Reconsidering sufficient and optimal test design in acute toxicity testing

Tjalling Jager[1]

[1] Dept. of Theoretical Biology, VU University Amsterdam, de Boelelaan 1085, NL-1081 HV, Amsterdam, the Netherlands
E-mail: tjalling.jager@vu.nl

This is a post-peer-review, pre-copyedit version of an article published in Ecotoxicology:

Jager T. 2014. Reconsidering sufficient and optimal test design in acute toxicity testing. Ecotoxicology 23:38-44.

The final authenticated version is available online at http://dx.doi.org/10.1007/s10646-013-1149-7. See also the terms of use of Springer: https://www.springer.com/gb/open-access/publication-policies/aam-terms-of-use.

Abstract

In dose-response analysis, regression analysis and hypothesis testing are the main tools of choice. These methods, however, have specific requirements for the design of acute toxicity experiments. To produce meaningful results, both approaches require a constant exposure concentration over the duration of the test, and regression analysis makes an additional demand for at least two doses with partial mortality at the end of the test. These requirements, however, result from the limitations of the statistical techniques, which only use the observations at the end of the test. In practice, most standard protocols for acute testing prescribe that observations are made at several points in time (often daily). In this contribution, I demonstrate how dynamic modelling can make use of this information to produce robust estimates of LC50 as function of time, with confidence intervals, from data sets that violate the requirements for standard dose-response analysis. This form of modelling invites an entirely different, more flexible, view on experimental design, which could lead to a more efficient use of test animals and, at the same time, a stronger support for environmental risk assessment as well as the science of ecotoxicology.

Keywords TKTD modelling; Survival; Experimental design; GUTS; LC50

Introduction

Which acute toxicity tests are sufficient to extract summary statistics for environmental risk assessment, and which test design is optimal? The answer to these questions obviously depends on the summary statistic and how it is to be derived from the experimental observations. In dose-response analysis, regression analysis offers many advantages over hypothesis testing (e.g., Landis and Chapman 2011). For survival data, the LC*x* is generally the preferred summary statistic, which is the estimated concentration resulting in *x*% mortality (relative to the control) after a specified exposure duration, at a constant exposure concentration. Regression analysis requires at least two concentrations with partial mortality (Hoekstra 1991). Unfortunately, the current OECD test design for fish (OECD 1992) often results in only one concentration with partial mortality (or even none) at the end of the test, raising doubts on the efficiency of this design (Rufli and Springer 2011), and even on the general appropriateness of the LC*x* as a summary statistic (Green et al. 2012). Another practical problem that the LC*x* shares with other standard summary statistics is that it is well-defined only for a constant exposure concentration. Therefore, aquatic test guidelines usually include the requirement of 'constant conditions' in their validity criteria (OECD 1992), and experimenters go to great lengths to ensure that this criterion is met (e.g., using semi-static or flow-through procedures). This is no small challenge, especially when facing compounds that degrade, adsorb or accumulate. When the exposure concentration decreases over time, or when exposure is pulsed, an LC*x* can still be calculated from the observations at the end of the test, but it becomes a rather meaningless number (Jager 2011).

Indeed, standard regression approaches require constant exposure and multiple concentrations with partial mortality. However, these requirements relate to limitations of the statistical technique, and not to the appropriateness of the LC*x* or the test design. Standard regression approaches overlook an important source of information: we do not just have the observations at the end of the test. In fact, most standard protocols prescribe that survival is scored over regular time intervals; for example, in the acute fish test, we have four days worth of observations on mortality. To unlock this information, we need a method that describes survival as a function of concentration *and* time (i.e., a response surface). Such methods exist, and have even been included in OECD/ISO guidance under the designator 'biology-based methods' (OECD 2006). For survival, time-explicit models can be quite simple, and have been extensively applied and tested (for a list of papers using hazard models, see www.debtox.info/papers_survival.php). Recently, the various existing survival models have been unified into a single coherent framework, GUTS (Jager et al. 2011). Such methods are not only able to deal with lack of partial mortality, but also there is no need for the exposure concentration to be constant over the test duration (Ashauer et al. 2010b; van Ommen Kloeke et al. 2012). Model parameters can still be estimated from time-varying exposure, and a pulsed design might even be preferable in some cases (Albert et al. 2012).

In this contribution, I will demonstrate how a simple model from the GUTS framework can be used to extract model parameters from data sets that violate the requirements for standard dose-response methods. Subsequently, these model parameters will be used to calculate the classic LC50 estimate, as a continuous function of exposure time, with confidence interval. It is important to stress that LC50 is not a model parameter of GUTS, but rather a model output. Three survival data sets will be used as examples to illustrate how dynamic modelling makes better use of the available data, as a starting point for questioning the current design of acute

toxicity tests. Here, I will restrict the discussion to the endpoint survival, although many of the arguments also hold for sub-lethal endpoints (which, however, require different model approaches, see Ashauer et al. 2011a).

Methods

Experimental toxicity data

Two data sets for fathead minnow (*Pimephales promelas*) were taken from the reports of the Center for Lake Superior Environmental Studies, namely for naphthalene (Geiger et al. 1985) and 3,4-dichloro-1-butene (Geiger et al. 1988). For naphthalene, the test design comprised 50 individuals at 4 treatments (incl. the control), whereas for dichlorobutene, there were 20 individuals at 6 treatments (incl. the control). Observations on mortality were made every day for a total exposure duration of 4 days. For the exposure concentration, I used the average measured exposure concentrations, corrected for recovery. There was no mortality in the control, so background mortality was not included in the model. For pulsed exposure, I used a data set for the amphipod *Gammarus pulex* exposed to diazinon (Ashauer et al. 2010b). From the three treatments in the original study, I only selected one (two pulses with an interval of 7 days between the pulses) to illustrate the possibility of extracting model parameters from a single treatment. The data comprise 70 individuals followed over 22 days. The background hazard rate was estimated from the mortality of 60 control individuals and fixed to the best estimate (0.0245 d^{-1}) when fitting the treatment data. The original survival data are provided as supplementary material.

Model approach

The survival data were analysed using a special case of the GUTS framework (Jager et al. 2011), namely the simple hazard model with scaled toxicokinetics (Bedaux and Kooijman 1994; Jager and Kooijman 2009). Scaled toxicokinetics is preferred here, because no information on body residues is required. Other cases from the GUTS framework can be used (e.g., adding damage or a tolerance distribution) in a similar analysis. However, the focus here is on data requirements and experimental design, not on the specific choice of model.

Three parameters need to be estimated from the data: a scaled threshold concentration for effects (z, the external concentration that does not lead to exceedance of the internal threshold after prolonged exposure), a dominant rate constant (k_e, the first-order combination of toxicokinetics and, possibly, repair of damage), and a killing rate (k_k, the proportionality constant for the strength of the effect above the threshold). If there is background mortality, this process needs to be included, with at least one additional model parameter. More details on the model and the parameters can be found in Jager et al. (2011).

Several implementations are available to perform these calculations (see www.debtox.info/software.php), and here I used the Matlab version. Here, I follow a Bayesian approach for the parameter optimisation and credible intervals, but as I assume uniform prior distributions, the results will be very similar to maximum likelihood estimation. The probability of observing a specific number of survivors over time, given a set of model parameters, follows from the multinomial distribution (Jager et al. 2011). Using the multinomial distribution, there is no dependency in the observations over time: the observation that is used is, for each individual,

the time interval in which it dies (in the calculation, the expected and predicted deaths in each discrete observation interval are used). However, dependencies might arise due to interactions between the individuals in a tank (e.g., when the death of one individual affects the probability to die for the survivors). A sample from the posterior parameter distribution was obtained using the slice sampler implementation in Matlab (5000 samples). This sample was subsequently used to calculate credible intervals on the parameter estimates and the model predictions for survival and LC50 over time (see also Ashauer et al. 2010a; Jager and Zimmer 2012).

Results and discussion

Model fits and LCx predictions

In dynamic models, the LC*x* is not a parameter but a model prediction. Using the three model parameters estimated from the data (Table 1), it is straightforward to make predictions for the LC*x* at *y* days of exposure, for any *x* and *y*, with credible intervals (Fig. 1-3). Instead of the LC*x* at the end of the test, the threshold for effects (*z*) can itself also be used as a robust (and, in principle, time-independent) summary statistic (Kooijman 1996). In hazard models, the LC*x* (for $0<x<100$) will asymptotically approach the threshold with increasing exposure duration (as can be seen in Fig. 1 and 2). The value of the threshold will thus equal the incipient LC50 (Jager et al. 2006; Jager and Kooijman 2009)

Alternatively, the calibrated model can be used to predict survival over time, for any specified exposure pattern (Ashauer et al. 2013; Ashauer et al. 2011b), but here I focus on the estimation of the LC50 to demonstrate how dynamic modelling can easily be used within the current risk assessment schemes.

Table 1 Parameter estimates and model predictions with 95% credible intervals. For diazinon, the predicted LC*x* values are extrapolations for the case of constant exposure.

	P. promelas, Naphthalene	*P. promelas*, Dichlorobutene	*G. pulex*, Diazinon
	Estimated model parameters		
Threshold for effects (z)	44.1 (41.7-45.3) µM	34.9 (27.0-41.6) µM	4.82 (4.33-27.2) nM
Killing rate (k_k)	0.102 (0.0567-0.140) $\mu M^{-1}d^{-1}$	0.736 (0.732-0.739) $\mu M^{-1}d^{-1}$	0.0322 (0.0172-4.132) $nM^{-1}d^{-1}$
Dominant rate constant (k_e)	5.53 (4.43-30.2) d^{-1}	0.226 (0.168-0.287) d^{-1}	0.0725 (0.0686-0.307) d^{-1}
	Model predictions		
LC10 at end of test	44.4 (42.2-45.6) µM	60.6 (57.2-63.2) µM	7.19 (6.28-27.1) nM
LC50 at end of test	46.1 (44.6-47.1) µM	64.3 (61.2-66.5) µM	9.78 (9.34-27.3) nM

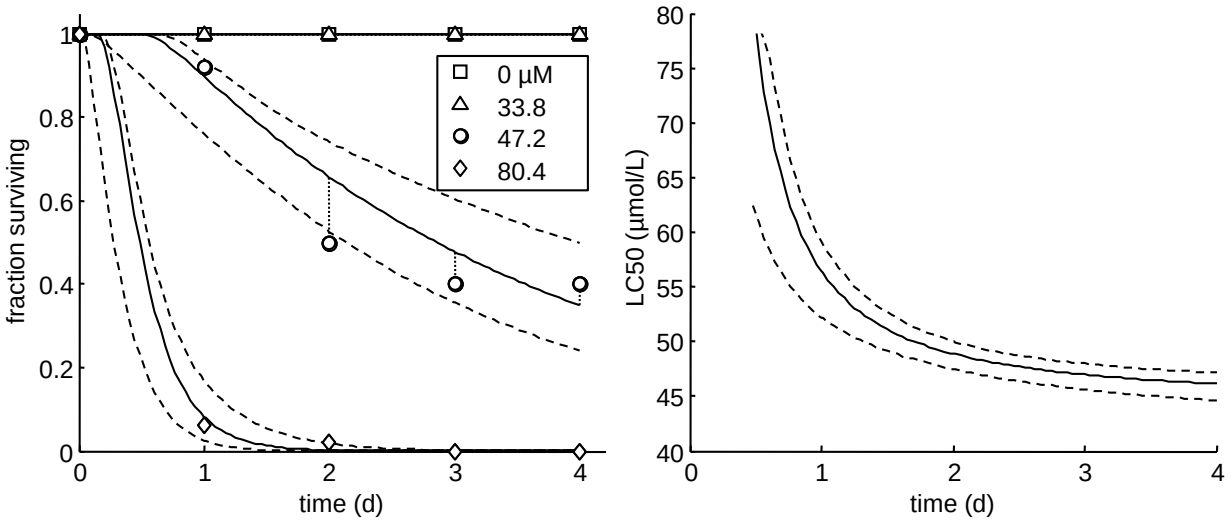

Fig. 1 Model fit on survival data for fathead minnow exposed to naphthalene (left), and predicted LC50 over time (right). Broken lines mark 95% credible intervals on model lines. Dotted lines connect data points to the corresponding model curve.

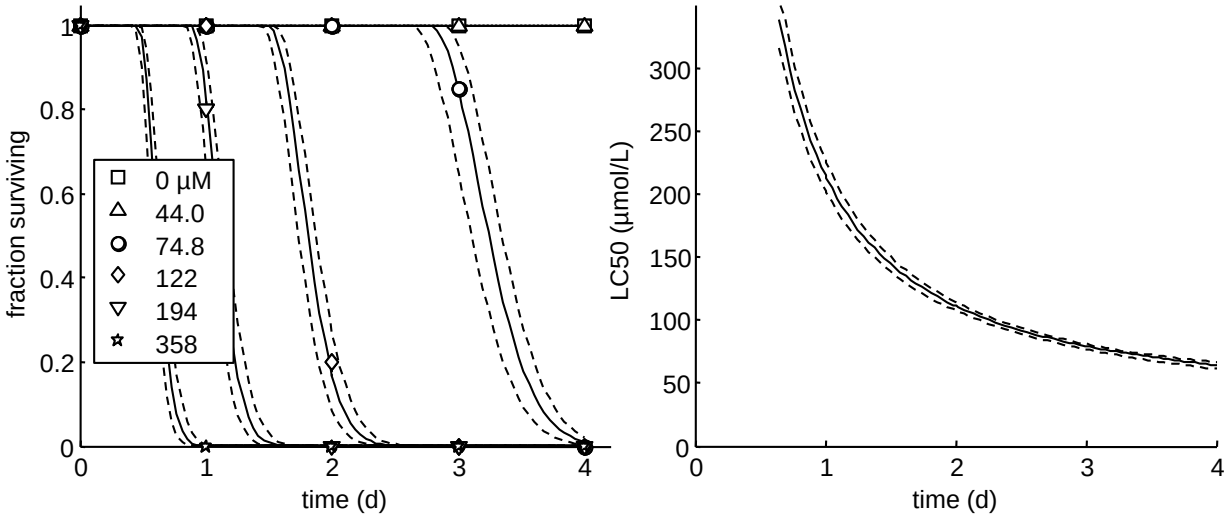

Fig. 2 Model fit on survival data for fathead minnow exposed to dichlorobutene (left), and predicted LC50 over time (right). Broken lines mark 95% credible intervals on model lines.

For naphthalene, there is a single treatment with partial mortality at the end of the test, and for dichlorobutene, there is no such concentration (Fig. 2). Therefore, these data sets are not amenable to standard regression methods. For a dynamic model, the absence of partial mortality at the end of the test does not preclude the estimation of the model parameters, as the observations on all time points are used together in an integrated analysis. Just three parameters are required to explain the complete survival pattern over time. In this case, the credible intervals on the parameters and on the LC50 are very tight, especially for dichlorobutene (Fig. 2). This is partly a result of the substantial number of fish in these tests (in total 200 for naphthalene and 120 for dichlorobutene). For dichlorobutene, even though there are no partial mortalities at the end of the test, the mortality pattern is actually extremely informative: there is no control mortality, one treatment has no effect, and four treatments result in complete mortality at

different points in time. The complete pattern allows for a precise estimation of the model parameters, which in turn allows for very precise LC50 estimations. For naphthalene, the LC50 at the end of the test is already very close to the incipient value (the threshold in Table 1), whereas for dichlorobutene, the LC50 can be expected to drop by almost a factor of two, had exposure continued beyond the 4-day test duration.

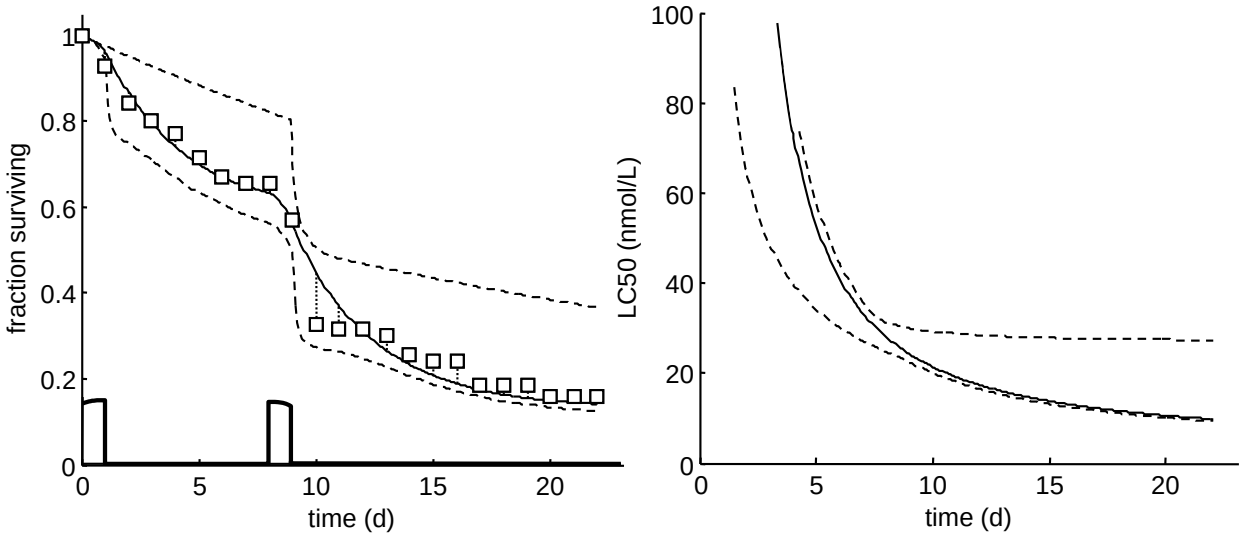

Fig. 3 Model fit on survival data for amphipods exposed to two pulses of diazinon (left, thick lines indicate the exposure pattern), and predicted LC50 for constant exposure over time (right). Broken lines mark 95% credible intervals on model lines. Dotted lines connect data points to the corresponding model curve.

For diazinon, the data set comprises just one treatment, with 70 individuals exposed to two 1-day pulses (Fig. 3). In this case, the credible intervals are rather wide, which reflects the substantial background mortality, and the somewhat poor fit to mortality from the second pulse (survival drops more steeply than expected from the first pulse). It must be stressed here that the model is fitted to the mortality in each time interval and not to the actual number of survivors at each observation time. The right panel of Fig. 3 shows how the LC50 is predicted to decline over time, had the amphipods been exposed to a constant concentration of diazinon.

Interestingly, the credible intervals are not symmetric around the best model curve in this case. This reflects the fact that this mortality pattern can be described by two radically different model parameterisations. Background mortality is substantial in this experiment, so the observed mortality due to the first pulse might simply be control mortality. This interpretation requires a high value for the threshold, as well as for both rate constants, to yield only rapid mortality at the second pulse. Alternatively, initial mortality might be caused by diazinon, requiring a low value of the threshold, and low rate constants, to provide a smoother mortality pattern at both pulses. The best model fit results from the latter interpretation, but the first is still statistically plausible. The fact that there are two distinctly different explanations for the observed toxicity pattern results in an oddly-shaped credible interval (note that the upper and lower lines of the interval are not model curves themselves but cut off 95% of the model predictions).

In this analysis, I fitted the background mortality separately and fixed it for the subsequent analyses. While this helps to make a transparent case for the purpose of this paper, it is not a

generally advisable procedure. For diazinon, it is likely that fitting the controls and treatments simultaneously would have resulted in wider credible intervals.

My reanalysis of these previously published data sets was only possible as the original survival data were made available by the authors. As LC*x* (or EC*x*) values make a poor summary of a toxicity data set (Jager 2011), providing the raw experimental data as supplementary material to publications should be encouraged.

Model uncertainty

Of course, the realism of the LC*x* estimates produced here, as well as their intervals, rests on the assumption that the model is correct (which is not different from any other statistical approach). The credible intervals on the parameter estimates and the predictions reflect the uncertainty in the parameter estimates, and not the uncertainty due to the model choice. For example, the two most popular model mechanisms for mortality are stochastic death (SD) and individual tolerance (IT) (Jager et al. 2011). Here, I selected SD, but in practice, both mechanisms can often explain the same survival patterns over time with comparable goodness of fit (Jager et al. 2011; Nyman et al. 2012), and it might well be that both mechanisms play a role at the same time (Baas et al. 2009; Newman and McCloskey 2000). In this case, the data for naphthalene (Fig. 1) will be better described by an IT model, because the mortality at 47.2 µM levels off after day 2. Selecting the most appropriate model is of course essential for meaningful model predictions and credible intervals.

Another model uncertainty relates to processes that are not included in the model. For example, many species biotransform compounds, which influences toxicokinetics (Ashauer et al. 2012), and the resulting metabolites may also contribute to the toxic effect. If detailed information is available, this can be accommodated in a dynamic model (e.g., Kretschmann et al. 2011), but in most toxicity tests we only have information about mortality over time.

Consequences for experimental design

The use of dynamic models for dose-time-response analysis invites an entirely different view on optimal test design. Neither the expected partial mortalities at the end of a test, nor the constancy of the exposure concentration is an issue in this case. Dynamic models, as used here, simply do not have such restrictions. As long as the exposure profile over time is known (or can be estimated), and survival is followed over time, the model parameters can be estimated (if sufficient mortality occurs). These model parameters can subsequently be used to estimate an LC*x* for constant exposure duration, or to make different model predictions. Relaxing the requirements of partial mortality and constant exposure may drastically simplify experimental design for many compounds.

At this moment, it is not so clear what an optimal design for dynamic modelling is, but it likely depends on the chemical in question. More observations over time will always improve the identification of model parameters, without requiring more test animals. For fast-acting chemicals, a shorter experimental duration may suffice (with more observations per day), whereas slower-acting compounds may be better served by a prolonged experimental duration. Experimental duration, in general, will not influence the best estimate of model parameters, but it will influence the confidence with which they can be estimated. The same can be said for number of test animals and number of test concentrations. It is possible to obtain meaningful

parameter estimates with a group of animals in a single treatment (as in Fig. 3), or from a range of concentrations with one individual each, as long as mortality is followed over time. However, fewer test animals and less test concentrations will not only lead to larger confidence intervals; they also make it more difficult to identify deviations from the model assumptions, such as multiple mechanisms of action (van Ommen Kloeke et al. 2012).

Application of dynamic models also allows for a more dynamic view on test design; there is no reason why the test design cannot be modified *during* the test itself. For example, when rapid mortality is observed in several doses, it might make sense to move the surviving individuals to clean water during the test. Similarly, when there is very little mortality, one or more of the exposure concentrations can be increased during the test. However, it must be stressed that any extrapolation beyond the experimental situation hinges on the realism of the model. Every model is a simplification of reality, and therefore 'wrong'. It is possible that low-level long-duration exposure reveals a different mechanism of action than high-level short duration (see e.g., van Ommen Kloeke et al. 2012), and prediction of mortality due to pulsed exposures from constant exposure (and vice versa) is not without problems (Nyman et al. 2012). Therefore, it makes sense to design toxicity test in such a way as to mimic the expected exposure situation in the field. For a detergent, a low, rather constant, concentration for a longer duration would be more relevant than for a pesticide, where a pulsed exposure design might make more sense. Pulsed exposure may even be preferable over constant exposure for the identification of particular model parameters (Albert et al. 2012).

Conclusions

Toxicity is a process that depends on concentration and on time, which is hardly a new insight (Sprague 1969). Dynamic modelling is the most suitable approach to analyse toxicity data over time, and to extract meaningful summary statistics (Ashauer and Escher 2010; Jager et al. 2006). Here, I stress an advantage of major practical benefit: we can relax the requirements for constant exposure and partial mortality, as long as we have observations over time. The three data sets in Fig. 1-3 cannot be analysed with standard dose-response regression, but dynamic modelling can still be used to derive meaningful and robust LC50 estimates, as a function of time, with confidence interval. Therefore, dynamic methods are particularly useful for first-tier risk assessments, to make optimal use of all available (possibly non-standard) toxicity data. Here, I focussed on estimating LC50 values, as this statistic is used in current risk assessment frameworks. However, the time-independent model parameters also offer the far more interesting possibility to predict mortality resulting from specific concentration profiles over time (Ashauer et al. 2013; Ashauer et al. 2011b). Implementation of such applications would, however, require more drastic revision of the risk assessment procedures.

Dynamic modelling invites a whole new and flexible view on test design. First of all, using all of the data over time substantially increases the statistical power of the analysis, which can be translated into a reduction of the number of test animals. Furthermore, the same summary statistics can be derived from a broad range of experimental set-ups that would be unsuitable for standard approaches. It is even straightforward to combine several data sets in a simultaneous model fit (Jager et al. 2006; van Ommen Kloeke et al. 2012). In general, it makes sense to tailor test design to the chemical and its expected exposure pattern in the field, which would require a major break with tradition in test guideline development.

Even the simplest form of dynamic modelling is more complex than fitting a dose-response curve (or hypothesis testing). However, the dynamic effect model used here is a lot simpler than the fate models that *are* routinely applied in first-tier risk assessments. At this moment, however, the ball is not just in the court of the developers of guidelines for risk assessment and toxicity testing: the use of standard test protocols and dose-response regression still dominates scientific research in ecotoxicology, and science has to lead by example.

Acknowledgements

This research has been financially supported by the European Union under the 7th Framework Programme (project acronym CREAM, contract number PITN-GA-2009-238148).

References

Albert C, Ashauer R, Künsch HR and Reichert P (2012) Bayesian experimental design for a toxicokinetic-toxicodynamic model. J. Stat. Plan. Infer. 142:263-275.

Ashauer R, Agatz A, Albert C, Ducrot V, Galic N, Hendriks J, Jager T, Kretschmann A, O'Connor I, Rubach MN, Nyman AM, Schmitt W, Stadnicka J, Van den Brink PJ and Preuss TG (2011a) Toxicokinetic-toxicodynamic modeling of quantal and graded sublethal endpoints: a brief discussion of concepts. Environ. Toxicol. Chem. 30:2519-2524.

Ashauer R, Caravatti I, Hintermeister A and Escher BI (2010a) Bioaccumulation kinetics of organic xenobiotic pollutants in the freshwater invertebrate *Gammarus pulex* modeled with prediction intervals. Environ. Toxicol. Chem. 29:1625-1636.

Ashauer R and Escher BI (2010) Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J. Environ. Monit. 12:2056-2061.

Ashauer R, Hintermeister A, Caravatti I, Kretschmann A and Escher BI (2010b) Toxicokinetic and toxicodynamic modeling explains carry-over toxicity from exposure to diazinon by slow organism recovery. Environ. Sci. Technol. 44:3963-3971.

Ashauer R, Hintermeister A, O'Connor I, Elumelu M, Hollender J and Escher BI (2012) Significance of xenobiotic metabolism for bioaccumulation kinetics of organic chemicals in *Gammarus pulex*. Environ. Sci. Technol. 46:3498-3508.

Ashauer R, Thorbek P, Warinton JS, Wheeler JR and Maund S (2013) A method to predict and understand fish survival under dynamic chemical stress using standard ecotoxicity data. Environ. Toxicol. Chem. 32:954-965.

Ashauer R, Wittmer I, Stamm C and Escher BI (2011b) Environmental risk assessment of fluctuating diazinon concentrations in an urban and agricultural catchment using toxicokinetic-toxicodynamic modeling. Environ. Sci. Technol. 45:9783-9792.

Baas J, Jager T and Kooijman SALM (2009) Estimation of no effect concentrations from exposure experiments when values scatter among individuals. Ecol. Modell. 220:411-418.

Bedaux JJM and Kooijman SALM (1994) Statistical analysis of bioassays based on hazard modelling. Environ. Ecol. Stat. 1:303-314.

Geiger DL, Call DJ and Brooke LT (1988) Acute toxicities of organic chemicals to fathead minnow (Pimephales promelas). Volume IV. Center for Lake Superior Environmental Studies, University of Wisconsin-Superior, Superior, WI, USA.

Geiger DL, Northcott CE, Call DJ and Brooke LT (1985) Acute toxicities of organic chemicals to fathead minnows (*Pimephales promelas*) Volume II. University of Wisconsin-Superior, Superior, Wisconsin, USA.

Green JW, Springer TA and Stavely JP (2012) The drive to ban the NOEC/LOEC in favor of ECx is misguided and misinformed. Integr. Environ. Assess. Manag. 9:12-16.

Hoekstra JA (1991) Estimation of the LC50, a review. Environmetrics 2:139-152.

Jager T (2011) Some good reasons to ban EC*x* and related concepts in ecotoxicology. Environ. Sci. Technol. 45:8180-8181.

Jager T, Albert C, Preuss TG and Ashauer R (2011) General Unified Threshold model of Survival - a toxicokinetic-toxicodynamic framework for ecotoxicology. Environ. Sci. Technol. 45:2529-2540.

Jager T, Heugens EHW and Kooijman SALM (2006) Making sense of ecotoxicological test results: towards application of process-based models. Ecotoxicology 15:305-314.

Jager T and Kooijman SALM (2009) A biology-based approach for quantitative structure-activity relationships (QSARs) in ecotoxicity. Ecotoxicology 18:187-196.

Jager T and Zimmer EI (2012) Simplified Dynamic Energy Budget model for analysing ecotoxicity data. Ecol. Modell. 225:74-81.

Kooijman SALM (1996) An alternative for NOEC exists, but the standard model has to be abandoned first. Oikos 75:310-316.

Kretschmann A, Ashauer R, Hitzfeld K, Spaak P, Hollender J and Escher BI (2011) Mechanistic toxicodynamic model for receptor-mediated toxicity of diazoxon, the active metabolite of diazinon, in *Daphnia magna*. Environ. Sci. Technol. 45:4980-4987.

Landis WG and Chapman PM (2011) Well past time to stop using NOELs and LOELs. Integr. Environ. Assess. Manag. 7:vi-viii.

Newman MC and McCloskey JT (2000) The individual tolerance concept is not the sole explanation for the probit dose-effect model. Environ. Toxicol. Chem. 19:520-526.

Nyman AM, Schirmer K and Ashauer R (2012) Toxicokinetic-toxicodynamic modelling of survival of *Gammarus pulex* in multiple pulse exposures to propiconazole: model assumptions, calibration data requirements and predictive power. Ecotoxicology 21:1828-1840.

OECD (1992) OECD guideline for testing of chemicals 203. Fish, acute toxicity test. Organisation for Economic Cooperation and Development (OECD), Paris, France.

OECD (2006) Current approaches in the statistical analysis of ecotoxicity data: a guidance to application, Series on Testing and Assessment, No. 54. Organisation for Economic Cooperation and Development (OECD), Paris, France.

Rufli H and Springer TA (2011) Can we reduce the number of fish in the OECD acute toxicity test? Environ. Toxicol. Chem. 30:1006-1011.

Sprague JB (1969) Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res. 3:793-821.

van Ommen Kloeke AE, Jager T, van Gestel CAM, Ellers J, van Pomeren M, Krommenhoek T, Styrishave B, Hansen M and Roelofs D (2012) Time-related survival effects of two gluconasturtiin hydrolysis products on the terrestrial isopod *Porcellio scaber*. Chemosphere 89:1084-1090.