EU contract on:

Biology-Based Modelling for the Analysis of Ecotoxicity Data for Risk Assessment Purposes

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Contents

Introduction

The environmental risk assessment of chemicals is based on a comparison of predicted concentrations in the environment (exposure assessment) and the predicted effects on populations or ecosystems (the effects assessment). The exposure part of the risk assessment procedures is based on process-based models. Experimental data for the chemical (such as hydrophobicity, vapour pressure and degradability) are used to estimate process parameters of the fate models (such as partition coefficients, volatilisation and degradation rates). Corrections for temperature and environmental conditions (such as soil organic matter content) are made, and different release scenarios can be calculated. The fate models make assumptions on the dominant distribution processes in the environment to provide a reasonable estimate of the exposure concentrations for ecosystems and humans. In contrast, the effects assessment functions more like a primitive scoring system. The results from the standard tests receive (arbitrary) assessment factors, depending on the availability and type of data, to yield a "safe" level for the environment. No attempt is made to identify the processes underlying the effects, and no attempts are made to use scientific insights to extrapolate from the test result to the field situation (e.g., different temperatures, different time-scales of exposure, and time-varying concentrations). Even though such extrapolations are uncertain, they *are* performed for the exposure assessment. This mismatch in approach between the exposure and effects assessment needs to be recognised. Clearly, there is room for substantial improvement in the effects side of chemical risk assessments.

For the effects assessment, experimental toxicity tests are performed according to OECD test protocols. The data from these tests are analysed in a statistical manner to yield a No-Observed Effect Concentration (NOEC, using hypothesis testing), or the concentration yielding a certain percentage of effect (LC50 or EC*x*, using regression analysis). Over the last decade, the NOEC (no-observed effect concentration) has been heavily criticised (Crane and Newman, 2000; Laskowski, 1995; Van der Hoeven, 1997), and the OECD even recommended to cease the use of this statistic (OECD, 1998). The critique mainly focuses on the fact that "no statistically significant effect" does not mean that there is no effect. In fact, the level of effect at the NOEC is regularly 10–34% and in extreme cases can approach 100% (Crane and Newman, 2000). The higher the variability in the data, the higher the NOEC, thus rewarding sloppy experimentation. The EC*x* (the estimated concentration where *x*% effect is expected) is an improvement of the NOEC in the sense that all data at one time point are used, and the point estimate does not depend on the variability in the data. Nevertheless, this approach also suffers from limitations (Jager et al., 2006; Kooijman, 1996). The main problem with the current approaches for EC*x* is that they do not consider the processes behind the data, but merely focus on interpolating to an *x*% effect level with an empirical statistical model (such as the log-logistic curve). As a result, there is no consistent way to deal with measurements over time, measurements of more endpoints from the same test, tests with time-varying exposure (e.g. due to degradation), etcetera. Further, there are no opportunities for educated extrapolations to the population level, to time-varying exposure, or to other environmental conditions (e.g. other temperatures or limiting food levels).

A better starting point for chemical risk assessment is formed by so-called biologically-based models. These models make explicit assumptions regarding the processes behind the toxic effect. The DEBtox method was developed at our department, ten years ago, as spin-off of the work on the theory of dynamic energy budgets (DEB) by the department's head (Prof. Bas Kooijman). To our knowledge, the DEBtox method is the only consistent approach at this time for both lethal and sub-lethal endpoints. A limited version of the DEBtox method has

been implemented in a user-friendly software tool with the same name (see http://www.bio.vu.nl/thb/deb/deblab). This implementation was focussed on the data provided from the standard test protocols, and therefore does not include many of the possibilities that the theory has to offer (e.g., the simultaneous analysis of multiple test endpoints, see Jager et al., 2004).

Because the DEBtox method departs from the processes behind the toxic effect, it explicitly includes time in the analysis. However, the method is simple enough to be able to work with standard test data (as obtained from tests following OECD guidelines). The DEBtox method works best when toxicity data are available for more than one point in time. This is not really problematic, for the standard OECD protocols often prescribe such measurements. For example, the acute *Daphnia* test protocol prescribes that immobilisation is determined after both 24 and 48 hours of exposure (although the data at 24 hours are not normally used in the risk analysis). For *Daphnia* reproduction, the protocol prescribes that observations on juveniles and adult survival are made at least three times per week, although in practice only the cumulative results after 21 days are used. The DEBtox method uses all the results from all time points to estimate model parameters (such as a no-effect concentration, NEC). This has several advantages; firstly, because all time points are used, the parameters can be estimated with greater accuracy (in general, DEBtox requires much less parameters to be estimated per data point than regression approaches, when data in time are available). Secondly, the model parameters of DEBtox can be used to estimate the EC*x* at any point in time. It can thus be shown how EC*x* changes over the duration of the experiment (which can reveal quite unexpected patterns; see Alda Álvarez et al., 2006b), and even predict what will happen after exposure longer than the test duration. Finally, because the data are used more efficiently, biologically-based methods offer great opportunities to diminish testing needs and animal lives. For example, model simulations at our department (J. Baas, unpublished results) have shown that (at least in theory) model parameters can be accurately estimated from survival experiments using a single animal per test concentration, as long as the animals are followed through time.

The discussion of the limitations of current approaches for dose-response analysis, and the opportunities that a biologically-based method has to offer, have been extensively discussed in a recent paper from our group (Jager et al., 2006), which is attached to this proposal.

At this moment, biologically-based methods are not used within the regulatory context. Virtually all risk assessment use LC50s and NOECs after a fixed exposure time. However, the biologically-based methods are discussed (next to the more classical approaches of hypothesis testing and regression analysis) in a recent ISO/OECD guidance document on the statistical analysis of ecotoxicity data (OECD, 2006). It is the purpose of this contract to introduce these methods to the regulatory community, and explore the possibilities for implementation in risk assessment methodology.

Tasks in the project; elaboration of the Technical Annex

Task 1.1. TCNES presentation

The first task in the project would be to prepare and give a presentation at a meeting of the Technical Committee on New and Existing Substances (TCNES). Because the committee members may not be familiar with DEBtox or the details of dose-response analysis, the presentation will focus on the limitations of the current procedures and the potential benefits of a biologically-based approach, without going into mathematical detail. In the introduction of this offer, we already presented our thoughts on this issue. Elements of this discussion will be presented to the TCNES. The discussion of the limitations of current approaches for dose-response analysis, and the opportunities that a biologically-based method has to offer, have been extensively discussed in a recent paper from our group (Jager et al., 2006), which is attached to this offer. We propose to give a summary of this discussion at the TCNES meeting, with one or two example data sets (following current OECD guidelines). We will analyse the data both using regression analysis and DEBtox to illustrate the difference in philosophy and the benefits of using a biologically-based approach.

Most importantly, the presentation will introduce and explain the structure of the workshop to be held at a later point in time (see Task 1.2). It will be clarified in this presentation that the goals of the workshop are to familiarise EU experts with the DEBtox approach, discuss the potential of such methods to address regulatory needs, and identify research needs and user requirements for a new version of the DEBtox software.

Task 1.2. Organisation of a workshop

The detailed organisation of the workshop and the background documents will have to be done in close collaboration with the ECB. However, we will elaborate on our ideas for this workshop below.

Background documents (or separate chapters of a single document)

- Presenting the DEBtox approach. This document will present the concepts behind the DEBtox method. This document will not discuss the mathematics behind the models (which is the purpose of the next document), but will explain the general philosophy, basic assumptions, and history of the method. These concepts are easy to understand, even for non-specialists. Just like the process-based fate models that are used in risk assessment, the user does not necessarily need to know the (mathematical) details behind the models, as long as the concepts and assumptions are clear.
- Background of the DEBtox models. Which assumptions are made in the models, and how are the DEBtox models related to the full DEB theory. The DEBtox models represent a simplification of the DEB theory that is valid under certain conditions. The mathematical derivation and the preconditions have, however, not been presented in the open literature yet. Although the derivation is rather technical, it will provide the necessary transparency that is needed for the regulatory context, and will show that the DEBtox approach results in only a few equations. This document can be seen as an update to the models and methods described in the DEBtox book (Kooijman and Bedaux, 1996a), which has also been published in a series of papers (Bedaux and Kooijman, 1994; Kooijman and Bedaux, 1996b, , 1996c; Kooijman et al., 1996).
- A limited set of examples to illustrate how the DEBtox method functions. In view of the purpose of this proposal, the examples will use data from standard OECD test protocols.

Examples will be selected in consultation with ECB. These examples are intended to clarify the methods used, how they work, and how they describe the data. This is particularly important, as the approach deviates considerably from the more familiar regression models.

- A manual for the current DEBtox IT tool. A very short manual has already been provided in the DEBtox book (Kooijman and Bedaux, 1996a), but more detailed guidance is needed on how to perform various analyses in a step-wise manner. The DEBtox software offers efficient ways to deal with problems in the test data that require further guidance. For example, in the case of mortality in a *Daphnia* reproduction test, the OECD guideline prescribes the removal of the replicate. This is not necessary in the DEBtox analysis, as all the data in time are used, up to the point where the animal dies. Emphasis will be placed on accessing the full possibilities of the tool (e.g., on how to make iso-effect plots and profile likelihoods, and how to interpret them), but also on the limitations. A section will be added describing what options are available to adapt the tool in the future. During the workshop, these options can be discussed, and new options added as a result of the demands of potential users. This document may at a later stage serve as the start for the development of a new version of the DEBtox IT tool (which is not foreseen in the current offer).
- A discussion document on how to implement biologically-based methods in the regulatory risk assessment context. In this document, it will be discussed how these methods can currently be used to support the risk assessment of new and existing chemicals. That is to say, using the data as currently provided using the OECD guidelines (in Task 2.2, the possibilities for amending the test guidelines will be discussed). For example, there are several possibilities for summary statistics, e.g., the NEC may replace the NOEC, and DEBtox can be used to generate EC*x* values for any selected exposure duration (and even extrapolate to longer durations). On the other hand, the DEBtox parameters can also be used to derive the predicted impact of a certain PEC in the field on the exposed populations (e.g., by predicting actual mortality over time, or through the population growth rate). These options will be explained, and their pros and cons, and feasibility, will be discussed.

The workshop

Again, the detailed organization of the workshop will have to done in close cooperation with the ECB; here we will lay down our current ideas for this workshop.

- The workshop will start with an explanation of the goals of the workshop: familiarise EU experts with the DEBtox approach, discuss the potential of such methods to address regulatory needs, and identify research needs and user requirements for a future new version of the DEBtox software.
- A short presentation will be given on the DEBtox model, its history, and its underlying concepts. This presentation can be short, as this will be more extensively treated in the background document. We do not envisage an extensive discussion of the DEBtox concepts at this workshop, although further explanation can of course be given if questions arise among the participants. The critical discussion within the working group should focus on the potential for implementation of DEBtox in the regulatory context, as specified under the forth bullet point below.
- An explanation and demonstration of the current software will be given, focusing on the analysis of data from standard test protocols. Next, the participants can work with the DEBtox IT tool themselves at the workshop. This is the best way to really get acquainted

with a new method. It would be most helpful if the participants bring along some real data sets whose analysis can be included in the workshop programme. These examples can be discussed within the group. Special attention will be given to the interpretation and application of the outcome of the DEBtox analysis.

- Discussion of the document on the implementation of biologically-based methods in the regulatory context. In the view of the participants, how does the DEBtox approach match with the regulatory needs? What would be suitable output of the IT tool to use for effects assessment? Which of the presented risk characterisation options in the document are feasible and useful? What further research would be needed?
- Discussion on needs for the DEBtox IT tool. How far does the current IT tool reflect the needs of the regulators? The theory offers far more possibilities than currently implemented in the DEBtox tool. There is already a version of the DEBtox approach which includes some of these possibilities to assess more complicated data sets, but this is only available in the form of MatLab scripts (and thus lacks user-friendliness). The extended version includes options such as the simultaneous analysis of multiple endpoints (such as survival and reproduction in the standard 21-day *Daphnia* test). These options may be discussed in relation to a possible future update of the DEBtox software.

After the workshop, a summary of the discussions will be prepared. This summary will include the participant's opinions on the suitability of biologically-based methods in a regulatory context, and possibly, identify additional research that would be needed to optimise this suitability. Further, this document will summarise the participant's opinions on the most suitable way to use the output of DEBtox in the risk characterisation. Finally, the summary will identify the user requirements for a new version of the DEBtox IT tool, in as far as these arose during the workshop.

Task 2.1. Background document on QSAR development

As explained in the technical annex, QSARs for toxicity are generally based on a training set of LC50, NOEC or EC*x* values. Because of the limitations of these summary statistics, the resulting QSARs suffer from the same problems. An example to explain this point: acute toxicity tests have fixed exposure durations (2 days for *Daphnia*, 4 days for fish). However, the toxicokinetics depend on the chemical (especially hydrophobicity) and the species (particularly the surface:volume ratio). For chemicals with a low hydrophobicity, the organisms are rapidly in equilibrium with the medium, and we tend to observe the incipient LC50 after the standard test duration. However, for more hydrophobic compounds, the standard test duration may be insufficient to obtain the incipient LC50; the LC50 may decrease further if we choose to extend the test duration. For *Daphnia*, the types of compound where we run into problems may be different than for fish, because the test duration is arbitrarily standardised without properly accounting for differences in toxicokinetics between these species. Clearly, the use of LC50s after a fixed exposure time can lead to bias in the resulting QSARs, and does not help the comparison of species.

As an additional example, it seems that chemicals classified as "reactive" have slower development of the toxic effects in time, compared to narcotics with the same hydrophobicity (the pattern of fish survival in time mimics that of more hydrophobic compounds). The reason is not yet clear, but this may point at kinetic limitations due to the activation process to reactive metabolites. This phenomenon needs to be studied in further detail, but may serve to illustrate that a 4-day toxicity test with fish may be too short, even for hydrophilic compounds, which can be a source of bias in QSARs.

For sub-lethal endpoints such as reproduction, and especially body growth, the problems are even worse as EC*x* values can reveal rather strange patterns in time, even when the exposure concentration is kept constant (Alda Álvarez et al., 2006b). Both the toxicokinetics and the physiological processes underlying the toxic effect are responsible for these patterns.

The parameters from DEBtox do not suffer from such problems, as they are independent of the exposure duration (although longer test durations may be needed in some cases to accurately identify the parameter values). Therefore, they form a better starting point to identify patterns in the data and compare species. An example has already been provided by Prof. Bas Kooijman earlier (Kooijman et al., 1998), showing that the DEBtox parameters for narcotic compounds tend to have predictable relationships with hydrophobicity. The interesting thing is that it may not be needed to actually perform (linear) regressions of model parameters against hydrophobicity (or other descriptors), as it may be possible to predict the actual relationships from first principles, because the DEBtox parameters have a direct physiological interpretation (Kooijman et al., 1998).

A problem in the development of QSARs using DEBtox could be that a DEBtox analysis requires the raw data from toxicity tests, which are often difficult to obtain. Most literature and databases report only the summary statistics, whereas a DEBtox analysis requires the raw survival observations in time. A positive exception are the reports from the Center for Lake Superior Environmental Studies (see Russom et al., 1997). A broad range of industrial chemicals was tested on fathead minnows, also providing the raw data for survival in time. This is a very coherent data set (all tests are performed under similar conditions, and the exposure concentrations where measured back in all exposures). We are not aware of a similar data compilation for other organisms or other endpoints, and therefore we will focus on this data set for survival in fathead minnows. However, if a coherent set of chronic data is available, this could be treated also. At least, we will discuss some of the options for sub-lethal QSARs, using the experience gained for nematodes in our cooperation with the University of Wageningen (Alda Álvarez et al., 2005; Alda Álvarez et al., 2006a; Alda Álvarez et al., 2006b).

It should be noted that it is not the purpose of this discussion document to produce actual validated QSARs. The document will clarify the potential bias made in traditional QSARs owing to the limitations in the underlying data, and show how a biologically-based approach can improve upon these methods. However, we will use some of the data for fathead minnows (and possibly available data for other organisms) to provide a first shot at actually producing estimation routines.

Task 2.2. Background document on improvement of test protocols

The current test protocols were defined with the derivation of classical summary statistics in mind (e.g., NOEC and LC50). Even though the DEBtox IT tool is able to work with test data according to these protocols, they are not optimal, and adaptation of the protocols in combination with DEBtox analysis can lead to serious improvement of the efficiency of risk assessment. The protocols may be loosened in some parts (e.g., using less animals), and additional requirements may be added elsewhere (e.g., prolonging test durations for certain compounds). In other words, the test protocols would be different, when keeping an analysis with DEBtox in mind. The areas that will be investigated will be the following:

- Number of test animals. Because biologically-based models explicitly use all data in time, more information is used in the analysis. As a result, it may be possible to decrease the number of test animals, while retaining the same degree of accuracy. We propose to test

the applicability of a reduction in number of test animals by using a few real data sets, and randomly select sub-samples with fewer animals from the data set. In this way, it can be investigated how the parameter estimates depend on the number of animals (at least for these selected data sets). Furthermore, we will look at the results from simulation studies, performed earlier (Andersen et al., 2000), and which are currently performed at our department, within the framework of the NoMiracle EU-project. The amount of budget allocated to this document does not allow for an extensive, and statistically sound, simulation study to be performed. Nevertheless, the presented examples will provide a good feel for the potential of reducing animal use.

- Flexibility in acute test duration. For some chemicals, it may be needed to extend the test duration (as already proposed by Sprague, 1969). Preferably, a test should continue until no additional mortalities occur (relative to the control). For chemicals with more rapid kinetics, it is advisable to take more observations in time, especially within the first 24 hours. This will be clarified with some examples of fish acute toxicity data for fathead minnows.
- Reversibility of effects. When time-varying exposures are expected in the field (e.g., due to batch production of chemicals), it is advisable to use a test protocol that aims to demonstrate the reversibility of effects. For example, by transferring the organisms after the test to clean medium and follow their performance. The DEBtox approach assumes by default that effects are fully reversible when the internal concentration decreases, which may not be generally true, but is supported by a DEBtox analysis of experiments with *Daphnia* exposed to the insecticide fenvalerate (Pieters et al., 2006).
- Partial life-cycle testing. The most valuable type of data for sub-lethal assessment in a DEBtox context is partial life-cycle data. With this we mean that more endpoints (survival, growth and reproduction) are followed over a part of the life cycle (from juvenile to adult and including several reproductive events). The current *Daphnia* 21-day reproduction test is already very close to these specifications. The standard protocol prescribes the observation of reproduction and survival in time, but also advices to measure body size at the end of the experiment. If body size is measured at several more time points during the test, this would help to identify the physiological mode of action of the compound, and make for an excellent data set to extrapolate to population effects, also under food limitation (Jager et al., 2004).
- For vertebrates, partial life-cycle testing is very costly (both in financial terms, as in terms of animal lives), and therefore not justifiable for many chemicals. Therefore, it has to be investigated whether *in vitro* testing can be used to derive the DEBtox parameters. Although this is an interesting direction of thought, it is outside the scope of this discussion document.

The discussion on the possibilities to adapt the standard test protocols will also be guided by the results of the workshop; i.e., on the most suitable implementation of DEBtox in the regulatory context. If participants of the workshop have a strong preference for a particular use of DEBtox in risk assessment, this may lead to different demands on the optimal test protocol.

General description of the department

The main object of the research programme of the Department of Theoretical Biology (headed by Professor S.A.L.M. Kooijman) at the Vrije Universiteit in Amsterdam can be labelled as quantitative bio-energetics. This topic has been chosen because of its relevance for a wide variety of biological specialisms; the availability of nutrients and energy is frequently the most important limiting factor in the development and functioning of living systems. The aim of the research programme is to develop a quantitative and coherent theory for energy and mass transduction that links theories concerning all levels of organization, from membrane physiology to ecosystem dynamics. The Dynamic Energy Budget theory, developed by Prof. Kooijman, offers good opportunities for use in ecotoxicology, for example, to describe toxicity of chemicals in relation to toxicokinetics, nutrition, and in combination with other stressors. This application resulted in the development of the DEBtox method for the analysis of toxicity data, which was recently accepted by the ISO and OECD as one of the valid methods to analyse such data. Our department has not only developed the DEB theory and DEBtox, but also ran/runs a series of PhD and postdoc projects on its further development (see list below). Furthermore, the department has produced a long list of papers in the open literature as well as other publications on DEBtox (see list below), and organises courses on DEB and DEBtox (see www.bio.vu.nl/thb/deb/course).

The department runs an open-ended project called Biomass (which stands for biomathematical assessments). In this project, department members can be hired by external parties to provide scientific services (see www.bio.vu.nl/thb/ncem/biomass). The Biomass project is embedded in the Netherlands Center for Environmental Modeling (CEM-nl), in which the department of Theoretical Biology cooperates with the Radboud University of Nijmegen and the Institute of Environmental Sciences in Leiden (see http://www.cem-nl.eu). The contract, when awarded, will be fulfilled as part of the Biomass activities.

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Relevant projects and publications of the dept. Theoretical Biology

Current and previous related projects of the department

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