_o/V_s)$ is the abundance ratio and where f represents the disappearance of the compound without organisms. With the toxicokinetics equation (2), this leads to the following system of equations:

$$\frac{dc_i(t)}{dt} = k_e(c_e(t) - c_i(t))$$

$$\frac{dc_e(t)}{dt} = N(t)ak_e c_i(t) - g(t, c_e(t)),$$

where $g(t, c_e(t)) = f(t, c_e(t)) + N(t)ak_e c_e(t)$. The function g describes the disappearance from the environment, while the term $N(t)ak_e c_i(t)$ represents the compound excreted by the organism.

The description of the external concentration provides an estimate for k_e. There are only three parameters left (k_i, NEC, d) to be estimated from the survival data. Since we only have to estimate three parameters instead of the original four, this increases the accuracy of the estimates of the NEC.

Once the decrease in the external concentration is described satisfactorily, the parameters are estimated in the same way as in the spline model.

The deduction model

In the deduction model, the internal concentration is directly deduced from the external concentration. The underlying assumption is that the total amount of the compound in the experimental environment is constant. Any compound that is not in the environment must therefore be inside the organism. This is only likely to occur in laboratory situations in small, static, exposure chambers. The gist of the approach is that for such experiments more information can be extracted from the data in a relatively simple way. In this way, no model for the kinetics is needed and the parameter k_e need not be estimated. This may improve the precision of the estimation of the remaining parameters. The assumption of the deduction model is, as in the function model for bioconcentration, that the compound is not metabolised and that the BCF has a large value. The equation to deduce the internal concentration from the external one is:

$$\frac{dc_i(t)}{dt} = -\frac{1}{N(t)a}\left(\frac{dc_e(t)}{dt} + f(t, c_e(t))\right) \qquad (7)$$

with a and $N(t)$ as defined previously; f represents the variations of the external concentration without organisms. We use the same cubic spline fitting method as for the spline model to fit the external concentration data. We also use maximum likelihood method to estimate the parameters.

Application of the model proceeds in three steps. First, one measures variations of external concentration without organisms. Next, one measures the variations of the concentration in the solution with organisms and estimates the toxicity parameters from the survival data, based on the internal concentration. Finally, the BCF has to be estimated, to convert the internal threshold to the NEC. To estimate the BCF, one needs to measure the internal concentration at least once.

TESTS OF THE MODELS FOR ESTIMATES OF NECS

The present section first compares the different models discussed previously on the basis of computer simulation studies to appreciate the differences between the estimates. Then, two sets of experimental data will be used to illustrate the interest of the methods developed in this paper.

Physical disappearance of the compound

We first compare the spline model, the function model and DEBtox for constant concentrations (using the mean value of the external concentration) for a physical disappearance of the compound with a disappearance rate constant $\alpha = 0.3\ d^{-1}$ and with initial concentrations $0, 4, 8, 16, 30, 50\ mg\ l^{-1}$. The solution is assumed to be renewed every 3 days, and the external concentration is measured every day. We chose the values $NEC = 2\ mg\ l^{-1}$, $k_e = 0.2\ d^{-1}$, $b_\dagger = 0.05\ l\ mg^{-1}\ d^{-1}$, $d_\dagger = 0.01\ d^{-1}$ and an initial number of 50 organisms. These values are consistent with several estimates in Bedaux and Kooijman (1994).

We studied the performance of the estimation with a Monte-Carlo simulation. We generated 1000 data sets using the theoretical survival curve with the parameter values in Table 1. For each data set, we then estimated the (known) parameters values. Table 1 presents means and standard deviations of the 1000 estimates. It shows that DEBtox with mean external-concentrations provides biased estimates. Nevertheless, the estimated NEC is not too different from the theoretical value (the difference is only 10% of the theoretical value) because the kinetics is relatively slow ($k_e T = 0.6$). In such a case, the internal concentration is not very different from the concentration expected with the mean value of the concentration. On the contrary, for high elimination rate, the estimated NEC may differ considerably from the theoretical value.

The use of the function model does not lead to a better estimate of the NEC than the use of the spline

Table 1. Results of the Monte-Carlo simulation with exponential disappearance of the compound

	Parameter	Mean estimates	Standard deviation
Theoretical values	NEC	2	
	k_e	0.2	
	$b_\dagger$	0.05	
	$d_\dagger$	0.01	
DEBtox mean concentrations	NEC	1.83	0.22
	k_e	0.26	0.064
	$b_\dagger$	0.056	0.0084
	$d_\dagger$	0.011	0.0010
Spline model	NEC	1.99	0.24
	k_e	0.194	0.043
	$b_\dagger$	0.0535	0.010
	$d_\dagger$	0.0099	0.00096
Function model	NEC	1.99	0.23
	k_e	0.193	0.042
	$b_\dagger$	0.0534	0.0094
	$d_\dagger$	0.011	0.00097

Table 2. Results of the Monte-Carlo simulation for disappearance of the compound due to its bioconcentration

	Parameter	Mean estimates	Standard deviation
Theoretical values	NEC	3	
	k_e	0.3	
	$b_\dagger$	0.03	
	$d_\dagger$	0.004	
Spline model	NEC	3.12	0.53
	k_e	0.30	0.077
	$b_\dagger$	0.033	0.007
	$d_\dagger$	0.004	0.0016
Function model	NEC	3.10	0.37
	$b_\dagger$	0.031	0.006
	$d_\dagger$	0.0041	0.0016
Deduction model	NEC	2.97	0.3
	$b_\dagger$	0.032	0.0035
	$d_\dagger$	0.0041	0.0015

model, even if the data should favour the function model. This is a direct consequence of the good description provided by cubic splines, even for a small number of measurements (here, four between two renewals).

Making assumptions about the disappearance of the compound is always risky, while the results presented here show that there is little benefit to expect. The only exception might be if the main cause of disappearance is the organism itself. Then, the method provides not only a better description of the data, but also information about the kinetics. We will discuss this in the next section.

Disappearance of the compound due to bioconcentration

In this section, we apply the estimation methods for disappearance of the compound due to high bioconcentration. The parameter a as defined previously was chosen in such a way that $aN(0) = 1.5$, with $N(0) = 40$, the initial number of organisms. We chose $NEC = 3$ mg l^{-1}, $k_e = 0.3$ d^{-1}, $b_\dagger = 0.03$ l mg^{-1} d^{-1}, $d_\dagger = 0.004$ d^{-1} and initial concentrations of $0, 2, 4, 8, 16, 30$ mg l^{-1}. The external concentration is assumed to have been measured every day. Table 2 presents the results of the Monte-Carlo simulation.

The estimate of the NEC is quite good in each case. However, the standard deviation for the NEC-estimate based on the Monte-Carlo simulation data is much smaller when using the deduction model compared to the spline model. For the deduction model, it can be about 50% of that of the spline model. This is to be expected: when the elimination rate is estimated from the concentration data, there are less parameters to be estimated from the survival data, and the accuracy of the latter estimates should thus improve.

The slight difference between the function model and the deduction model can easily be explained. With the former, the internal concentration is deduced from the estimated values of k_e and a, which can be slightly different from the theoretical one, because they depend on the survival data which follow a stochastic process. On the other hand, with the deduction model we evaluate the internal concentration directly from the concentration data.

Comparison with experimental data

Measurements of external concentration in bioassays for toxicity are hard to find in the literature. For this reason, the following two examples are more illustrations than tests of the models. However, they do show the consistency of the theoretical aspects presented above.

Effects of insecticide diazinon on terrestrial isopod Porcellio scaber. We illustrate the function model using *Porcellio scaber* survival data with exponential degradation of the organophosphorus insecticide diazinon in the soil (data from Widianarko and Van Straalen, 1996). Unfortunately, the actual concentration of the product in the soil was not measured and the degradation rate (0.116 d^{-1}) was estimated from the survival data. We adapted this estimate to compare the analysis of Widianarko and Van Straalen with our own approach. This is of interest because Widianarko and Van Straalen assumed from the outset, the NEC and the background hazard rate to be zero. Our analysis offers the opportunity to test this assumption. Table 3 presents the data and Table 4 the estimates based on these data.

The confidence interval we provide for the NEC is based on the log likelihood profile presented in Fig. 1 (see e.g. Aitkin *et al.*, 1989, p. 190). This interval is obtained as follows. First, we intersect the profile

Table 3. Number of survivors as a function of time and initial concentration of diazinon for *Porcellio scaber*

Day of measurement	Initial concentration ($\mu g/g^{-1}$)						
	Control	2.00	2.83	4.00	5.66	8.00	11.31
0	50	50	50	50	50	50	49
7	48	45	47	43	38	27	23
14	47	40	43	38	32	9	10
21	47	37	39	34	28	9	6
28	46	33	38	33	28	8	6
35	45	31	37	30	27	8	6
42	44	30	35	30	27	8	6

Table 4. Parameter estimates for the toxicity of diazinon on *Porcellio scaber*

	Parameter	Estimate	Confidence interval
Estimation	$NEC(\mu g\ g^{-1})$	0.67	$0.3 < NEC < 1.75$
	$k_e(d^{-1})$	0.25	
	$b_\dagger(g\ \mu g^{-1} d^{-1})$	0.026	
	$d_\dagger(d^{-1})$	0.0052	

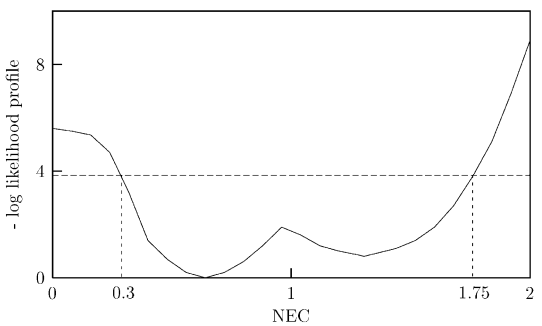

Fig. 1. Minus log-likelihood profile obtained with the data of Table 3. The dotted line represents $y = 3.84$.

with the line $y = 3.84$. The value 3.84 is the value of a test statistics that follows a chi-square distribution with one degree of freedom used to construct a 95% confidence interval. Then we find the x-coordinates of the intersection points. All NEC values between those two points constitute the confidence interval for the NEC, i.e. the NEC values that cannot be rejected at $\alpha = 0.05$ on the basis of the data set. As zero is outside the constructed interval, the NEC is significantly different from zero.

Bioconcentration as the source of disappearance of PAH in the solution. To evaluate the toxicokinetics parameters and the bioconcentration of six polycyclic aromatic hydrocarbons (anthracene, phenanthrene, pyrene, benzo[e]pyrene, benzo[a]pyrene and benzo[ghi]perylene) in the freshwater isopod *Asellus aquaticus*, Van Hattum (1999) measured the external and internal concentrations of the test compounds. A substantial decline of the external concentration of the test compound was found. Table 5 provides the measures of concentration of pyrene in the solution

Table 5. Measures of concentration of pyrene in the solution without[a] and with[b] organisms (*Asellus aquaticus*) and distribution of the compound between water and isopods

Day	Concentration[a]	Concentration[b]	Distribution (%)	
	(μgl^{-1})	(μgl^{-1})	Water	Isopods
0	16	15.7		
1		11.1		
1.75		8.95		
2.31		8.65	55	26
3.08	15	8.75		
5		8.05		
6.02		7.6		
7.2	14	7.8	50	37

[a] Without organisms.
[b] With organisms.

Table 6. Estimated Toxicokinetics parameters and estimate of r, the disappearance rate, on the basis of the concentration of pyrene in the solution given by Table 5, for the experiment with organisms. The BCF has the measured value 2050 l kg^{-1}

	Parameter	Estimate
No physical disappearance	*BCF*	2770 l kg^{-1}
	k_e	0.47 d^{-1}
	a	1
Physical disappearance	*BCF*	2140 l kg^{-1}
	k_e	0.6 d^{-1}
	a	0.77
	r	0.033 d^{-1}

and the distribution of the compound between water and organism. It appears that the main cause of disappearance is uptake by the test organisms.

We used equations (2) and (7) to estimate the toxicokinetics parameters with and without the possibility of a background exponential disappearance of the compound. We only used external-concentration data with organisms in the solution. Table 6 presents the results. The comparison between measured and estimated bioconcentration factor and between measured and estimated abundance ratio (the measure of a is the ratio of the distribution in isopods and in water, here 0.74) suggests the presence of a small abiotic disappearance (see Table 5). This conclusion and the estimated rate of disappearance are in good agreement with an experiment conducted previously without organisms. The function model thus offers an appropriate tool to analyse the data.

DISCUSSION

Time-varying concentrations present a sticky problem in the analysis of data from bioassays. Our Monte-Carlo simulations show that replacing the time-varying concentrations by their mean values leads to biased estimates of the toxicity parameters. As an alternative, we propose different models to tackle the problem for one important parameter, the NEC. Simulations show that there is no statistical

problem inherent to the models; tests against experimental data suggest that they can satisfactorily describe real data.

The spline model is a general technique to estimate the toxicity parameters for any kind of variations of the external concentration. Assumptions about the disappearance (function model) do not generally lead to better estimates of the toxicity parameters. Only if few data for the external concentration are available, or if the organism is partially responsible for the disappearance, will the function model outperform the spline model.

The deduction model provides an estimate of toxicity parameters by deducing the internal concentration directly from the external concentration. Hence, no assumptions regarding the toxicokinetics are involved, which is the main advantage of the method. The method can be very useful for complicated toxicokinetics, as for instance exhibited by heavy metals. In this way, Luoma (1977) observed a saturation of Hg uptake by a polychaete (*Nereis succinea*) and a small shrimp (*Palaemon debilis*), probably due to a saturation of the carrier-mediated transport. The weak point of the method is that it assumes the compound not to be metabolised and to be highly bioconcentrated. Nevertheless, a bioconcentration factor of 100 should suffice to reliably apply the method, a value for the BCF that is not excessively high. Another restriction is that the compound should not be metabolised. However, this restriction applies to all methods currently available, so it is not specific to our model.

In this article, we only deal with lethal effects but all the models developed here can also be used to analyse sublethal effects, such as effects on growth or reproduction as Kooijman and Bedaux (1996) did for DEBtox for constant concentrations. This allows to deduce population consequences from known effects on individuals. Effects of chemical compounds are modelled by a change in the parameters of the dynamic energy budget (DEB) of an organism (Kooijman, 2000). The DEB model specifies the rules that organisms use for energy allocation to maintenance, growth, development and reproduction.

In this paper, we assume that there is a unique bioconcentration factor. In reality, the ultimate ratio between the internal concentration and the external concentration kept constant can depend on this concentration (see for instance Vighi (1981) who calculated different BCFs of lead with *Daphnia magna* for two different concentrations in the solution). Nevertheless, assuming that this ratio does not vary much in the range of variation of the concentration, this problem can be circumvented by assuming that there is a particular BCF for each tank. This will not change anything for the spline model and the function model without high bioconcentration of the compound, for the BCF is an hidden variable in these models. For the other cases, it is necessary to divide the internal concentration by the measured BCF, for each tank, before the estimation of NEC.

The extension from constant to time-varying concentrations is just one possibility to expand on the DEBtox model. Other possibilities are variations of parameters values among individuals and interactions between compounds. This should increase relevance but also complexity of the models. Such extensions are theoretically possible, but the successful application depends on the availability of adequate data.

We conclude that the models proposed in this paper are good tools to deal with time-varying concentration in a proper way. The advantages and inconveniences have been presented for each of them. It is now up to the user to choose which one is the most appropriate weighing the desired accuracy of the estimates and the costs of the measurements involved.

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