Predicting effects of fluctuating or pulsed exposure to pesticides on aquatic organisms

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Abstract

Aquatic non-target organisms are exposed to fluctuating concentrations and sequential pulses of contaminants. It is necessary to predict toxic effects from such patterns of exposure so that aquatic risk assessments can be improved. The theoretical base and applicability of available models are evaluated and reduced to a few basic concepts. Process oriented simulation of effects emerges as the most promising tool and subsequently a process-based model, the Threshold Damage Model (TDM), is developed. The TDM links exposure with effects. Survival of the aquatic invertebrate *Gammarus pulex* following exposure to three pesticides with contrasting modes of action (chlorpyrifos, pentachlorophenol and carbaryl) and a variety of exposure patterns is simulated.

Measured internal concentrations of the pesticides in *Gammarus pulex* facilitate the subsequent estimation of toxicokinetic parameters. Parameterization of toxicodynamic model parameters is based on survival experiments of up to 28 days length with sequential pulsed exposure patterns. For evaluation, the TDM is compared to a simpler model based on time-weighted average concentrations (TWA).

After successful application to sequential pulsed exposures to all three compounds separately, the two models are extended to suit multiple compounds. The TDM predicts that recovery of damage to *Gammarus pulex* from exposure to chlorpyrifos takes longer than that from exposure to carbaryl and consequently that the sequence of exposure can matter. This hypothesis is confirmed experimentally and the simulation of recovery dynamics by the TDM facilitates a process-based ecotoxicological explanation.

Overall, the TDM outperforms the TWA model. It simulates effects on aquatic organisms from fluctuating or sequential pulsed exposure to contaminants and can extrapolate to realistic exposure patterns. The toxicodynamic parameters reflect the mode of action. The calculation of total recovery times is illustrated and implications for ecotoxicology as well as applications in risk assessment are discussed.
Acknowledgements

I am deeply grateful to Prof. Colin Brown for having supervised the present PhD and, more generally, for facilitating my research by providing advice, resources and continuous support as well as giving me the freedom to develop and follow my own research interests. Grateful thanks are also due to Dr. Alistair Boxall for his advice and support.

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In addition, I am grateful to the European Union for funding this research and I would also like to express my gratitude to all those people in Silsoe and York who made me feel so very welcome.

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Author’s declaration

Chapters 2 to 6 have been written as papers for international peer-reviewed journals. The current publication status of the papers is presented in Table 0-1. All these papers have been reworked, so that they are presented in a consistent style and format in this thesis. For those papers, which have been published, copyright rests with the publishers.

Table 0-1. Status of the five papers presented in this thesis with respect to the publication process.
All papers authored by: Roman Ashauer, Alistair BA Boxall and Colin D Brown.

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<th>Title</th>
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<td>Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides</td>
<td><em>Environmental Toxicology and Chemistry</em></td>
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<tr>
<td>Uptake and elimination of chlorpyrifos and pentachlorophenol into the freshwater amphipod <em>Gammarus pulex</em></td>
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<td>Simulating toxicity of carbaryl to <em>Gammarus pulex</em> after sequential pulsed exposure</td>
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<td>In press (2007)</td>
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</table>
The work in this thesis was undertaken as a PhD student in the EcoChemistry team, first at Cranfield University in Silsoe (March 2004 – September 2004) and then at the Central Science Laboratory in York and the University of York (October 2004 – March 2007). The candidate was contributing to and funded by the European Union research project HAIR (HArmonised Indicators for pesticide Risk). This research was funded under the EU framework VI programme, project number SSPE-CT-2003-501997.

All papers presented in this thesis have joint authorship. This reflects the contributions of the co-authors in their roles as supervisor (Prof. Colin Brown) and advisor (Dr. Alistair Boxall). The papers have been written by the candidate as leading author, however it should be noted that the papers have gained in quality through suggestions, advice and editing from the co-authors. Chapters 2 to 4, which are published, have also benefited from the comments of the anonymous referees as part of the review process.
1. Introduction

Pollution of aquatic systems

The assessment of pollutants in aquatic systems is a major challenge for human society. More than one-third of the earth’s accessible renewable freshwater is used for purposes that are likely to contaminate it [1]. With a yearly pesticide production of five million tons per year [1], their intentional release into the environment and their inherent toxic potential, pesticides have a relatively large potential for effects on non-target organisms. They may reach waterbodies via various pathways, and typically aquatic organisms are exposed to sequential pulses with fluctuating concentrations [2, 3].

Patterns of exposure to pesticides

Fluctuating concentrations or sequential pulses of pesticides in streams have been reported frequently [4-11]. Exposure patterns from such studies are specific for that particular location, application pattern, crop, soil, climate and hydrological condition. Nevertheless the general causes for such exposure patterns are known [12]. First, temporal staggering of environmental pathways such as spray drift and repeated drainage or runoff events can lead to repeated losses of pesticide from the same field, hence creating sequential pulses of concentrations in adjacent water bodies. Fate processes in the water bodies itself such as sorption to sediment, degradation and flow dilution cause further fluctuations in the exposure profile. Secondly, repeated applications of pesticides on the same field or fields in the same catchment can contribute to sequential exposure pulses in a ditch or stream.

Pesticide usage data are collected biannually by CSL [13]. A brief analysis of these data for usage of chlorpyrifos in the orchards of Kent reveals that more than 99% of the farms surveyed had more than one application of chlorpyrifos in the year 2004. Nine percent of all subsequent applications per farm occurred on the same day, 18% within two weeks of the first application on that farm (Figure 1-1).
Figure 1-1. Cumulative percentages of repeated applications on the same farms. Data for chlorpyrifos in orchards in Kent (year 2004).

The example of chlorpyrifos usage in orchards illustrates that the repeated application of the same compound on different fields of a farm could potentially cause several exposure events in a stream or ditch that is adjacent to several of those fields.

Figure 1-2. Mean dates (●) as well as 25th and 75th percentiles (+) of chlorpyrifos applications in orchards on the x-axis. Percentage of fields with subsequent applications on y-axis (from left to right: 1st, 2nd, 3rd, 4th, 5th and 6th application).

Analysis of these usage data at field level reveals that about 66% of all applications are followed by at least one subsequent application on the same field (Figure 1-2). The timing of sequential applications of chlorpyrifos on the same
field in orchards is not evenly distributed over the year, but instead clustered in less than twelve weeks during summer.

**Linking exposure to effects**

The difficulties of relating fluctuating or sequential pulsed exposure patterns to toxicity data for the purpose of risk assessment have been recognized before [2, 3, 14, 15] but to date no satisfying solution is available [16]. The underlying problem is that effects are traditionally measured at constant concentrations over set durations and expressed on the basis of concentration in the water. Modified test designs with pulsed exposure patterns such as those discussed by Boxall et al. [14] can only address a limited number of exposure patterns; hence any generally applicable extrapolation method to relate laboratory toxicity test data to realistic exposure profiles must rely on modelling. Toxicokinetic and toxicodynamic modelling has been proposed as a strong tool for mechanism-based ecotoxicology [17, 18, chapter 2]. The work in this thesis is the first application of toxicokinetic and toxicodynamic modelling to effects on aquatic organisms from fluctuating or sequential pulsed exposure.

**Aims and objectives**

The overall aim of the PhD is to develop a method to predict effects of fluctuating or sequential pulsed exposure to pesticides on aquatic organisms. The detailed objectives are (i) to review and assess existing models that link exposure with effects; (ii) to select, modify or develop a suitable model for dynamic simulations of effects following fluctuating or sequential pulsed exposure; (iii) to develop a method to parameterize the model and (iv) to test the modeling approach with a model organism and three pesticides of contrasting modes of action.
**Format of presentation**

The aims and objectives described above have been addressed in five stand-alone papers, which constitute the main part of this thesis.

*Chapter 2* is a critical review of existing models that may be used to predict effects on aquatic organisms resulting from time-varying exposure to pesticides. When the theoretical basis of the models and their applicability to fluctuating concentrations is evaluated and compared, only two models emerge as the most promising concepts. In chapter 4 those two models are further developed and merged into a new ecotoxicological model.

*Chapter 3* provides the toxicokinetic parameters for chlorpyrifos and pentachlorophenol. Internal concentrations of those two pesticides are measured in *Gammarus pulex* and toxicokinetic parameters are determined by fitting a one-compartment single first-order model.

*Chapter 4* presents a new, process-based model, the Threshold Damage Model (TDM) that links exposure with effects. Survival experiments using chlorpyrifos and pentachlorophenol together with the freshwater invertebrate *Gammarus pulex* are carried out with various exposure patterns. These provide the basis for estimating the toxicodynamic parameters as well as extensive model evaluation and comparison with two simpler models based on time-weighted average concentrations (TWA). The theoretical base, data needs and potential for extrapolation are discussed.

*Chapter 5* contains toxicokinetic and toxicodynamic experiments with carbaryl and *Gammarus pulex*. The data are used to estimate model parameters for carbaryl and to compare the TDM to one of the TWA models. Measurements of internal concentrations in the survival experiment are used to test the toxicokinetic sub-model. The ecotoxicological interpretation of the toxicodynamic parameters is discussed for pentachlorophenol, carbaryl and chlorpyrifos. Calculation of total recovery times provides an example for the use of the TDM in risk assessment.
Chapter 6 broadens the modelling approach to sequential pulses of multiple compounds by extending the TDM and the TWA model to suit multiple toxicants. The TDM predicts that recovery of damage to *Gammarus pulex* from exposure to chlorpyrifos takes longer than that from exposure to carbaryl and consequently that, under certain conditions, the sequence of exposure matters. Experimental evidence confirms this hypothesis and implications for risk assessment are discussed.

Chapter 7 contains a rigorous assessment of the relationship between the TDM and important ecotoxicological models that have previously been reported. Finally the findings of the previous chapters are discussed in the context of risk assessment and possibilities for future research are outlined.
References


2. Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides

Introduction
Pesticides are biocides that are broadly applied within the open environment. Thus they have a relatively large potential for effects on non-target organisms. Aquatic risk assessment to regulate the use of pesticides generally compares predicted environmental exposure concentrations with effects measured at constant concentrations and expressed on the basis of concentration in the water. In reality, pesticide exposure in water varies over time or occurs in pulses [1]. The effects from pulsed exposure will differ from those observed under the conditions of constant exposure that are used in standard laboratory tests [2]. The importance of recovery periods between successive pulses has been recognized by several authors [1-7].

As pulsed exposures have clear relevance to real situations, it is necessary to understand and simulate their effects. We need a theoretically sound model to relate pulsed or time-varying field exposures to laboratory effect data. Experimental approaches are available, including pulsed exposure studies [2] and toxicity tests undertaken over different time periods. The advantage of a modeling approach lies in the ability to extrapolate to a wide range of field exposure scenarios. Models for this purpose have been developed and tested over a period of more than 20 years. We evaluate and compare the theoretical basis of these models and their applicability to the simulation of effects from fluctuating exposures. Based on this, we select the most appropriate models and propose modifications. Finally, we discuss how these models can be used and highlight the advantages of an approach based on biological processes.

Our objective is to predict the effects of repeated and fluctuating pesticide exposure on aquatic organisms. To achieve this, a model should be dynamically applicable and have a meaningful prediction endpoint such as lethality, feeding rate, or growth. Some of the available methods are excluded because they do not permit a dynamic simulation of effects. Examples are the statistical approaches described by Kalbfleisch et al. [8]. Mainly graphical methods are provided in a

When we relate field exposures to laboratory exposure effect data, we face the problem that we do not have time series of the exposure concentrations at a scale of ecological relevance for pesticides. It is expensive and time consuming to generate this kind of data, and extrapolation to other scenarios is difficult because aquatic exposure conditions are highly specific. Very few studies have been reported that investigate fate of pesticides in small flowing water bodies such as ditches and streams [9-11]. Thus, there is little information about processes such as kinetics of sorption and desorption, volatilization, or hydrological dilution and how these modify exposure over time. In place of measured time series for pesticide concentrations, we generally use fate and exposure models [1, 12] to predict aquatic exposure. These models have generally been evaluated against measurements for peak concentrations of pesticides in surface runoff or drainflow at edge-of-field [13-15]. Where calibration is undertaken, this is also normally for peak concentration [12]. Thus, the link to real time series is largely assumed, and use of the models is probably limited at present to generating generalized scenarios of time-varying concentrations. The review of approaches to predict effects from time-varying exposure that is presented below should be seen within the context that much remains to be done to characterize the nature of the exposure profile before predictions for field situations become a realistic possibility.

**The time – concentration – effect relationship**

**Toxicokinetics and toxicodynamics**

A compound generally has to enter the organism and reach the site of action to exert an effect. Simulation of effects arising from pulsed exposure is only possible if the concentration in the organism or, for more complex organisms, at the site of action, is simulated. Toxicokinetics describe the time course of the compound in the organism (e.g., rates of uptake and elimination). Toxicodynamics describe the dynamics of injury and recovery in the organism. The whole concept can be formalized according to Rozman and Doull [16]:

---

*Chapter 2*
Toxicity $T$ is defined as: $T = f(E, K, D)$

- Exposure: $E = f(\text{concentration, time})$;
- Toxicokinetics: $K = f(\text{uptake, elimination})$;
- Toxicodynamics: $D = f(\text{injury, recovery})$.

Taking fish as an example of a complex organism, there are models available that can simulate the internal distribution of a compound. For a review of these pharmacokinetic models see Barron et al. [17]. However, our understanding of these internal distribution processes for most aquatic organisms is too rudimentary to be useful in models. The demand for estimation of numerous parameters by these more complex models restricts the generality of their use. For these reasons, and because uptake and elimination from most aquatic invertebrates are satisfactorily described by the simpler model with one compartment and first-order kinetics, we will concentrate on the latter approach. The whole body concentration is assumed to be a sufficient description of the concentration at the target site concentration (e.g., [18-22]). The processes of uptake and elimination control the internal (whole body) concentration. They are usually described with first-order rate constants that lump several uptake and elimination processes into one parameter. The ratio of the uptake and elimination rates is the bioconcentration factor (BCF) at toxicokinetic steady state.

The internal concentration is a better basis for assessing the intrinsic toxicity of a compound than external concentrations [23, 24]. The toxicodynamics describe the processes leading to the toxic effect. The concentration at the target site (or as a replacement the internal concentration) acts as driving force for the toxicodynamics. Toxicodynamics are determined by the mode of action so that this component must account for the reversibility of the damage. If one rate is used for injury or damage accrual, then this is again a simplification of the underlying mechanisms that lumps several parallel or serial processes into one. It is a universal approach though, assumed to be applicable to different mechanisms of toxic action, repair and recovery.

Toxicokinetic and toxicodynamic models must be combined with time-to-death or hazard modeling to predict toxicity time courses [25]. In other words, the toxicokinetics and toxicodynamics need to be integrated into one model to
describe the exposure-effect relationship. This rationale is widely supported [24, 26-28]. Escher and Hermens [24] believe that “a mechanistic perspective improves any attempt to set up predictive models.” They see physiologically-based toxicokinetic-toxicodynamic models as a method to account for “the complexity of pathways and mechanisms of receptor-mediated toxicants.” Toxicokinetic-toxicodynamic models aim to simulate effects under nonsteady state (dynamic) field situations.

One-compartment single first-order kinetics

A standard approach for modeling toxicokinetics is used by several of the models. An assumption is that the toxicokinetics are not affected by toxic stress, i.e., the toxic effects of the compound do not alter the uptake and elimination kinetics. Accumulation of the compound according to first-order kinetics is described by:

\[
\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C - k_{\text{out}} \times C_{\text{int}}
\]  \hspace{1cm} (2-1)

where \(C_{\text{int}}\) is the internal concentration [Amount × Mass\(^{-1}\)], \(C\) the concentration in the water [Amount × Volume\(^{-1}\)] and \(k_{\text{in}}\) and \(k_{\text{out}}\) the uptake rate constant [Volume × Mass\(^{-1}\) × Time\(^{-1}\)] and the elimination rate constant [Time\(^{-1}\)], respectively. Under the assumption of constant environmental concentration \(C\) and \(C_{\text{int}}(0) = 0\), integration yields the internal concentration at time \(t\):

\[
C_{\text{int}}(t) = \frac{k_{\text{in}}}{k_{\text{out}}} \times C \times \left(1 - e^{-k_{\text{out}} \times t}\right)
\]  \hspace{1cm} (2-2)

Equation 2-1 is integrated numerically in simulations with time-varying external concentration. The use of one-compartment single-first order toxicokinetics has the advantage that both the uptake and the elimination rates are measurable, and that estimation methods for both parameters exist [29-36]. The reader is referred to Kooijman and Bedaux [26], Landrum et al. [37], or Gobas and Morrison [38] for further discussion of one-compartment single first-order kinetics. Toxicokinetic models for fish were reviewed and compared by Barber [39].
Classification of compounds according to target sites and modes of action

There is high interspecies variability in the abundance of target sites and metabolism or repair mechanisms. Still, the behaviour at the same target site is thought to be relatively constant across different biological systems [24]. A classification system for the mode of action thus reduces the number of models required to describe effects for a large number of compounds with different modes of action such as pesticides. Information on the location of the target site helps to ensure that the toxicokinetic model is appropriate. The mode of action determines the degree of reversibility, and description of toxicodynamics must account for this.

A widely used system of four classes of mode of action for aquatic toxicity was suggested by Verhaar et al. [40, 41]. Class 1 chemicals (nonpolar narcotics) affect the organism only by interaction with lipids of bio-membranes. As partitioning is the driving force, the toxic potency is dependent on the hydrophobicity (log $K_{ow}$) and dissociation of the chemical. This mode of action is named baseline or minimum toxicity [41, 42] and is assumed to be completely reversible. The effect is directly related to the membrane concentration and the time-toxicity relationship is determined by the toxicokinetics [24]. This forms the basis for several modelling approaches, including the critical body residue (CBR) approach [23] or PULSETOX [5]. Class 2 compounds possess hydrogen bond donor acidity and they are slightly more toxic than predicted by baseline toxicity. Effect concentrations are generally about 5 to 10 times lower than for nonpolar narcotics [41]. Class 3 and 4 chemicals have effect concentrations that are generally about 10 to $10^4$ times smaller than baseline toxicity predicts and are much more variable [24]. Class 3 compounds react nonselectively with certain chemical structures, whereas class 4 compounds interact with certain receptor molecules [41]. The degree and speed of reversibility of the toxic reaction in these two classes determines which modelling approach is appropriate. For detailed discussion of mode of action, target site, and classification of compounds, the reader is referred to Escher and Hermens [24].
Rate limiting steps
The time scale of the toxicokinetics is controlled by the elimination rate constant, while that of the toxicodynamics is determined by the recovery/repair rate constant. Two cases can occur [16]:

\[
\text{recovery rate} < \text{elimination rate} \quad \Rightarrow \quad \text{Toxicodynamics are rate determining} \\
\text{recovery rate} > \text{elimination rate} \quad \Rightarrow \quad \text{Toxicokinetics are rate determining}
\]

It is hypothesized that at the same level of toxic effect the product of concentration and time is constant after steady state has been reached in both the toxicokinetics and the toxicodynamics [16]. This special relationship between exposure time and effect concentration is called Haber’s law or response-reciprocity [1]. The method of time-weighted averages is also based on Haber’s law, as well as all the methods, mainly graphical, described by Handy [4]. These graphical methods depend on derivation of a mean exposure concentration through various means. Then the likely toxicity is read from toxicity plots constructed from standard toxicity data.

Predicting chronic toxicity
The assessment of effects of long-term exposure in realistic scenarios will almost always involve repeated, varying exposure concentrations for biodegradable compounds like pesticides that enter the aquatic environment in discrete episodes. Chronic toxicity is not necessarily caused by a different mode of action and should not be assumed so until proven [23]. Acute to chronic ratios are related to the time dependence of toxicity and vary strongly between modes of action [24]. This variation results from different toxicokinetics and different toxicodynamics. Thus explicit modelling of these processes will strengthen the prediction of long-term effects.

Evaluation of modelling concepts
In the following we distinguish between models that include toxicokinetics as a first step and toxicodynamics as a second (two-step models), and those models that distinguish only one step (one-step models). The discussion of one-step models will show that they are not our first choice to model the effects from pulsed exposures. However, they are described because they show the history of attempts to tackle the issue of pulsed exposure. Furthermore, the advantages of the
two-step models become clearer in the context of these early models. Descriptions and dimensions of all parameters can be found in Appendix A.

Table 2-1. Summary of the direct-link models.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Link to effects</th>
<th>Dynamic application possible?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haber’s law</td>
<td>( A_x = C \times t_d )</td>
<td>Yes, with limitations</td>
</tr>
<tr>
<td>Power term models</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) ( A_x = C^y \times t_d )</td>
<td>a) Yes, with limitations</td>
<td></td>
</tr>
<tr>
<td>b) ( A_x = C \times t_d^y )</td>
<td>b) No</td>
<td></td>
</tr>
<tr>
<td>Probit plane model</td>
<td>( Z = b_0 + b_1 \times \ln C + b_2 \times \ln t )</td>
<td>No</td>
</tr>
<tr>
<td>Exponential mortality</td>
<td>( \mu = \frac{1}{t_{\text{test}}} \ln \left(1 + \left(C/\text{LC50}\right)^b\right) )</td>
<td></td>
</tr>
<tr>
<td>based on logistic function</td>
<td>( n(t) = n_0 \times e^{-\mu t} ) {for constant C}</td>
<td>Yes</td>
</tr>
<tr>
<td>Survival analysis</td>
<td>( \ln t_d = f(C) + \varepsilon )</td>
<td>No</td>
</tr>
<tr>
<td>Cumulative episodic exposure</td>
<td>( CEC = \sum C \times \frac{t_{\text{tot}}}{t_a} )</td>
<td>Yes</td>
</tr>
</tbody>
</table>

All models establish a direct link between exposure and effect. Toxicokinetics and toxicodynamics are not included.

A: species specific constant for x% mortality; C: concentration in water; y: power term; \( t_d \): time to death; Z: probit of the response; \( b_0, b_1, b_2 \): regression constants; CEC: cumulative exposure concentration; \( t_{\text{tot}} \): total time; \( t_a \): acceptable duration; \( t_{\text{test}} \): intervals \( C_w > C_{\text{safe}} \); LC50: lethal concentration for 50% of individuals in toxicity test; \( t_{\text{test}} \): duration of the toxicity test; \( \mu \): mortality rate; b: slope parameter; n: number of animals alive; f(C): function relating the covariates (concentration) to \( t_d \); \( \varepsilon \): error term and scale parameter.

One-step models: Direct link between exposure and effect

The models in this section all seek to establish a direct link between exposure concentration and effect, without consideration of toxicokinetics or toxicodynamics. Table 2-1 gives an overview.
Power term models, Ostwald’s equation and time weighted averages. Several empirical models describe the concentration–time response relationship without dependence on toxicokinetics. Empirically derived toxicity models usually take the form of the power term models in Table 2-1. Equation (b) is known as Ostwald’s equation. Under non steady state conditions in toxicokinetics or toxicodynamics, the relationship between exposure and effect is thought to be: 

\[
\text{constant effect} = C \times t^y
\]  

[16]. From \( \text{constant effect} = C^a \times t^b \), the simpler function \( \text{constant effect} = C \times t^y \) can be derived by defining \( y \) as the ratio \( b:a \) [43].

The right hand side of the power term models can be viewed as a dose factor [44], which is the product of concentration and time weighted by a power term and represents the total exposure. Thus \( \int C(t)\,dt \) for Haber’s law and \( \int C(t)^y \,dt \) for power term model (a) in Table 2-1 represent the dose factor for fluctuating exposure. These can be calculated for any exposure scenario and then related to the dose factor from toxicity experiments:

\[
\frac{\int_0^t C(t)\,dt}{C_{\text{test}} \times t_{\text{test}}} = \frac{\% \text{ mortality predicted}}{\% \text{ mortality test}}
\]

\[
\Rightarrow S(t) = 1 - \frac{\% \text{ mortality test} \times \int_0^t C(t)\,dt}{C_{\text{test}} \times t_{\text{test}} \times 100}
\]

(2-3)

In this example \( C_{\text{test}} \) and \( t_{\text{test}} \) are the concentration and duration in a standard toxicity test at constant exposure concentration and \( S(t) \) is the probability of the test organisms to survive until time \( t \). This is actually a modification of the time-weighted average approach (see below) and can be extended further to tests with fluctuating concentrations by replacing the product of concentration and time by the integral of the exposure concentration over time.

This method yields some estimate of the effect from fluctuating exposure, but has severe limitations based on theoretical reasoning alone. First, for very long exposure durations, the dose factor might reach significant levels even if concentrations are below effect levels; second, the relationship of the dose factor (or product of concentration and time) to the response level is not defined and
requires arbitrary assumptions (e.g., linear relationship); and third, the model is not based on knowledge about biological processes.

When time-weighted average concentrations are used to deal with fluctuating exposures, the exposure concentration is integrated over time and then divided by the duration of the corresponding toxicity test. This method is inherently based on Haber’s law, and it is used in the risk assessment of pesticides to calculate exposure-toxicity ratios [45, 46].

**Probit plane models.** The underlying assumption of the probit models is that the concentration just sufficient to kill an individual is normally distributed for all individuals in a population [47]. The plot of the mortality response against log \( C \) or log \( t \) is then shaped like a cumulative normal distribution. The two-factor probit plane model [44, 48] is shown in Table 2-1. Such a model was used by Peterson et al. [49] to explain the time-concentration-effect relationship for carbaryl and the stream invertebrate *Cinygma* sp. Detra and Collins [50] showed that a three dimensional model of the type: \( \text{effect} = \ln t + a \times \ln C \) is in excellent agreement with acetylcholinesterase inhibition by parathion in midge larvae (*Chironomus riparius*). The equation of the probit plane model can be transformed into the power term model (a) in Table 2-1:

\[
Z = b_0 + b_1 \times \ln c + b_2 \times \ln t
\]

\[
\Leftrightarrow Z - b_0 = \ln \left( C^{b_1} \times t^{b_2} \right)
\]

\[
\Leftrightarrow \text{constant} = \ln \left( C^y \times t \right)
\]

\[
(2-3)
\]

where \( y = b_1 / b_2 \) [44, 48]. When \( b_1 \) and \( b_2 \) are equal then: \( C\times t = \text{constant effect} \), which is Haber’s law.

**Classical power term.** The power term model (a) in Table 2-1 has been used to model substances causing cumulative damage [1], and it is one of the three models tested by Meyer et al. [51]. The power term does not have a physiological meaning. It just improves the fit of the model to the data [51]. Meyer et al. found that predictions for pulse LC50s (lethal concentration for 50% of the population) based on the \( C \times t \) model were only reasonable for less than four pulses. Since the model of Mancini [3] approaches an incipient lethal level, Meyer et al. [51]
speculate that it will perform better than the $C \times t$ model for large numbers of pulses. Although Meyer et al. conclude that none of the three models tested simultaneously accounts for the underlying processes, they still believed them to be suitable for regulation of chlorine in power plant effluents [51].

**Margin of exposure.** The time dependence of an effect is dominated by the frequency of exposure at short toxicokinetic and toxicodynamic half-lives [52] as represented by the model: Margin of exposure, \( MOE = \frac{C \times t_y}{C \times t} \) or Margin of risk, \( MOR = \frac{C \times t}{C \times t^y} \), where $C \times t$ refers to the ideal exposure conditions and $C \times t^y$ describes the real world exposure. Large departures from continuous exposure will result in small $y$. This method mainly points out deviations from the special conditions of Haber’s law and does not provide a tool for assessment of fluctuating exposure.

**Exponential mortality based on logistic function.** Traas et al. [7, 53] used a model with exponential mortality that was based on information derived from standard toxicity tests with the logistic dose-response model. The model (see Table 2-1) uses a mortality rate $\mu$ [Time$^{-1}$], which is derived from standard toxicity experiments. The slope parameter $b$ equals $1/\beta$, where $\beta$ is the slope parameter in the classical logistic function and is estimated from experimental data with the logistic dose-response model [53]. A disadvantage of this model is that the mortality rate depends directly on the external concentration, and thus the model does not reflect uptake and elimination processes. This derivation of the mortality rate is not process oriented and is therefore difficult to interpret. The general problems associated with the logistic models are discussed by Kooijman and Bedaux [26]. By replacing the number of animals $n$ at time $t$ by the probability $S$ of survival until time $t$ and the mortality rate $\mu$ by the hazard rate $h$, this approach could be reformulated to show the similarity to the hazard models:

\[
S(t) = S_0 \times e^{-ht}
\]

which reduces to the hazard model if $S_0 = 1$ and the cumulative hazard $h \times t = H(t): S(t) = e^{-H(t)}$

\[
(2-4)
\]

\[
(2-5)
\]
The use of a mortality rate is an approach to describe the death process, quite similar to hazard modelling. The mortality rate here and the hazard rate in the hazard model are essentially describing the same process. Both represent the rate or probability of dying in an infinitely small time interval. Both are derived for use at the level of the organism, but become meaningful in an interpretation at the population level.

Survival analysis. Also known as time-to-event models, survival analysis models include both concentration and exposure duration as covariates (Table 2-1) and can predict the effect of combinations of these [54]. This type of modelling enhances the predictive capabilities of test results because it includes time and concentration as covariates [54]. However, it is not possible to use the model in a dynamic mode to predict effects of varying concentrations.

Cumulative episodic exposure. Morton et al. [55] combined effects assessment with exposure modelling. Analogous to species sensitivity approaches, they derived an ‘estimated safe concentration’ (ESC), which is the concentration at which only a certain percentage of species is likely to be affected. Integration only considers that portion of the area under the exposure curve where the concentration is above this ESC threshold (see Table 2-1). The acceptable duration $t_a$ must be specified based on toxicity experiments, the toxicokinetics or community aspects [55]. The cumulative exposure concentration (CEC) is the final risk estimate, essentially an average concentration for periods exceeding the ‘estimated safe concentration’, multiplied by a time factor. This cumulative exposure concentration is then read onto a species sensitivity distribution, yielding the percentage of species affected [55]. The underlying assumption of this concept is that $C \times t = constant\ effect$, i.e., that Haber’s law holds true. This is not usually the case, especially in situations with fluctuating concentrations. Furthermore, the concept does not assess whether the time between the pulses is sufficient for recovery and does not integrate the processes of uptake and elimination.

Suitability of the direct-link models to simulate effects from time-varying exposure. None of the direct-link models are suitable for simulating effects from varying exposure. Some are not applicable in dynamic simulations (see Table 2-1), while others fail to incorporate important process knowledge or are based on
weak assumptions. The method of time-weighted averages is used in pesticide risk assessment to calculate exposure-toxicity ratios [45, 46], but has severe limitations when it is modified to predict effects from fluctuating exposure over time.

**One-step models using the critical body residue concept**

The models in this section all derive a value for the critical body residue (CBR) from standard toxicity tests and use this measure to assess when lethality will occur for different exposure scenarios. The time dependence of toxicity is modelled solely through the toxicokinetics, and the differences between the models lie in their methods to establish the CBR (see Table 2-2).

*The critical body residue approach.* The CBR theory links lethality to aqueous concentrations and whole body residues [1]. The underlying rationale is that the toxicity resulting from pulsed exposure is primarily controlled by the accumulation and depuration rates for the substance. Inherent in the CBR concept is the assumption that the critical body residues are an acceptable surrogate for lethal dose [1]. Further assumptions are that the toxic action (toxicodynamics) is instantaneous and completely reversible. Evidence for the critical body residue concept has been summarized by McCarty and Mackay [23] and Leslie et al. [56]. Historically the CBR concept was used for various modes of action [5, 23], although Verhaar et al. [57] state that the approach should only be used for narcotics. The CBR approach also gained popularity for modelling the effects of pulsed exposure under the name PULSETOX [5]. PULSETOX is a simple one-compartment, single first-order kinetics model that calculates the whole body concentration in fish. This is then used to predict acute toxicity.

The CBR is thought to be constant for nonpolar (nonspecific) narcotics and “possibly” so for polar narcotics [57]. It is used as a dose metric and it can be calculated as shown in Table 2-2 from experiments with constant exposure concentration. The CBR can be interpreted as the internal concentration at the end of a toxicity test in which 50% of the individuals have died. Once the CBR and the parameters $k_{in}$ and $k_{out}$ are known, the equation in Table 2-2 can be rearranged to predict $LC50$ values for different durations. Essentially the $LC50$ is a function
of $e^{-k_{in}t}$ [57] and becomes constant for long exposure times after steady state is attained [25]: $LC50(\infty) = CBR / BCF$.

The validity of the CBR concept for narcotic compounds was questioned recently by Lee et al. [25], who found that the CBR model could not predict the time course of toxicity of polycyclic aromatic hydrocarbons in *Hyalella azteca*. Critical body residues have been found to decrease with increasing exposure time, even after attainment of toxicokinetic steady state [25, 57, 58].

**Critical body residue with power term.** The basic model of Mancini [3] is formulated more generally with the power term $y$ (see Table 2-2), because some data are better represented by a power expression for the concentration in water. This better fit is attributed to internal distribution processes, including deviations from first-order kinetics [3]. In a plot of $\ln(LC50)$ versus $\ln(t)$ this model will approach a horizontal asymptote that represents the incipient lethal level. Breck [59] replaced uptake and elimination by “damage and repair” in his terminology, but basically still used the model of Mancini [3].

**Reduction in life expectancy.** Yu et al. [60] present a model that expresses long-term nonspecific toxicity in terms of reduction of life expectancy per unit internal concentration. This approach is developed for the assessment of long-term effects. Compared with the CBR concept, this model is based on the assumption that internal lethal concentrations decrease with increasing exposure time. Thus the internal concentration at which 50% of the individuals die is calculated differently (Table 2-2). For the effective zero time, which is the time at which enough toxicant has accumulated at the target site to cause lethality, the concentration can be calculated from experimental data via backwards extrapolation. The time to death for zero concentration is the normal life expectancy of the organism. The model is first fitted to experimental data in order to obtain $a_c$. Internal lethal concentrations are calculated for different exposure times $t$, by replacing $LT50$ in the equation with $t$. The parameter $a_c$ does not have a biological meaning.
Table 2-2. Summary of the critical body residue (CBR) models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Toxicokinetics</th>
<th>Link to effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBR approach</td>
<td>$\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C - k_{\text{out}} \times C_{\text{int}}$</td>
<td>$\rightarrow 50%$ dead when $C_{\text{int}}$ reaches CBR</td>
</tr>
<tr>
<td></td>
<td>$CBR = \frac{k_{\text{in}}}{k_{\text{out}}} \times LC50 \times (1 - e^{-k_{\text{out}}t_{LC50test}})$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$= C_{\text{int}}(t_{LC50test})$</td>
<td></td>
</tr>
<tr>
<td>CBR with power term</td>
<td>$\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C^y - k_{\text{out}} \times C_{\text{int}}$</td>
<td>$\rightarrow 50%$ dead when $C_{\text{int}}$ reaches CBR</td>
</tr>
<tr>
<td></td>
<td>$CBR = \frac{k_{\text{in}}}{k_{\text{out}}} \times LC_{50}^y \times (1 - e^{-k_{\text{out}}t_{LC50test}})$</td>
<td></td>
</tr>
<tr>
<td>Life expectancy reduction</td>
<td>$\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C - k_{\text{out}} \times C_{\text{int}}$</td>
<td>$\rightarrow 50%$ dead when $C_{\text{int}}$ reaches $ILC50$</td>
</tr>
<tr>
<td></td>
<td>$ILC50 = a_c \times \ln \left( \frac{LT50}{NLT50} \right)$</td>
<td></td>
</tr>
<tr>
<td>Extended logistic model</td>
<td>$\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C - k_{\text{out}} \times C_{\text{int}}$</td>
<td>$\rightarrow$ death occurs when $C_{\text{int}}$ exceeds threshold</td>
</tr>
</tbody>
</table>

All use the same toxicokinetic model, apart from that with the power term.
The common idea in these models is that a certain internal concentration causes the effect. The models differ in the way that they derive this critical internal concentration.

$C_{\text{int}}$: internal concentration; $k_{\text{in}}$: uptake rate constant; $k_{\text{out}}$: elimination rate constant; $C$: concentration in water; $y$: power term; $ILC50$: internal lethal concentration (for 50% lethality); $a_c$: compound specific proportionality constant; $NLT50$: average normal lethal time (life expectancy); $LT50$: lethal time (exposure time in a toxicity test with 50% mortality); $CBR$: critical body residue; $LC50$: lethal concentration for 50% of individuals in toxicity test; $t_{LC50test}$: duration of the toxicity test.
Extended logistic model. Kooijman [61] defined a model in which the no-effect level parameter is treated as a probability with a log-logistic distribution. Death is assumed to occur when the internal concentration exceeds the internal threshold equivalent to the corresponding external no-effect concentration that is also log-logistically distributed [61]. This is similar to the CBR approach with a stochastic description of the critical body residue (as determined through the external no-effect level). The internal concentration is modelled using one compartment, first-order kinetics. For constant exposure concentration, the whole model can be summarized as Bedaux and Kooijman [62]:

\[
S(t, c) = \left( 1 + \left( \frac{\max(0; \left( C \left( 1 - e^{-k_{out} t} \right) - c_0 \right))}{LC50(\infty) - c_0} \right)^{\beta} \right)^{-1} \tag{2-6}
\]

where \( S \) is the survival probability, \( C \) is the concentration in water, \( k_{out} \) is the elimination rate constant, \( t \) is time, \( c_0 \) is the concentration in water at the no-effect level, \( LC50(\infty) \) is the LC50 for infinite exposure, and \( \beta \) is the slope parameter of the logistic distribution. The uptake rate constant would be an additional parameter for fluctuating concentrations. The statistical and methodological problems associated with logistic models are well summarized by Kooijman and Bedaux [26]. Furthermore LC50 and EC50 values are of little ecological importance and their relevance for risk assessment is limited [26]. Consequently, the logistic model is rejected for dynamic effects modelling or estimating safe concentrations.

Suitability of critical body residue models. The critical body residue models with and without power term, as well as the reduction in life expectancy model, cannot readily be applied to fluctuating concentrations, because it is not clear how to deal with internal concentrations that rise above the CBR (or internal lethal concentration) and then fall below the threshold. For example, PULSETOX assumes that a lethal effect results if the internal concentration exceeds the CBR [5]. This problem is inherent in all CBR models apart from the extended logistic model. It is not defined how internal concentrations just below the CBR affect the organism, or what time course of toxicity would result as the internal concentration approached and then exceeded the CBR. These models are able to estimate LC50 values for different exposure scenarios, but application for
dynamic simulations is limited. Another limitation of the CBR models is that they are intended for narcotic compounds only. The extended logistic model could be modified to simulate fluctuating exposure concentrations, but the author himself now rejects this approach in favour of the DEBtox model ([26], page 15). Although it is not possible to use the reduction in life expectancy model in its current form for dynamic effect simulations, the approach is interesting because it explicitly considers exposure time in relation to life expectancy of the organism.

Two-step models: Distinguishing toxicokinetics and toxicodynamics

*The critical area under the curve and critical target occupation models.* There are two names for the same model for irreversibly acting class 3 and 4 compounds: the critical target occupation (CTO) model [21] and the critical area under the curve (CAUC) [57]. Both assume that the compound-receptor interaction is instantaneous and irreversible and that no recovery occurs. Time dependency of toxicity is observed long after toxicokinetic steady state has been achieved, which is why the critical body residue model is not applicable [21]. By irreversibly reacting with a target site, the total amount of affected target sites becomes related to the magnitude of the effect [57]. Generally the magnitude of effects is related to the amount of covalently bound toxicants for irreversible receptor interactions. This amount increases as long as the receptor is exposed to the toxicant, and is therefore proportional to the reaction rate for the binding reaction and the total amount of the toxic compound that has reached the receptor since the beginning of the exposure. Legierse et al. [21] hypothesize for this mode of action that the critical target occupation should be constant for different compounds in the same species. The critical area under the curve (CAUC) is the time integral of the concentration at the target site and is calculated as follows [57]:

\[
CAUC = \int_0^t C_{int}(t) \, dt
\]  

(2-7)

The CAUC is compound-specific and the effect occurs when it is reached. After integration and replacing the aqueous concentration $c$ with the concentration for 50% effect $EC50$, the time dependence of $EC50$ for constant exposure scenarios becomes:
\[ EC50(t) = \frac{\text{CAUC}}{\text{BCF}} \times \frac{1}{t - (1 - e^{-\lambda t})/k_{\text{out}}} \]  
(2-8)

\( BCF \) is the bioconcentration factor and \( k_{\text{out}} \) is the elimination rate constant. This equation is likely to overestimate the toxicity of the compound because it assumes that \( EC50(\infty) = 0 \). This would only be true if there were no reversibility and no repair or recovery at all.

The \( \text{CAUC} \) translates into the \( \text{CTO} \) in the case of acetylcholinesterase (AChE) inhibition [21] by multiplication with the first-order rate constant for AChE inhibition \( k_{i} \):

\[ CTO = k_{i} \times \int_{0}^{t} C_{\text{int}}(t) \, dt = k_{i} \times \text{CAUC} \]  
(2-9)

The time dependence of the \( \text{LC50} \) is modelled slightly differently because Legierse et al. [21] use an incipient \( \text{LC50} \), the \( \text{LC50}(\infty) \), to account for compensating mechanisms put into action by an organism subjected to long-term exposure:

\[ \text{LC50}(t) = \frac{\text{CAUC}}{\text{BCF}} \times \frac{1}{t - (1 - e^{-\lambda t})/k_{\text{out}}} + \text{LC50}(\infty) \]  
(2-10)

Legierse et al. [21] estimated the parameters \( \text{CAUC} \) and \( \text{LC50}(\infty) \) by fitting to a range of constant exposure experiments where different exposure times resulted in different \( \text{LC50} \) values. The elimination rate constant was estimated using a quantitative structure-activity relationship (QSAR). In their comparison of the \( \text{CBR} \) and the \( \text{CTO} \) model, Legierse et al. [21] found that the \( \text{CBR} \) model overestimates toxicity at short exposure times and underestimates toxicity at long exposure times.

Whereas the critical target occupation could be the same for different irreversibly acting compounds, this is not true for the critical area under the curve. To simulate effects of fluctuating concentration with these models, one would first calculate the \( \text{CAUC} \) or \( \text{CTO} \) variables from constant exposure experiments and measurements for uptake and elimination rate constants. Then one could assess whether a fluctuating external concentration leads to a breach of the \( \text{CAUC} \) or \( \text{CTO} \) that would correspond to 50% mortality. The models in their original form
do not yield information on endpoints other than 50% mortality, although it is possible to derive the CAUC and CTO for different endpoints.

**The simple hazard model.** The hazard model is often used to link some metric of exposure to effect, usually internal exposure and death, respectively [26, 63-65]. The probability $S$ of an organism to survive until time $t$ is related to the hazard rate $h(t)$ by:

$$S(t) = \exp \left( - \int_0^t h(t) dt \right) \quad \Leftrightarrow \quad S(t) = e^{-H(t)}$$  \hspace{1cm} (2-11)

where $H(t)$ is the integrated hazard rate or cumulative hazard. The hazard rate is the probability that an organism dies per time interval [28]. The background hazard and the hazard caused by the toxicant can be summed [28, 64]. In the following, $h$ refers to the toxicant-induced hazard rate which is directly proportional to the internal concentration in the models of Jagers op Akkerhuis et al. [28] and Widianarko and Van Straalen [63]:

$$h(t) = \theta C_{in}(t)$$  \hspace{1cm} (2-12)

with $\theta$ being a proportionality constant. Application to fluctuating concentrations is straightforward once the proportionality constant $\theta$ has been estimated from a previous experiment and provided the toxicokinetic parameters are known.

**The threshold hazard model (THM).** The original threshold hazard model is part of the more general DEBtox concept [26]. The DEBtox theory assumes that there is no (detectable) effect or damage done to the organism up to a certain internal concentration of a toxicant, the so-called no-effect level [26]. The rationale for the no-effect level in DEBtox is that “even if each molecule has an effect, regulation systems in the individual cancel these effects” ([22], page 205). After long, constant exposure the internal threshold concentration is linked to an external threshold $c_0$ by: $C_{\text{internal threshold}} = c_0 \times k_{\text{in}} / k_{\text{out}}$ [26]. This is only valid when toxicokinetic steady state between the organism and the surrounding water has been achieved. Use of an external threshold concentration in the original DEBtox model is part of an effort to minimize the number of parameters, but this works only for constant exposure scenarios. Elimination of the uptake rate constant from the model is not valid for fluctuating concentrations because the organism and the
surrounding water are not at toxicokinetic steady state. The same argument
discounts the approach of Pery et al. [64] in using mathematical methods to
describe time-varying exposure concentrations so that the basic DEBtox model
could be used. The DEBtox model is fitted to survival data from toxicity tests
under constant exposure concentrations. The three parameters that are determined
- the killing rate, the external no-effect threshold concentration, and the
elimination rate constant - are not sufficient for dynamic simulation with
fluctuating exposure concentration. The uptake rate constant is also required and it
then becomes easier to use internal threshold concentrations for pulsed or varying
exposure. We refer to this slightly modified model as the threshold hazard model
(THM).

The toxicokinetics are simulated with one-compartment simple first-order
kinetics. The hazard rate \( h \) [Time\(^{-1}\)] is assumed to be proportional to the
exceedance of the internal concentration \( C_{\text{int}} \) [Amount \( \times \) Mass\(^{-1}\)] over the internal
threshold concentration \( C_{\text{internal threshold}} \) [Amount \( \times \) Mass\(^{-1}\)]. The survival function is
then related exponentially to the hazard and a killing rate constant \( k_{k2} \) [Mass \( \times 
\text{Amount}^{-1} \times \text{Time}^{-1} \)] is introduced:

\[
h(t) = k_{k2} \times \max\{C_{\text{int}} - C_{\text{internal threshold}}, 0\} \]

(2-13)

The integration of the hazard rate yields the cumulative hazard \( H \) [-], which is
used to calculate the survival probability \( S \) [-]:

\[
H(t) = \int_0^t h(t) \, dt \quad (2-14)
\]

\[
S(t) = e^{-H(t)} \quad (2-15)
\]

The characteristics of the THM are illustrated in Figure 2-1. An inherent
implication of the no-effect threshold is that organisms are simulated as
recovering completely and instantly as soon as the concentration falls below the
threshold [64]. The DEBtox theory does not include a repair or recovery process,
so time is only relevant for the toxicokinetics, not for the toxicodynamics [26, 65].
If the model performs well on the data from constant exposure experiments, it is
expected to be suitable for risk assessment of episodic pollution for that
combination of species and compound [65].
Figure 2-1. Simulations with the threshold hazard model using an arbitrary pulse scenario to illustrate the model characteristics.

Uptake and elimination are simulated with one-compartment single first-order kinetics. The cumulative hazard and the survival probability are calculated with Equations 2-14 to 2-16. The graphs show two illustrative exposure pulses and the resulting internal concentration, cumulative hazard and survival probability. The parameter values are purely illustrative \((k_{in} = 0.2, k_{out} = 0.1, k_{k2} = 0.002, C_{internal threshold} = 5)\). The cumulative hazard and the survival probability remain constant while the internal concentration is below the threshold.

The damage assessment model (DAM). A model including toxicokinetics and toxicodynamics with no a priori assumption about the reversibility of the toxicodynamics was developed recently by Lee et al. [66]. An observed decrease in lethal body residues after toxicokinetic steady state was reached indicated that polycyclic aromatic hydrocarbon (PAH) toxicity in *Hyalella azteca* was not determined solely by toxicokinetics [25]. The authors concluded that the toxicodynamics were a further time-dependent step. This observation can be described by models with two rate-limiting steps: the bioconcentration process (toxicokinetics) and the damage-recovery process (toxicodynamics). Both these processes are modelled with first-order kinetics in the damage assessment model [66]. In contrast to the threshold hazard model, the recovery/repair process can be viewed as an explicit description of those “regulation systems” ([22], page 205) that cancel out the effect below an observable threshold.
Lee (http://www.bio.vu.nl/thb/deb/essays/Lee2003.pdf) proposed to use the DAM for pulsed exposures, but the method was not fully developed.

The internal concentration is modelled within the DAM using the one-compartment single first-order approach. In a second step, damage accumulates in proportion to the accumulated body residue and damage repair (or recovery) is proportional to the accumulated damage [66].

\[ \frac{dD}{dt} = k_a C_{int} - k_r D \]  

where \( D \) is the damage [-], \( k_a \) is the rate constant for accrual of damage [Mass × Amount\(^{-1}\) × Time\(^{-1}\)], and \( k_r \) is the rate constant for damage recovery or repair [Time\(^{-1}\)]. The characteristics of the DAM are illustrated in Figure 2-2. With \( D(0) = 0 \) and a constant concentration \( C \), the damage function is:

\[ D(t, C) = k_a \frac{k_{in}}{k_{out}} C \left( e^{-k_r t} - e^{-k_{out} t} + \frac{1 - e^{-k_r t}}{k_r} \right) \]  

It is assumed that death occurs when the damage reaches a certain critical level.

The link between damage and survival rate is established through a hazard model:

\[ H(t, C) = k_3 D(t, C) \]  
\[ S(t, C) = e^{-H(t, C)} \]

where \( H(t) \) is the hazard function [-], \( k_3 \) is a dimensionless coefficient and \( S(t) \) is the survival probability [-].

The CAUC and CTO models and the CBR model are special cases of the more general DAM. The constant CBR model is similar to the DAM when the rate constant for repair/recovery is very large (\( k_r \approx \infty \)) [66]. This is indicated by the CBR assumption of complete and rapid reversibility of effect. The difference is that the DAM translates the internal concentration into a survival probability using an exponential function. In the case of \( k_r \approx \infty \), the DAM is also identical to the simple hazard model. The CAUC and CTO models assume irreversible binding and the equivalent DAM model is represented by a very small repair/recovery rate (\( k_r \approx 0 \)) [66]. The DAM is more flexible and more general than the other three models because it does not make any a priori assumptions about the reversibility of the interaction between toxicant and target site. Lee et al.
[66] found that the DAM could fit time-dependent toxicity data for *Hyalella azteca* exposed to flourene, phenanthrene, and pyrene better than the constant CBR or the CAUC model without toxicity threshold. Both, the CAUC model with toxicity threshold and the DAM, described the data well.

**Figure 2-2. Simulations with the damage assessment model using an arbitrary pulse scenario to illustrate the model characteristics.**

Uptake and elimination are simulated with one-compartment single first-order kinetics. The damage and the survival probability are calculated with Equations 2-17, 2-19, and 2-20. The graphs show two illustrative exposure pulses and the resulting internal concentration, damage and survival probability. The parameter values are purely illustrative ($k_{in} = 0.2, k_{out} = 0.1, k_3 = 0.005, k_3 = 0.4, k_4 = 0.01$). The damage decreases between the pulses and after the last pulse, causing the survival probability to increase.

The damage accumulation process is largely governed by $k_i$, which is related to the mode of toxic action [66]. Since the toxicodynamics are independent of the toxicokinetics, the latter should be measured or estimated separately if the toxicodynamics are inferred from a toxicity experiment. One consequence of the repair/recovery process is that damage can decrease after the end of an exposure pulse (Fig. 2-2). This causes the survival probability to increase. An increasing survival probability is difficult to interpret because it describes the number of organisms surviving to time $t$, and this number cannot increase once the
organisms die. If a nonlethal endpoint is used instead of survival, recovery of the endpoint can occur and this model behavior becomes desirable.

**Proposal for a modified damage assessment model.** The rate constant \( k_a \) [Mass \( \times \) Amount\(^{-1} \times \) Time\(^{-1} \)] and the coefficient \( k_3 \) [-] can be combined to \( k_a \times k_3 = k_{k1} \), a killing rate constant [Mass \( \times \) Amount\(^{-1} \times \) Time\(^{-1} \)]. By replacing \( k_3 \) and \( k_a \) with \( k_{k1} \) and using \( H = k_3 \times D \), it is possible to rewrite the toxicodynamics of the DAM as follows:

\[
\frac{dD}{dt} = k_a C_{\text{int}} - k_i D \quad \times k_3
\]

\[
\Leftrightarrow \frac{dH}{dt} = k_a k_3 C_{\text{int}} - k_i H
\]

\[
\Leftrightarrow \frac{dH}{dt} = k_{k1} C_{\text{int}} - k_i H
\]

With constant concentration \( C, C_{\text{in}}(0) = 0 \) and \( H(0) = 0 \), the hazard function is then:

\[
H(t, C) = k_{k1} \frac{k_{\text{in}}}{k_{\text{out}}} C \left( e^{-k_{\text{in}} t} - e^{-k_{\text{out}} t} + \frac{1 - e^{-k_i t}}{k_i} \right)
\]

This equation combined with that for the survival probability function enables calculation of the survival probability directly for a given constant exposure concentration \( C \) and a time \( t \). There are four rate constants in the modified damage assessment model. Two of them \( (k_{\text{in}}, k_{\text{out}}) \) could be derived independently from the literature, estimation methods or measurements. There remain two rate constants \( (k_{k1} \) and \( k_i) \) to be derived from direct experiments or indirect parameter estimation.

It is necessary to introduce a restriction concerning the hazard rate. Since, by definition, the hazard rate cannot be negative it has to be:

\[
\frac{dH}{dt} = \max\{k_{k1} C_{\text{int}} - k_i H; 0\}
\]

With this definition, the hazard will increase monotonously and the survival probability will decrease monotonously (Figure 2-3). This solves the difficulty with increasing survival probability in the original DAM. Another justification for the introduction of a threshold for recovery is the fact that death does not permit recovery and thus provides a threshold by definition.
The graphs show the cumulative hazard and the survival probability. Uptake and elimination are simulated with one-compartment single first-order kinetics. The cumulative hazard and the survival probability are calculated with Equations 2-23 and 2-20, respectively. The parameter values are again purely illustrative \((k_{in} = 0.2, k_{out} = 0.1, k_{k1} = 0.002, k_r = 0.01)\). The hazard increases monotonously and the survival probability decreases monotonously.

The repair/recovery process is now restricted dependent on hazard and internal concentration. Although the modified DAM does not include damage explicitly, it seems counterintuitive to have a repair/recovery process included in the model, but not to permit full repair or recovery. The reason is a mix of two different scales in the model: the survival probability is expressed at the scale of the whole organism and cannot increase because death is irreversible. Damage (or hazard) is modelled at the suborganism scale and is reversible. As survival probability and damage (or hazard) are linked, the recovery/repair process is restricted. The larger the hazard, the earlier the repair/recovery process will be restricted because large hazards lead to high repair/recovery rates in the model which might cause the term \(k_{k1}C_{int} - k_r H\) to become negative.

Theoretically, the toxicity threshold within the DAM should be defined as a time-integrated exposure reflecting accumulated damage [66]. The internal threshold concentration in the modified DAM can be seen as: \(k_r \times \frac{H(t)}{k_{k1}}\). The hazard rate is
zero below the threshold concentration. The threshold thus depends on the hazard \( H(t) \). The threshold would be higher after longer exposure.

One should use the modified damage assessment model without the restriction of the hazard rate only when simulating non-lethal endpoints. These can fully recover, so that the hazard rate can be negative. Instead of Equation 2-23 one would use:

\[
\frac{dH}{dt} = k_{k1}C_{in} - k_rH
\]  

(2-23)

**A summary of the modified DAM:**

\[
\frac{dC_{in}(t, C)}{dt} = k_{in}C(t) - k_{out}C_{in}(t)
\]  

(2-24)

\[
\frac{dH(t, C)}{dt} = \max\{k_{k1}C_{in}(t, C) - k_rH(t, C); 0\}
\]  

(2-25)

\[
S(t, C) = e^{-H(t, C)}
\]  

(2-26)

If \( C \) is constant, \( C_{in}(0, C) = 0 \) and \( H(0, C) = 0 \) then the survival probability \( S \) is:

\[
S(t, C) = e^{-k_{k1}\frac{C_{in}(t)}{k_{out}}\left(\frac{e^{-k_r-t} - e^{-k_{out}}}{k_r - k_{out}}\right)}
\]  

(2-27)

The symbols are: hazard \( H \), external concentration \( C \), internal concentration \( C_{in} \), uptake rate constant \( k_{in} \), elimination rate constant \( k_{out} \), killing rate \( k_{k1} \) and repair/recovery rate \( k_r \).

**Suitability of the two-step models.** The critical area under the curve model and the critical target occupation model do not predict the probability of survival resulting from fluctuating exposure. Furthermore, they are made redundant by the damage assessment models. The link to survival probability is made in the hazard models. Table 2-3 provides an overview of the hazard models. These models as presented here do not include background mortality. Background mortality is easily included in all hazard models by adding a constant control mortality rate to the hazard rate. The possibility of combining damage accrual, repair/recovery, and a threshold into one model is not shown in Table 2-3. If it is justified by experimental data or by the understanding of toxicodynamic processes, this model
can be constructed easily. It would have three toxicodynamic parameters. (See chapter 4.)

The simple hazard model is a special case of the damage assessment models. It is preferable not to make \textit{a priori} assumptions about the reversibility of the toxic action. Reversibility can be inferred from experimental data by using the modified damage assessment model if survival is the measured endpoint.

\textbf{Table 2-3. The toxicodynamics can be modelled with different hazard models: Possible combinations of damage accrual, repair/recovery, and a threshold.}

<table>
<thead>
<tr>
<th>Damage assessment model</th>
<th>Modified damage assessment model</th>
<th>Simple hazard model</th>
<th>Threshold hazard model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard rate is proportional to accumulated damage</td>
<td>accumulated damage</td>
<td>internal concentration</td>
<td>internal concentration above threshold</td>
</tr>
<tr>
<td>Recovery modelled through damage repair</td>
<td>repair/recovery of cumulative hazard</td>
<td>instant recovery assumed</td>
<td>instant recovery assumed</td>
</tr>
<tr>
<td>Threshold</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Toxicodynamic parameters</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

All four models use one-compartment simple first-order toxicokinetics with two parameters (uptake and elimination rate constants). The possibility of combining damage accrual, repair/recovery and a threshold into one model with three toxicodynamic parameters is not shown here.

The validation status of both the damage assessment model and the threshold hazard model is limited. The damage assessment model has not been tested on survival data from fluctuating exposures. The damage assessment model was used
to predict values of lethal residue at 50% mortality for various exposure durations in four studies [66-69]. The lethal residues were then compared to measured values. Thus only the special case of $S = 0.5$ and $C = \text{constant}$ was evaluated for various durations. The compounds used were narcotics and the organism was *Hyalella azteca* in all studies and additionally *Chironomus tentans* and *Diporeia spp.* in Schuler et al. [68]. The original version of the threshold hazard model as used in the DEBtox concept has been tested many times [26, 22, 65, 70], but only with data from toxicity tests with constant exposure concentration. The modified damage assessment model and the threshold hazard model have not been tested yet.

**Discussion**

**Mechanistic character and complexity of the models**

The models in this paper can be compared according to their complexity, represented by the numbers of parameters, and mechanistic character (percentage of parameters describing a biological process) as in Figure 2-4. The different parameters are listed in Table 2-4. The threshold concentrations in the extended logistic model and the threshold hazard model are not classed as parameters describing a process, even though they have a biological meaning. Similarly, the killing rates are describing a process, but at the same time include a proportionality coefficient, which is why they are not classed as process parameters.
Figure 2-4. Characterization of the models in terms of ‘complexity’ (number of parameters) and mechanistic character (percentage of parameters describing a biological process).

PTM=Power Term Models, EM=Exponential Mortality based on logistic function, CEE=Cumulative Episodic Exposure, CBR=Critical Body Residue, CAUC=Critical Area Under the Curve models, SHM=Simple Hazard Model, CBRy=Critical Body Residue model with power term, LER=Life Expectancy Reduction, ELM=Extended Logistic Model, modDAM=modified Damage Assessment Model, THM=Threshold Hazard Model, DAM=Damage Assessment Model, CTO=Critical Target Occupation Model.

The number of parameters itself has to be viewed with respect to the question posed. The optimal number of parameters is a balance between describing the dominant processes and introducing parameter redundancy. Parameter redundancy makes estimation of parameters more difficult and increases the likelihood of parameter correlation. A model does not have enough parameters when some of the important processes that play a role in the question addressed are not accounted for. Models are preferred that have the smallest number of parameters giving an adequate description of the system and where the greatest proportion of parameters are directly measurable [71]. Independent measurement of parameters is more robust than estimating parameters via inverse modelling (i.e., fitting the whole model with several parameters to a limited set of measured endpoints that are only indirectly related to some parameters). Using the simplest appropriate model will also minimize errors [1, 71].
Table 2-4. Parameters necessary to run the different models in predictive mode (where possible for fluctuating concentrations).

<table>
<thead>
<tr>
<th>Model</th>
<th>Number of parameters</th>
<th>Parameters describing a biological process</th>
<th>Dependent parameters</th>
<th>Other parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power term</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative episodic exposure</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Life expectancy reduction</td>
<td>3</td>
<td>$k_{in}, k_{out}$</td>
<td>$a_c$</td>
<td></td>
</tr>
<tr>
<td>Critical body residue</td>
<td>3</td>
<td>$k_{in}, k_{out}$</td>
<td>$CBR$</td>
<td></td>
</tr>
<tr>
<td>Critical body residue with power term</td>
<td>4</td>
<td>$k_{in}, k_{out}$</td>
<td>$CBR$</td>
<td>$y$</td>
</tr>
<tr>
<td>Extended logistic model</td>
<td>5</td>
<td>$k_{in}, k_{out}$</td>
<td></td>
<td>$c_0, LC50(\infty), \beta$</td>
</tr>
<tr>
<td>Exponential mortality based on logistic function</td>
<td>1</td>
<td></td>
<td></td>
<td>$\beta$</td>
</tr>
<tr>
<td>Critical area under the curve</td>
<td>3</td>
<td>$k_{in}, k_{out}$</td>
<td>$CAUC$</td>
<td></td>
</tr>
<tr>
<td>Critical target occupation</td>
<td>5</td>
<td>$k_{in}, k_{out}, k_i$</td>
<td>$CTO$</td>
<td>$LC50(\infty)$</td>
</tr>
<tr>
<td>Simple hazard model</td>
<td>3</td>
<td>$k_{in}, k_{out}$</td>
<td>$\theta$</td>
<td></td>
</tr>
<tr>
<td>Threshold hazard model</td>
<td>4</td>
<td>$k_{in}, k_{out}$</td>
<td>$c_0, k_{k2}$</td>
<td></td>
</tr>
<tr>
<td>Modified damage assessment model</td>
<td>4</td>
<td>$k_{in}, k_{out}, k_s$</td>
<td>$k_{k1}$</td>
<td></td>
</tr>
<tr>
<td>Damage assessment model</td>
<td>5</td>
<td>$k_{in}, k_{out}, k_s, k_a$</td>
<td>$k_3$</td>
<td></td>
</tr>
</tbody>
</table>

The total number of parameters includes dependent parameters. Parameters: $k_{in}$: uptake rate constant, $k_{out}$: elimination rate constant, $k_i$: repair/recovery rate constant, $k_s$: damage accrual rate constant, $A$: species specific constant for x% mortality, $CEC$: cumulative exposure concentration, $a$: compound specific proportionality constant, $CBR$: critical body residue, $CAUC$: critical area under the curve, $CTO$: critical target occupation, $k_i$: first order rate constant for inhibition of acetylcholineesterase, $y$: power term, $ESC$: estimated safe concentration, $t_a$: acceptable duration, $C_0$: no effect level water concentration, $LC50(\infty)$: ultimate $LC50$, $\beta$: slope parameter of the logistic model, $\theta$: proportionality constant, $k_{k1}$ and $k_{k2}$: killing rates, $k_3$: coefficient.
Chapter 2

Models suitable to simulate effects from fluctuating exposure

The aim of simulating the effects of realistic exposure scenarios with repeated pulses or fluctuating concentrations can best be achieved through dynamic modelling. Models based on descriptions of biological or chemical processes are preferred for predictive modelling because they allow for meaningful interpretation of the results and are more justifiable. A discussion of the many advantages of a process-oriented approach can be found in Kooijman and Bedaux ([26], page 13). The models should include the one-compartment single first-order approach for simulating the toxicokinetics. This widely used concept describes the processes of uptake and elimination with an appropriate level of complexity. Furthermore, uptake and elimination rates can be measured or estimated. The models that fulfil these criteria are compared in Table 2-5. Only two concepts cover all suitable approaches: the two versions of the DAM and the threshold hazard model. The critical body residue model and the CTO/CAUC models are already represented in the DAM, as is the simple hazard model, which can also be seen as a threshold hazard approach with a zero threshold.

The modified damage assessment model and the threshold hazard model are considered to be the best models for modelling the effects of varying pesticide exposure to aquatic organisms. The modified DAM has four parameters, and three of those describe a biological process directly. The threshold hazard model also has four parameters, where two describe a biological process directly and a third one has a biological meaning. These are uptake and elimination for both models, and either repair/recovery for the modified damage assessment model or internal threshold for the threshold hazard model. The killing rate constant is the fourth parameter in both models and it describes an actual process. In the modified DAM, the killing rate constant links the internal concentration and hazard rate, whereas in the THM it links the difference between the internal concentration and the threshold to the hazard. The killing rate constant will not have the same value in the two models.
Table 2-5. Comparison of the two-step models.

The toxicokinetics are one-compartment single first-order in all these models, but the toxicodynamics are different.

<table>
<thead>
<tr>
<th>Model</th>
<th>Toxicokinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>All two-step models</td>
<td>[ \frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} \times C(t) - k_{\text{out}} \times C_{\text{int}}(t) ]</td>
</tr>
<tr>
<td><strong>Toxicodynamics</strong></td>
<td></td>
</tr>
<tr>
<td>CAUC/CTO model</td>
<td>CAUC = \int_0^t C_{\text{int}} , dt = \text{constant}</td>
</tr>
<tr>
<td>Simple hazard model</td>
<td>[ h(t) = \theta C_{\text{int}}(t) ]</td>
</tr>
<tr>
<td>Threshold hazard model (THM)</td>
<td>[ h(t) = k_{k2} \times \max[C_{\text{int}} - C_{\text{internal threshold}}; 0] ]</td>
</tr>
<tr>
<td>Damage assessment model (DAM)</td>
<td>[ \frac{dD(t)}{dt} = k_a C_{\text{int}}(t) - k_r D(t) ]</td>
</tr>
<tr>
<td>modified DAM</td>
<td>[ h(t) = \max[k_{k1} C_{\text{int}}(t) - k_r H(t); 0] ]</td>
</tr>
<tr>
<td>Link to survival probability in hazard and damage assessment models</td>
<td>[ S(t) = e^{-H(t)} ]</td>
</tr>
</tbody>
</table>

\( C_{\text{int}} \): internal concentration; \( k_{\text{in}} \): uptake rate constant; \( k_{\text{out}} \): elimination rate constant; \( C \): concentration in water; \( CAUC \): critical area under the curve; \( \theta \): coefficient; \( h \): hazard rate; \( H \): cumulative hazard; \( S \): survival probability; \( k_{k2} \): killing rate constant; \( D \): Damage; \( k_a \): damage accrual rate; \( k_s \): coefficient; \( k_r \): repair/recovery rate constant; \( k_{k1} \): damage rate constant (damage proportional to hazard); \( t \): time.
The modified damage assessment model includes toxicodynamics without any a priori assumption about reversibility. This flexibility in the toxicodynamics means that the classification of chemicals according to their toxic mode of action is less critical: the modified damage assessment model fits narcotics and reactive- or specifically-acting compounds. The reversibility of the toxic action does not need to be classified for our modelling purpose because the toxicodynamics in the modified damage assessment model can fit any degree of reversibility. The toxicodynamics parameters that are derived from fitting the model will indicate the degree of reversibility for that combination of species and compound. Thus the modified damage assessment model is a tool to understand and compare the ecotoxicity for different combinations of compound and species.

Currently none of the models identified as suitable above has been evaluated with respect to predicting effects from fluctuating or pulsed exposures. Comparison of the predictive power of the various models requires datasets that provide information on toxic effects resulting from repeated exposure pulses or fluctuating concentrations, as well as measuring the toxicokinetics. In particular, the simulation of recovery processes must be evaluated with data from toxicity experiments with repeated exposure. Some data are available on toxic effects from pulsed exposure to pesticides (Appendix B), and other studies have investigated uptake and elimination of pesticides (Appendix C). However, there are only a few experiments with repeated exposure pulses and various recovery times in between, and in each case we lack the corresponding toxicokinetic data for the same compounds and organisms. Furthermore, there is a complete lack of toxicity time series in the field that could serve as a benchmark for model predictions.

The greatest need to support research into effects of fluctuating concentrations on aquatic organisms is thus for laboratory experiments with contrasting pesticides that can support evaluation of modelling approaches. The experiments must measure uptake and elimination rates alongside toxicity tests with repeated exposure pulses.
Comparing the modified DAM and the THM

Limitations. All of the models discussed above simplify the system that they describe. For example, all would need to be modified to include growth dilution and other factors (e.g., metabolism) if these processes were known to be significant. The assumption within the damage assessment model of first-order kinetics for both rate-limiting steps is a simplification. This is widely accepted for the toxicokinetics, but there is little empirical evidence to support the assumption for the toxicodynamics. The assumption of only two rate-limiting steps lumps several processes together. The modified damage assessment model does not represent one toxic mode of action, but rather tries to generalize over many modes of action. This theoretical applicability over a great number of compounds and species still has to be proven, as the number of studies in which the DAM has been evaluated is limited. Also, the model may be out-performed by more specific models for a given combination of compound and species.

The hazard rate in the threshold hazard model returns straight to either zero or the background hazard as soon as the concentration falls below the threshold. This description of ‘recovery’ is achieved purely via the toxicokinetics and cannot take into account observations that show real recovery at the site of action (e.g., acetylcholinesterase inhibition [72, 73]). The fitted elimination rate constant in the threshold hazard model will thus overestimate the real (measurable) elimination rate constant if repair/recovery takes place.

Possible drawbacks of the modified DAM and THM models are that the parameter estimation process is not trivial and depends to a large extent on the quality of the available data. Currently, it is not possible to determine the parameters for a large number of compound-organism combinations because the necessary datasets are not available. Both models need to be tested on experimental data from long-term experiments with repeated pulsed exposure if they are to be accepted as tools for risk assessment.
**Threshold.** The situation where the hazard rate is zero occurs in the THM when the concentration is below the threshold. The corresponding situation in the modified DAM is when \((k_1 \times C_{\text{int}} - k_r \times H) \leq 0\). The hazard rate is then also set to zero as sufficient elimination and repair has taken place. A threshold is observed when the concentration in the water or the organism is above zero, but the hazard rate is zero. This situation can be modelled with both the THM and the modified DAM approach, but the model structure means that the modified DAM cannot yield a threshold if there has not been any previous exposure.

**Endpoints.** Two types of endpoints can be simulated: those at the level of an organism such as survival probability, feeding rate, or other behavioural responses, and those on a sub-organism level such as enzyme activity. Those endpoints that exhibit recovery, i.e., feeding rate, behavioural responses, and enzyme activities, can only be modelled using the damage assessment model, as this is the only model that includes a conceptualization of recovery or repair. Ideally the internal accumulated damage \((D)\) should be linked to enzyme inhibition or other measures of toxic effect at the target site, and the survival probability can be replaced by feeding rate reduction or other endpoints. Sub-lethal endpoints are the most appropriate for the derivation of recovery times. Survival can be simulated with the THM or the modified DAM.

**Mixture toxicity.** Theoretically, the modified damage assessment model and the threshold hazard model could be used to predict the toxicity of mixtures of non-interacting compounds. Once the parameters have been established for each compound, the toxicity of compounds with different modes of action can be predicted by summing up their respective hazard rates. The same approach would work for compounds with the same mode of action, although it could be simplified: in the case of compounds with the same mode of action, the toxicodynamic parameters should be very similar (ideally the same). Consequently, one could estimate the toxicodynamic parameters of several compounds from the behaviour of one known substance. The internal concentrations of a mixture of similarly acting compounds could be summed and one set of toxicodynamic parameters applied.
Environmental standard setting. The modified DAM is very flexible for environmental standard setting. The inclusion of recovery/repair processes makes the model realistic enough for the derivation of “environmental safe scenarios” instead of “environmental safe concentrations.” These scenarios would explicitly consist of concentration, exposure time, and, if required, time between pulses. These could also be derived from the DEBtox model, but the interpretation is less straightforward because the DEBtox conceptualization lacks explicit repair/recovery.

Conclusion

The relationship between exposure time, exposure concentration, and toxic effects is specific for each combination of species and compound. These differences are attributed to two groups of processes: the toxicokinetics and the toxicodynamics. Those models that explicitly reflect this two-step concept have the greatest potential to simulate the effects from varying exposure concentrations. The model parameters have biological meaning, and thus facilitate a better understanding of the ecotoxicity of different compounds and species, and even that of mixtures of non-interacting compounds.

Out of the large number of models available, many (i.e., all the one-step models) are not fit for the purpose of modelling the effects of varying exposure concentrations. The two-step models are fit-for-purpose, but several are only applicable to a specific mode of action. The threshold hazard model and the damage assessment model are generalized forms and make the others redundant. A modified damage assessment model is proposed because the original DAM is over-parameterized and has an inconsistency when modelling survival. The threshold hazard model and the modified damage assessment model represent the optimum descriptions that we have at present. These dynamic process-oriented hazard models deal with fluctuating exposure concentrations and ideally include recovery processes. This makes them first choice for simulating effects of pulsed exposures with varying concentrations and durations.
Predictions from these process-based hazard models are more useful than traditional methods such as LC50 or NOEC (no observed effect concentration) values from standard toxicity tests, because the models allow a quantitative prediction of the effects for various exposure scenarios, including those that occur in reality. The possibility to model lethal and sub-lethal effects with these models gives applications within risk assessment, standard setting, and ecological modelling.
References


Chapter 2


(43) Miller FJ, Schlosser PM, Janszen DB. 2000. Haber's rule: A special case in a family of curves relating concentration and duration of exposure to a fixed level of response for a given endpoint. *Toxicology* 149:21-34.
Chapter 2


Chapter 3

3. Uptake and elimination of chlorpyrifos and pentachlorophenol into the freshwater amphipod 
Gammarus pulex

Introduction

In order to protect natural populations of aquatic organisms it is necessary to understand and predict the adverse effects arising from exposure to chemical contaminants. Chemicals enter aquatic systems via various pathways and in many cases the aquatic organisms are exposed to repeated pulses or fluctuating concentrations [1, 2]. A compound generally has to enter the organism and reach the site of action to exert an effect [3-5]. If we understand the uptake and elimination dynamics (toxicokinetics) of contaminants in aquatic organisms we can predict the time course of concentrations in the organisms. This allows us to assess exposure to contaminants at the site of action and may lead to a better understanding of the resulting effects.

In this study we determined uptake and elimination of chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and pentachlorophenol in the freshwater amphipod Gammarus pulex. This organism is of ecological importance because it is involved in detritus processing in streams [6], it is widely distributed and links primary producers to higher-order organisms [7]. Gammarus pulex is frequently used in biomonitoring studies [6, 8], in laboratory toxicity studies [9, 10] and in microcosm experiments [11, 12]. Although there are toxicokinetic data on other freshwater invertebrates such as Hyalella azteca [4, 5, 13], no uptake or elimination rates for Gammarus pulex have been measured or published. The objectives of this study were to: i) establish a method to measure uptake and elimination rates of organic compounds in Gammarus pulex; ii) measure uptake and elimination rates of two model compounds in Gammarus pulex; and iii) assess whether the one-compartment single first-order model is an appropriate representation of the toxicokinetics of these compounds in the organism. The pesticides pentachlorophenol

(PCP) and chlorpyrifos (CPF) were chosen as model compounds. Pentachlorophenol is an uncoupler of oxidative phosphorylation and can cause accelerated cell respiration and heat output in aquatic freshwater invertebrates [14]. Chlorpyrifos is an organophosphate insecticide and causes toxicity through inhibition of the enzyme acetylcholinesterase [15]. Measurement of the uptake and elimination rates of these compounds in *Gammarus pulex* is the first step in a series of experiments designed to assess effects from pulsed or fluctuating exposure to pesticides.

**Materials and Methods**

**Organisms and exposure water**

*Gammarus pulex* were collected from a small stream, Bishop Wilton Beck, in Bishop Wilton (E479600, N455000), ca. 20 km northeast of York, UK. Organisms were collected on 15 February and 27 May 2005 for the chlorpyrifos and pentachlorophenol experiment, respectively. Streamwater was collected at the same time and stored at 5°C until the start of the experiments (1 March 2005 for chlorpyrifos and 3 June 2005 for pentachlorophenol). Prior to the experiments, the organisms were kept in aerated streamwater under the same conditions as in the experiments and were fed in excess with re-hydrated horse-chestnut leaves.

A mixture of males and females was used in the experiments. Average wet weight of the *Gammarus pulex* was 13.04 mg (n = 80, SE = 0.79 mg) in the chlorpyrifos experiment and 18.55 mg (n = 84, SE = 0.57 mg) in the pentachlorophenol experiment. Assuming equal proportions of male and female *Gammarus pulex* of the same average age we can estimate the age of the organisms from their wet weight [16]. The average age was estimated at 140 and 158 days in the CPF and PCP experiments respectively. The lipid content of seven replicates with 20 *Gammarus pulex* in each replicate (collected on 27 May 2005, analysed on 7 June 2005) was estimated by change in dry weight after lipid extraction with diethyl-ether [17]. The ratio of dry weight to wet weight was 0.21 (SE = 0.04) and the lipid content was 1.34% of wet weight (SE = 0.09).
Measuring the lipid content of the organisms in bioconcentration studies is desirable for several reasons: i) bioconcentration is associated with partitioning of hydrophobic compounds into lipids, so a correlation between lipid content and bioconcentration is often found; ii) variation in lipid contents over the course of a year can be a source of variation in uptake and elimination; and iii) bioconcentration factors are more meaningfully compared across species when expressed on a lipid basis.

**Chemicals**

The $^{14}$C-radiolabelled chemicals used were [pyridine-2,6-$^{14}$C] chlorpyrifos (99% purity, 32 Ci/mol, lot # 050107) and $[^{14}$C(U)] pentachlorophenol (99% purity, 11.9 Ci/mol, lot # 050112). Both compounds were purchased from American Radiolabeled Chemical, Inc. (St. Louis, U.S.A.). Unlabelled pentachlorophenol was purchased from Sigma-Aldrich Ltd. (Gillingham, UK, 99.5% purity, lot # 330-97A).

Pentachlorophenol is a weak acid (pKa = 4.74 [18]) that is largely in the ionized form at the pH in our study (pH range: 8.48 – 9.15, measured with a Hanna pH 213 and HI 1131 electrode, Hanna Instruments). The partitioning of PCP (log Kow = 5.01 for the neutral species [18]) between water and octanol phase has been found to be pH dependent, with the apparent log octanol/water partition coefficient being 2.75 at pH 8.9 [19].

**Experiments**

The experiments comprised an uptake phase and an elimination phase. The organisms were first exposed to spiked solutions for three days (73.5 and 72.45 hours for CPF and PCP respectively), then rinsed in de-ionised water and transferred to clean water for the remaining three days of the experiments. Experiments were carried out under static conditions in 600-mL pyrex beakers filled with 500 mL of exposure solution. Eight beakers were kept in a cooling tank with water as coolant to maintain constant temperatures. The temperatures were 16°C ± 2°C and 12°C ± 2°C in the pentachlorophenol and the chlorpyrifos experiment, respectively. The light regime was a cycle of 12 hours light and 12 hours dark. Seven replicate beakers were treated and one control beaker was used in each experiment. All beakers were sealed with
parafilm and aerated with pressurised air through Pasteur pipettes (dissolved O₂ ranged between 8.8 and 9.2 mg/L in the pentachlorophenol experiment, measured with HI 9142 Dissolved Oxygen Meter, Hanna Instruments).

The nominal concentration of total pentachlorophenol in the uptake phase was 70 µg/L. This solution was prepared by mixing 7 µg of radiolabelled pentachlorophenol with 63 µg of unlabelled pentachlorophenol per liter. The concentration of 70 µg/L pentachlochlorophenol in the dosing solution corresponds to one tenth of the concentration for 50% mortality amongst Gammarus pulex after 48 hours static exposure to pentachlorophenol (700 µg/L [20]). Four liters of filtered streamwater were spiked with pentachlorophenol and then distributed into the beakers. The chlorpyrifos (80 µL of 2134 Bq/mL of 14C-chlorpyrifos in methanol) was added directly to beakers that already contained 500 mL of streamwater. The nominal concentration of chlorpyrifos in the uptake phase was 0.1 µg/L, corresponding to the concentration for 10% mortality of Gammarus pulex after 48 hours in a mesocosm experiment [11]. The toxicity of CPF to Gammarus pulex varies from a 48 hr – LC₅₀ of 0.08 µg/L and a 96 hr – LC₁₀ of 0.02 µg/L in laboratory experiments to a 48 hr – LC₅₀ of 0.3 µg/L and a 48 hr – LC₁₀ of 0.1 µg/L in mesocosm experiments [11].

Streamwater was filtered through glass wool immediately before the start of the experiments to remove suspended solids. The carrier solvent for the PCP and the CPF was methanol. The initial methanol concentrations were 0.016% in the chlorpyrifos experiment and 0.009% in the pentachlorophenol experiment. We assume that the methanol evaporated quickly and that it had no effect on the Gammarus pulex.

There were 20 Gammarus pulex in each beaker at the beginning of the pentachlorophenol experiment whereas in the chlorpyrifos experiment the number of Gammarus pulex at the start of the experiment varied from 14 to 20 organisms per beaker. Mortality was 15.4% over the total duration of the chlorpyrifos experiment and 5.7% in the pentachlorophenol experiment. We assume that neither the initial number of organisms nor the mortality disturbed the uptake and elimination measurements because the ratio of solution to Gammarus pulex mass was very large.
(the mass of 20 *Gammarus pulex* in a beaker was only 0.05% of the total mass in the system). Mortality in the single control beaker was 0% in the PCP experiment and 53% in the CPF experiment (i.e. 8 out of 15). The control mortality in our test system is usually between 2 and 20% after six days (mean 7%; data from six unpublished toxicity tests, each with five replicate control beakers and with 10 *Gammarus pulex* per beaker), thus the mortality in the control beaker of the CPF experiment is an anomaly, partially due to the small number of organisms and lack of replication. Nevertheless, the observed mortality of 15.4% over six days in the treated beakers of the CPF experiment is within the expected range because our nominal concentration of 0.1 µg/L in the uptake phase corresponds to the concentration for 10% mortality of *Gammarus pulex* after 48 hours in a mesocosm experiment [11].

The animals were fed with an excess of re-hydrated horse-chestnut leaves (2-3 leaves of approx. 3 cm² per beaker). Brown horse chestnut leaves were collected directly from the trees in autumn 2004 and stored dry until the experiments. Leaf material was soaked in de-ionised water at least 10 days before the experiments. Samples were taken at the beginning and end of the uptake phase to determine sorption to leaf material. Fresh leaf material was added for the elimination phase.

**Sampling and analysis of water**

Water samples (5 mL) were taken from each beaker at 0, 3, 7, 12.25, 24.25, 48.5, 72.5, 77.5, 81.5 and 145.5 hours after the start of the CPF experiment. Water samples (1 mL) in the PCP experiment were taken from each beaker at 0, 3.25, 7.25, 13, 25, 49, 61, 72, 72.45, 80.45, 97.45, 120.5 and 145 hours. Water samples were analysed immediately after collection. Radioactivity was quantified with liquid scintillation counting (Beckman LS6000 TA Liquid Scintillation Counter, Beckman Instruments Inc., Fullerton, USA) after adding 15 mL of Ecoscint A scintillation cocktail (National Diagnostics, Hessle, UK) to the 5 mL chlorpyrifos samples and 10 mL Ecoscint A to the 1 mL pentachlorophenol samples. Samples were counted three times for 5 minutes. Sample counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected with the external standard ratio method. The limit of detection (LOD) in aqueous samples was
0.007 µg/L for CPF and 0.72 µg/L for PCP. The limit of quantification (LOQ) in aqueous samples was 0.015 µg/L for CPF and 1.47 µg/L for PCP. Concentrations in water were below the limit of detection after 3.23 days and 3.40 days for chlorpyrifos and after 3.02 and 3.35 days for pentachlorophenol. These concentrations were set to 0 in the parameter estimation. Any concentrations between the limit of detection and the limit of quantification were used in the parameter estimation with their measured values. This occurred for CPF after 6.06 days and for PCP after 4.06, 5.02 and 6.04 days.

Sampling and analysis of *Gammarus pulex* and leaf material

One *Gammarus pulex* was sampled from each of the seven replicate beakers after 3, 7, 13.25, 24.25, 48.5, 72.5, 77.5, 81.5, 98.5, 122.5 and 145.5 hours in the CPF experiment and after 3.25, 7.25, 13, 25, 49, 61, 72.45, 75.45, 80.45, 97.45, 121 and 145 hours in the PCP experiment. Each *Gammarus pulex* was removed from the beaker, blotted dry, weighed on a precision balance (XS205, Mettler-Toledo Inc.) and frozen at –20°C until analysis. Similarly, leaf material was carefully blotted dry, weighed and frozen until analysis. To digest the *Gammarus pulex* we added approximately 1.5 mL Soluene-350 tissue solubiliser (Packard BioScience B.V., Groningen, The Netherlands) to the frozen organisms. Then the samples were placed in a water bath at 60°C for 24 hours. The samples were cooled and 10 mL Hionic Fluor scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands) were added. After mixing and settling of the insoluble exoskeletons, the supernatant of the samples was carefully poured into liquid scintillation (LSC) vials. Approximately 3 mL Hionic Fluor were added to the original vial, shaken and carefully decanted into the LSC vial. This was repeated twice in order to completely extract the compound. Repeated extraction (for testing purposes) showed that no radioactivity remained in the exoskeletons after this procedure.

To extract sorbed pesticide from the samples of leaf material, 10 mL of Ecoscint A scintillation cocktail were added to the leaf material. Then the samples were placed in the waterbath at 60°C for 24 hours. After cooling, the scintillation cocktail was decanted into LSC vials. The remaining leaf skeletons were rinsed twice with ca. 3
mL Ecoscint A and all scintillation cocktail added to the LSC vials. Repeated extraction (for testing purposes) showed that no radioactivity remained on the leaf skeletons after this procedure.

The *Gammarus pulex* and leaf samples were analysed by liquid scintillation counting as described above. The LOD for an average sized *Gammarus pulex* in each experiment was 3.2 and 44 ng/g wet weight for CPF and PCP, respectively. The LOQ was 6.4 and 90 ng/g wet weight for CPF and PCP, respectively.

**Data analysis**

Average concentrations and standard errors of the seven replicates were calculated for each sampling time. Uptake and elimination rate constants were estimated using ModelMaker version 4 (AP Benson, Wallingford, UK). Accumulation of the compounds according to first-order kinetics was described with a one-compartment single first-order model:

\[
\frac{dC_{\text{int}}}{dt} = k_{\text{in}} \times C - k_{\text{out}} \times C_{\text{int}}
\]  

(3-1)

where *t* is the time [Time], *C*~\text{int}~ is the internal concentration [Amount × Mass\(^{-1}\)], *C* the concentration in the water [Amount × Volume\(^{-1}\)] and *k*\(_{\text{in}}\) and *k*\(_{\text{out}}\) the uptake rate constant [Volume × Mass\(^{-1}\) × Time\(^{-1}\)] and the elimination rate constant [Time\(^{-1}\)], respectively. The bioconcentration factor (BCF) at steady state is defined as \(k_{\text{in}} / k_{\text{out}}\). Using the one-compartment single-first order model (equation 3-1) for the toxicokinetics assumes that the internal concentration for the whole body is a good approximation of the concentration at the target site. The flux into the organism is proportional to the concentration in the water and the flux out of the organism is proportional to the concentration in the organism. The reader is referred to Kooijman and Bedaux [21], Landrum et al. [22] or Gobas and Morrison [23] for further discussion of the one-compartment single first-order model.

Equation (3-1) was fitted to the measured internal concentration using the measured concentration of the compounds in water as the driving variable. Uptake and elimination were fitted simultaneously using the Levenberg-Marquardt algorithm in
ModelMaker together with simulated annealing. The minimised function was the weighted residual sum of squares (wRSS) defined as [24]:

$$wRSS = \sum \frac{(M_i - O_i)^2}{\varepsilon_i^2} \quad (3-2)$$

where $O_i$ are the observed internal concentrations and $M_i$ the corresponding model values calculated with equation (3-1). The standard errors of the observed values were used as error estimates $\varepsilon$. ModelMaker integrates equation (3-1) numerically, so it can accommodate fluctuating concentrations in the water phase.

Starting values for the parameters $k_{in}$ and $k_{out}$, as required by the fitting algorithm, can be estimated with quantitative structure activity relationships (QSARs, see Table 3-1). Hendriks [25] gives an estimation method for $k_{out}$. The parameter $k_{in}$ can then be calculated as [26]:

$$k_{in} = \text{l lipid content} \times K_{ow} \times k_{out}. \quad (3-3)$$

### Table 3-1. Starting values for the parameters of the one-compartment single first-order toxicokinetic model for pentachlorophenol (PCP) and chlorpyrifos (CPF) in *Gammarus pulex* as estimated by QSARs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>PCP</th>
<th>CPF</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>log Kow</td>
<td>-</td>
<td>2.75 a)</td>
<td>4.7 b)</td>
<td>log Kow of PCP at pH 8.9</td>
</tr>
<tr>
<td>$k_{out, QSAR}$ c)</td>
<td>day$^{-1}$</td>
<td>22.7</td>
<td>0.57</td>
<td>with 1.34% lipid content and 13.04 mg average <em>Gammarus pulex</em> weight for CPF and 18.55 mg for PCP</td>
</tr>
<tr>
<td>$k_{in, QSAR}$ d)</td>
<td>L × kg$^{-1}$ × day$^{-1}$</td>
<td>171</td>
<td>385</td>
<td>equation (3-3)</td>
</tr>
<tr>
<td>BCF$_{QSAR}$</td>
<td>L × kg$^{-1}$</td>
<td>7.5</td>
<td>672</td>
<td>BCF = $k_{in} / k_{out}$</td>
</tr>
</tbody>
</table>

a) Ref. [19]  
b) Ref. [27]  
c) Ref. [25]  
d) Ref. [26]
Results

The two unknown parameters of the model, the uptake rate constant $k_{in}$ and the elimination rate constant $k_{out}$ were successfully determined in both experiments. Both fitting procedures were stable and led to unique parameter combinations. Variations in the parameter estimates resulting from different starting values (for testing purposes) were much smaller than the standard error of the parameter estimates. Using the estimates from the QSARs as starting values is preferred because it reduces the potential that unrealistic parameters are returned and makes the parameter estimation reproducible.

Figure 3-1. Uptake and elimination of chlorpyrifos in *Gammarus pulex*.

Mean (×) chlorpyrifos concentration (± 1 SE) in water (top), in *Gammarus pulex* (bottom) and the amount sorbed to leaf material (∅, bottom) over time. The solid line shows the fitted model (eq. 3-1) with $k_{in} = 747$ L kg$^{-1}$ day$^{-1}$ and $k_{out} = 0.45$ day$^{-1}$. 
Chlorpyrifos is volatile and the resulting decline in aqueous concentrations during the exposure phase (Figure 3-1) results in the concentration of chlorpyrifos in the *Gammarus pulex* reaching steady state faster than predicted for constant exposure conditions. Chlorpyrifos eliminated from the *Gammarus pulex* into the water phase is detected at day 6. The decline in the exposure phase does not hamper the parameter estimation as all the concentrations used for parameter estimation were measured apart from the initial concentration. The initial concentration was erroneously measured before the test solution in the beaker was well mixed. Thus the initial concentration was taken from another experiment that was carried out without feeding the *Gammarus pulex*. This second experiment failed due to cannibalism amongst the *Gammarus pulex* and is not reported.

![Figure 3-1. Uptake and elimination of pentachlorophenol in *Gammarus pulex*.](image1)

Mean (×) pentachlorophenol concentration (± 1 SE) in water (top) and in *Gammarus pulex* (bottom) and the amount sorbed to leaf material (○, bottom) over time. The solid line shows the fitted model (eq. 3-1) with $k_{in} = 89$ L kg$^{-1}$ day$^{-1}$ and $k_{out} = 1.76$ day$^{-1}$.
The uptake rate constant $k_{in}$ for chlorpyrifos was $747 \pm 61 \text{ L kg}^{-1} \text{ day}^{-1}$ (value for best fit ± SE) and the elimination rate constant $k_{out}$ for chlorpyrifos was $0.45 \pm 0.05 \text{ day}^{-1}$. The bioconcentration factor (BCF) was $1660 \text{ L kg}^{-1}$. The resulting fit of the model to the data is shown in Figure 3-1 and the indicators for the quality of fit are shown in Table 3-3.

The measured concentrations of pentachlorophenol in water (Figure 3-2) show only a small decrease over time. The uptake rate constant $k_{in}$ for pentachlorophenol was $89 \pm 7 \text{ L kg}^{-1} \text{ day}^{-1}$ (value for best fit ± SE) and the elimination rate constant $k_{out}$ was $1.76 \pm 0.14 \text{ day}^{-1}$. The resulting bioconcentration factor BCF is $51 \text{ L kg}^{-1}$. Steady state in the internal concentration is reached around day two, even though the measured data shows some variability at day two. The resulting fit of the model to the data is shown in Figure 3-2 and the indicators for the quality of the fit are shown in Table 3-3.

Table 3-2. Measured values for the parameters of the one-compartment single first-order toxicokinetic model for pentachlorophenol (PCP) and chlorpyrifos (CPF) in *Gammarus pulex*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>PCP</th>
<th>CPF</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{out}$ ± SE</td>
<td>day$^{-1}$</td>
<td>1.76 ± 0.14</td>
<td>0.45 ± 0.05</td>
<td>measured in this study</td>
</tr>
<tr>
<td>$k_{in}$ ± SE</td>
<td>L kg$^{-1}$ day$^{-1}$</td>
<td>89 ± 7</td>
<td>747 ± 61</td>
<td>measured in this study</td>
</tr>
<tr>
<td>$t_{95}$</td>
<td>days</td>
<td>1.7</td>
<td>6.7</td>
<td>time until 95% of steady state is achieved$^b$</td>
</tr>
<tr>
<td>BCF</td>
<td>L kg$^{-1}$</td>
<td>51</td>
<td>1660</td>
<td>this study</td>
</tr>
<tr>
<td>lipid weight</td>
<td>L kg$^{-1}$</td>
<td>3806</td>
<td>123880</td>
<td>this study, lipid content of wet weight</td>
</tr>
<tr>
<td>based BCF</td>
<td></td>
<td></td>
<td></td>
<td><em>Gammarus pulex</em> = 1.34%</td>
</tr>
</tbody>
</table>

These parameter values result from the best fit of eq. (3-1) to the observed internal concentrations. The BCF and $t_{95}$ are further characteristics of the toxicokinetics of PCP and CPF in *Gammarus pulex*.

a) SE: Standard error of mean

b) $t_{95} = \frac{-\ln 0.05}{k_{out}}$, under the assumption of constant exposure concentration
Both compounds accumulated in *Gammarus pulex* (Figures 3-1 and 3-2, Table 3-2). Pentachlorophenol reached steady state within the duration of both the uptake and elimination phases. Each phase lasted three days whereas the time to 95% of steady state for PCP (as calculated with the one-compartment single-first order model) was only 1.7 days (Table 3-2). With $k_{out} = 1.76$ the fraction of steady state achieved after 72 hours is 99%, calculated as: 

$$\text{fraction}_{\text{steady state}} = 1 - e^{-k_{out} \times \text{time}}.$$  

Although reaching steady state is not a requirement for the parameter estimation, it is one indicator that the one-compartment single-first order model is indeed an appropriate description of the toxicokinetics of PCP in *Gammarus pulex*. Chlorpyrifos approached steady state in the uptake phase, but only because the exposure concentration decreased (Figure 3-1). From the elimination phase and the time to 95% of steady state of 6.7 days it is apparent that steady state would not be reached within the three days of the experiment under constant exposure conditions.

**Discussion**

The QSAR over-predicts the elimination rates and under-predicts the BCFs for both compounds when compared to the measured values (Tables 3-1 and 3-2). The estimated uptake rate for PCP is larger than the measured value, whereas the reverse is true for CPF. The differences between the QSAR predictions and our measured values are within the uncertainty that has to be expected for QSAR predictions, because QSARs are derived from data of a broad range of compounds and species.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCP</th>
<th>CPF</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>wRSS $^a$</td>
<td>22.3</td>
<td>7.5</td>
<td>remaining wRSS</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>11</td>
<td>number of datapoints</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.81</td>
<td>0.93</td>
<td>fit of eq. (3-1) to data</td>
</tr>
</tbody>
</table>

$^a$ The wRSS is the weighted residual sum of squares (equation 3-2).
The different bioconcentration factors of PCP and CPF reflect the difference in hydrophobicity between the two compounds. The Kow of CPF is 89 times that of PCP (Table 3-1) and the BCF of CPF is 33 times that of PCP (Table 3-2). The concentrations of both compounds were higher in the Gammarus pulex than in the leaf material (Figures 3-1 and 3-2). Analysis of leaf material also shows that the higher concentration on the leaf material (second sampling date) did not exceed the early concentration by more than a factor of 1.54 in the CPF experiment and 1.75 in the PCP experiment. Distortion of the uptake process by sorption of the compounds to the leaf material was assumed to be negligible in our experiments.

The measured uptake and elimination rate constants for PCP in Gammarus pulex are of the same order of magnitude as those reported for PCP in other aquatic organisms. Nuutinen et al. [5] reported an uptake rate constant of $617 \pm 70$ L kg$^{-1}$ day$^{-1}$ (value ± standard deviation) and a (total compound) elimination rate constant of $1.63 \pm 0.29$ day$^{-1}$ (value ± standard deviation) for PCP in Hyalella azteca (BCF = 378 L kg$^{-1}$). Landrum et al. [28] measured uptake and elimination rate constants of $74 \pm 22$ L kg$^{-1}$ day$^{-1}$ (value ± SE) and $0.079 \pm 0.034$ day$^{-1}$ (value ± SE) respectively in Pontoporeia hoyi (BCF = 939 L kg$^{-1}$) and $6 \pm 4$ L kg$^{-1}$ day$^{-1}$ (value ± SE) and $0.047 \pm 0.015$ day$^{-1}$ (value ± SE) respectively in Mysis relicta (BCF = 128 L kg$^{-1}$). Our measured bioconcentration factor of 51 L kg$^{-1}$ is somewhat smaller, but this could be due to the relatively high pH in our exposure solution and the low lipid content of our Gammarus pulex. The Kow of PCP is pH dependent and can be much larger at pH values lower than those in our study [19]. Thus a lower pH would result in larger bioconcentration factors.

The chlorpyrifos experiment demonstrates that toxicokinetic parameters can be inferred for compounds that show a decline in concentration in the water phase, e.g. due to volatilization. Our measured BCF for chlorpyrifos in Gammarus pulex (1660 L kg$^{-1}$) is very close to the wet weight based BCF for Asellus aquaticus (1715 L kg$^{-1}$ [29]), but our lipid weight based BCF ($1.2 \times 10^5$ L kg$^{-1}$) is lower than that for Asellus aquaticus ($2.5 \times 10^5$ L kg$^{-1}$). Our BCF is higher than the BCF of $400 \pm 119$ L kg$^{-1}$.
(value ± standard deviation) for chlorpyrifos in the mussel *Mytilus galloprovincialis* measured by Serrano et al. [30].

There are three possible shortcomings in our method: i) any metabolites were not identified; ii) only one concentration level was tested for each compound; and iii) it is not clear how much the uptake and elimination behaviour of these compounds would differ under field conditions. Since we measured radioactivity, we have also detected any metabolites present in the organism. Only metabolites that are eliminated much slower than the parent compound would lead to a large error in our measured internal concentrations of the parent compounds. Thus, this issue seems of minor relevance for CPF and PCP in *Gammarus pulex*. It is known that uptake or elimination rates might vary with dosed concentration for some organisms, including chlorpyrifos in guppyfish [31]. Furthermore, the toxicokinetics might change when the organism is affected by the toxicity of the compounds, especially at larger concentrations, but the tested concentrations in our experiments were already close to the toxic range of concentrations for CPF and PCP in *Gammarus pulex*. One aspect of the uncertainty resulting from the extrapolation of laboratory results to toxicokinetics in the field can be estimated by an assessment of the seasonal variation in lipid content.

<table>
<thead>
<tr>
<th>Lipid content</th>
<th>BCF for PCP [L kg⁻¹]</th>
<th>BCF for CPF [L kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum a</td>
<td>0.84</td>
<td>32</td>
</tr>
<tr>
<td>Measured in this experiment</td>
<td>1.34</td>
<td>51</td>
</tr>
<tr>
<td>Maximum for males a</td>
<td>1.97</td>
<td>75</td>
</tr>
<tr>
<td>Maximum for females a</td>
<td>3.76</td>
<td>143</td>
</tr>
</tbody>
</table>

*a The minimum and maximum lipid contents were calculated from [17] with a dry weight to wet weight ratio of 0.21.*

The lipid content of *Gammarus pulex* varies seasonally [17] and it has to be assumed that uptake and elimination rates vary seasonally as well. If we assume that the BCF is directly proportional to the lipid content, we can derive an adjustment-factor for the
BCF by using the minimum and maximum lipid contents from reference [17]. The possible variation of the BCF is then readily calculated (Table 3-4). This variation in the BCF due to variation in lipid content (factor 4.5 between minimum and maximum for females) needs to be considered when making predictions for risk assessment. Additionally, variation in animal physiology, different growth stages, food availability and general activity can lead to further variation in uptake and elimination rates. Considering these unavoidable sources of uncertainty when extrapolating from laboratory measurements to field situations, we are confident that our measured uptake and elimination rates are sufficiently precise and robust to be used in aquatic risk assessment.

The method presented in this paper is suitable to determine the uptake and elimination rate constants for small aquatic invertebrates. Although the exoskeleton of Gammarus pulex does not completely dissolve in tissue solubiliser, the method extracts all of the radioactivity and thus the compound present in the organism. It is possible to describe and predict the time-varying concentration of chlorpyrifos and pentachlorophenol in Gammarus pulex using the one-compartment single first-order model. The model agrees with the observed data and the typical steady state plateau of the one-compartment single first-order model was observed in the pentachlorophenol experiment. The chlorpyrifos experiment was too short to reach steady state, in agreement with the model prediction of ca. 7 days to steady state for this compound. The parameter estimation process is stable and straightforward. Bioconcentration factors at steady state (or any point in time) can be readily calculated from the two parameters $k_{in}$ and $k_{out}$. 
References


Chapter 3


(21) Kooijman SALM, Bedaux JJM (1996) The analysis of aquatic toxicity data. VU University Press, Amsterdam, Netherlands


4. A new ecotoxicological model to simulate survival of aquatic invertebrates after exposure to fluctuating and sequential pulses of pesticides

Introduction

Pesticides have the potential to cause effects on non-target organisms because they are broadly applied within the open environment. They may reach water bodies via various pathways and typically aquatic organisms are exposed to sequential pulses with fluctuating concentrations [1]. Standard toxicity tests are performed at constant concentrations and fixed durations, so any extrapolation to more realistic patterns of exposure must rely on modeling. We need a theoretically sound framework for this fundamental problem in environmental risk assessment and appropriate mathematical models to relate fluctuating field exposures to laboratory effects data [2, 3]. Generally there are three steps involved. After selecting a model that simulates effects based on the time course of the contaminant concentration, the model parameters are estimated by calibration on experimental data and the model performance is evaluated against independent experimental data.

Modeling has the advantage that we can extrapolate to a wide range of field exposure scenarios. A variety of approaches have been developed [1, 4-6] and the importance of recovery periods between successive pulses has been recognized [7], even for compounds such as chlorpyrifos that have slow recovery [8-10].

We evaluated and compared the theoretical base of the available models and found that currently there is no generally applicable and validated method to link sequential or fluctuating exposure to effects [chapter 2]. Nevertheless, modeling is potentially a very powerful tool [11, 12] and two approaches were most suitable after some modifications [chapter 2]: one originates from the Damage Assessment Model [13] and the other from the DEBtox concept [11]. In this study we combine these approaches to form the Threshold Damage Model (TDM). This is a new, process-

based model and we use it to simulate the survival of an aquatic invertebrate 
(Gammarus pulex) after fluctuating and sequential pulsed exposure to two pesticides 
with contrasting modes of action (chlorpyrifos and pentachlorophenol).

The TDM is parameterized with previously measured uptake and elimination rate 
parameters [chapter 3] and data from pulsed toxicity tests with three different pulse 
patterns (experiments A and C). We compare the TDM with two simpler models 
based on time-weighted averages (TWA), one calibrated on pulsed toxicity tests 
(experiments A and C) and the other on LC50 data (lethal concentration for 50% of 
the population from standard toxicity tests). The comparison is based on the 
goodness-of-fit of the simulations, the predictive power in independent experiments 
(experiments B and D) and a critical evaluation of the model structures in the context 
of ecotoxicological processes.

Materials and Methods
Organisms and exposure water
Gammarus pulex (mixture of males and females, length ca. 5-10 mm), were collected 
from a small stream, Bishop Wilton Beck, ca. 20 km northeast of York, UK. 
Streamwater was also collected and stored at 5°C. Prior to experiments, organisms 
were kept for three to seven days in aerated streamwater under the same conditions as 
in the experiments and were fed in excess with re-hydrated horse-chestnut leaves.

Experiments
The four experiments are denoted A, B, C and D (Figures 4-1 to 4-4). The Gammarus 
were treated using different exposure patterns of either chlorpyrifos (CPF, A and B) 
or pentachlorophenol (PCP, C and D) and we observed survival over time.

All experiments were carried out under static conditions in 600-mL pyrex beakers 
filled with 500 mL exposure solution. Each experiment consisted of three treatments 
and one control group with five beakers each. Every beaker contained 10 Gammarus 
at the start of the experiments and the numbers of living Gammarus were counted 
daily. In additional experiments we used the same test system to obtain 48hr-LC50
values (concentration at which 50% of test organisms die within 48 hours) under constant exposure to chlorpyrifos and pentachlorophenol. More experimental details can be found in the supporting information.

**Modeling**

*The Threshold Damage Model (TDM).* The relationship between exposure duration, exposure concentration and toxic effect is specific for each combination of species and compound. Differences are attributed to two groups of processes: the toxicokinetics and the toxicodynamics [12, 14] that are explicitly reflected in the process-based model that we apply to link exposure with survival. Differential equations are used to simulate the survival over time in response to the changing exposure concentration as a driving variable. The rationale and benefits behind this approach are detailed in chapter 2, where we identified the modified Damage Assessment Model and the Threshold Hazard Model as the most suitable currently available models. These two models were based on the Damage Assessment Model [13] and the DEBtox model [11] and we now merge them into the Threshold Damage Model. Equations (4-1) to (4-4) constitute the Threshold Damage Model.

Equation (4-1) is the one-compartment first-order kinetics model, which simulates the toxicokinetics i.e. the time course of the internal concentration in relation to the concentration in the water phase surrounding the organism. Internal concentration acts as a replacement for the concentration at the target site. The times to steady state and complete elimination of the compound are driven by the elimination rate constant.

\[
\frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} \times C(t) - k_{\text{out}} \times C_{\text{int}}(t)
\]

where \(C_{\text{int}}\) is the internal concentration [Amount \(\times\) Mass\(^{-1}\)], \(C\) the concentration in the water [Amount \(\times\) Volume\(^{-1}\)] and \(k_{\text{in}}\) and \(k_{\text{out}}\) the uptake rate constant [Volume \(\times\) Mass\(^{-1}\) \(\times\) Time\(^{-1}\)] and the elimination rate constant [Time\(^{-1}\)], respectively.
Equation (4-2) simulates the first part of the toxicodynamics as an accrual and, in the second term of equation (4-2), the recovery or repair of damage:

\[ \frac{dD(t)}{dt} = k_k \times C_{in}(t) - k_r \times D(t) \]  \hspace{1cm} (4-2)

where \( k_k \) is a killing rate constant \([\text{Mass} \times \text{Amount}^{-1} \times \text{Time}^{-1}]\), \( k_r \) is the rate constant for damage recovery or repair \([\text{Time}^{-1}]\) and \( D(t) \) is damage \([-\]]. The differential of \( H(t) \), as used in equation (4-3) is the hazard rate, which is the probability of the organisms dying at a given time. In equation (4-3) the hazard rate rises above zero when a threshold for damage is exceeded:

\[ \frac{dH(t)}{dt} = \max[D(t) - \text{threshold},0] \]  \hspace{1cm} (4-3)

where \text{threshold} is a dimensionless threshold parameter \([-\]].

The killing rate constant is a combined parameter, describing both damage accrual and the proportionality factor to the hazard rate and reflecting the toxic potency of the compound. The recovery rate parameter lumps all processes leading to recovery, such as repair mechanisms on a cellular scale (e.g. reestablishment of a proton gradient along a membrane after uncoupling, reactivation of blocked enzymes or synthesis of new enzyme) or adaption of the physiology and other compensating processes. The toxicodynamic parameters in equations (4-2) and (4-3), and thus the speed of recovery, depend on the mode of action of the compound.

Only if a certain level of internal damage is exceeded will the whole organism respond with increased mortality, i.e. the hazard rate will increase. This threshold results from the change in scales: equation (4-2) describes processes on the scale of cells or sites of action, whereas the survival probability in equation (4-4) only has meaning at the scale of the whole organism.

In equation (4-4) we use the standard approach of linking hazard to survival:

\[ S(t) = e^{-H(t)} \times S_{\text{background}}(t) \]  \hspace{1cm} (4-4)

where \( S(t) \) is the survival probability \([-\] \( (\text{probability of an organism surviving until time } t) \) and \( S_{\text{background}}(t) \) is the survival probability resulting purely from the background (or control) mortality \([-\].

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Chapter 4

**Calibration of the Threshold Damage Model (TDM).** Averages of surviving *Gammarus* were calculated for each sampling time in each treatment. These, when expressed as the fraction surviving, represent the observed survival probability. The toxicodynamic parameters of the TDM $k_k$, $k_c$, and *threshold* were then determined by fitting the whole model to the observed survival in experiment A for chlorpyrifos and experiment C for pentachlorophenol (Table 4-1).

Equations (4-1) to (4-4) were fitted simultaneously to all three treatments using least squares optimization based on the Levenberg-Marquardt algorithm in ModelMaker version 4 (AP Benson, Wallingford, UK). The measured concentrations of the respective compound in the exposure solutions were the driving variable. The parameters $k_{in}$ and $k_{out}$ were taken from [chapter 3]. The ModelMaker 4 parameter settings are listed in the supporting information.

Before fitting the TDM, we accounted for background mortality by fitting equation (4-5) to the survival data of the control treatment in each experiment, thus correcting for changes in the background mortality between experiments.

$$S_{\text{background}}(t) = e^{-h_b \times t}$$  \hspace{1cm} (4-5)

where $h_b$ is the background hazard rate [Time$^{-1}$] and $t$ is time [Time].

This parameter estimation method requires external knowledge to exclude unrealistic parameter values and parameter values depend on the experiments selected for the fit. Therefore we also provide the parameter set that results from fitting the TDM to all available data, i.e. experiments A and B for CPF and C and D for PCP (Table 4-1, Figures in Appendix D). The result is a more robust fit and these parameters should be used for further simulations. Numbers in brackets {} are calculated with the parameter set based on all available data.
The Time-Weighted Averages (TWA) models. We compared the performance of the TDM with a modified time-weighted averages approach. When time-weighted average (TWA) concentrations are used to deal with fluctuating exposures, the exposure concentration is integrated over time and then divided by the duration of the corresponding toxicity test. This method is inherently based on Haber’s law and can be modified to simulate the survival over time if we assume a linear relationship between dose and effect [chapter 2]. The survival probability is then calculated as:

\[ S(t) = 1 - f_{\text{TWA}} \times \int_{0}^{t} C(t) \, dt \]  \hspace{1cm} (4-6)

where \( f_{\text{TWA}} \) is a scaling factor [Volume \times Mass^{-1} \times Time^{-1}] and \( C \) is the concentration in the exposure solution [Mass \times Volume^{-1}].

Calibration of the Time Weighted Averages (TWA) models. There are two ways of deriving the scaling factor \( f_{\text{TWA}} \), both tested here: (i) equation (4-6) can be fitted simultaneously to the survival data of experiments A for chlorpyrifos and C for pentachlorophenol (while accounting for background mortality as in the TDM); the best fit then yields the value of \( f_{\text{TWA}} \) (TWA pulses model); or (ii) \( f_{\text{TWA}} \) can be calculated from standard toxicity test data such as 48hr-LC50 values in a straightforward manner as follows (TWA LC50 model):

\[ f_{\text{TWA}} = \frac{\% \text{mortality in test}}{C_{\text{test}} \times t_{\text{test}} \times 100\%} \]  \hspace{1cm} (4-7)

where \( C_{\text{test}} \) and \( t_{\text{test}} \) are the concentration and duration in a standard toxicity test at constant exposure concentration.

Predictive simulations for model evaluation. After calibration the survival of Gammarus in experiments B and D was simulated with all three models (Figures 4-2 and 4-4). These simulations were driven only by the measured concentrations in the exposure solution (and adjusted for background mortality).
Results and Discussion

Estimated parameters of the TDM

The parameters of the TDM could be successfully estimated for both compounds (Tables 4-1 and 4-2). Plots of residuals, correlation and intermediate steps can be found in Appendix D. The recovery rate constant can be used to calculate the time until the organisms have recovered from the internal damage. If we simplify the toxicodynamics to a one-compartment first-order system by assuming that elimination is completed before recovery starts, then we can calculate the time for 95% recovery of damage \( t_{95} \) as follows:

\[
  t_{95} = \frac{1}{k_r} \ln(1 - 0.95)
\]

The 95% recovery times are then 21 days \( \{18 \text{ based on all available data}\} \) for chlorpyrifos and 0.09\( \{0.05\} \) days for pentachlorophenol. Times to 50% recovery are 5\( \{4\} \) and 0.02\( \{0.01\} \) days, respectively. The difference reflects the mode of action and has practical value for assessing the risk from sequential exposure events. Although it is known that certain levels of internal damage have to be exceeded before an effect is seen at the organism scale (e.g. enzyme inhibition; \[15, 16\]) we cannot relate the threshold parameter directly to any measured level of damage.

We chose PCP and CPF for their contrasting mode of actions. PCP uncouples oxidative phosphorylation by disrupting the proton gradient along membranes \[17\]. The subsequently accelerated heat output and waste of energy is the actual damage to the organism and has been modeled with a threshold approach for aquatic invertebrates \[18\]. The proton gradient is reestablished once PCP is eliminated from the membranes. Thus the fast recovery described by our recovery rate constant is plausible.

CPF inhibits the enzyme acetylcholinesterase (AChE). The inhibited enzyme can either be reactivated by dephosphorylation or undergo irreversible ageing \[19\]. Half-lives for diethylphosphorylated enzymes (as is the case with CPF) are typically between 50 and 100 days \[19\] but are generally dependent on the initial inhibition, the compound and the species \[15, 20\]. Overall recovery of AChE activity in the
organism is faster, e.g. within 14 days for fathead minnows [20], because of synthesis of new AChE [20]. Hence our time to recovery of 21{18} days again fits with the literature. The level of inhibited AChE required for effects at the organism scale varies (50-95%, [16]; >70% for mortality, [15]), but generally confirms the concept of a threshold for CPF toxicity to aquatic invertebrates [10].

The TDM worked equally well with fast (PCP) and slow recovery (CPF). We hypothesize that compounds with the same mode of action will have similar recovery rate constants, but the killing rate constant should vary according to the toxic potencies of the compounds. The threshold parameter should take similar values for compounds that target the same system in the organism (e.g. inhibition of AChE).

Further experiments with compounds of the same and different modes of action are necessary to test the above hypothesis, but if confirmed it can be used to estimate parameter values for new compounds with known modes of action.

Our method of estimating the toxicodynamic parameters from mortality is an inverse modeling approach; hence it depends on an appropriate model structure as well as low variability in the data. The latter could be improved by using more standardized tests (i.e. lab-cultured organisms, artificial water) and the former has to be critically examined when different organisms are used.
Table 4-1. Model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Chlorpyrifos&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pentachlorophenol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Units&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake rate constant</td>
<td>( k_{in} )</td>
<td>747 ± 61</td>
<td>89 ± 7</td>
<td>L × kg&lt;sup&gt;-1&lt;/sup&gt; × day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Elimination rate constant</td>
<td>( k_{out} )</td>
<td>0.45 ± 0.05</td>
<td>1.76 ± 0.14</td>
<td>day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Killing rate constant</td>
<td>( k_k )</td>
<td>0.27 ± 0.05</td>
<td>0.061 ± 0.08</td>
<td>g&lt;sub&gt;wet.w.&lt;/sub&gt; × day&lt;sup&gt;-1&lt;/sup&gt; × µg&lt;sub&gt;a.i.&lt;/sub&gt;&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recovery rate constant</td>
<td>( k_r )</td>
<td>0.14 ± 0.04</td>
<td>34 ± 42</td>
<td>day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Threshold</td>
<td>threshold</td>
<td>0.074 ± 0.015</td>
<td>0.21 ± 0.06</td>
<td>-</td>
</tr>
<tr>
<td>Scaling factor f_TWA pulses</td>
<td>( f_{TWA} ) pulses</td>
<td>393 ± 15</td>
<td>0.0208 ± 0.0009</td>
<td>L × mg&lt;sup&gt;-1&lt;/sup&gt; × day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Scaling factor LC50</td>
<td>( f_{TWA} ) LC50</td>
<td>74</td>
<td>0.045</td>
<td>L × mg&lt;sup&gt;-1&lt;/sup&gt; × day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Parameter value ± standard error from fit

<sup>b</sup> From chapter 3

<sup>c</sup> Parameter set for model testing (fit to experiment A for CPF and experiment C for PCP), parameter set from fit to all experiments (A and B for CPF, C and D for PCP) in brackets {}

<sup>d</sup> Scaling factor LC50 was calculated directly from 48hr-LC50s (CPF: 3.4 µg/L, PCP: 5.6 mg/L)

<sup>e</sup> g<sub>wet.w.</sub> is g wet weight and µg<sub>a.i.</sub> is µg of active ingredient
Figure 4-1. Chlorpyrifos concentrations and survival of *Gammarus pulex* in experiment A.

The survival plot shows the observed survival (○) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model predicting survival on the basis of the 48hr-LC50 (dotted line). The fit of the TDM and the TWA pulses model to this experiment result in the parameter set that we use for model evaluation in case of chlorpyrifos.
Figure 4-2. Chlorpyrifos concentrations and survival of *Gammarus pulex* in experiment B.

The survival plot shows the observed survival (O) and its standard error (n=5), the threshold damage model (solid line), the time-weighted averages model (dashed line) and the time-weighted averages model simulating survival on the basis of the 48hr-LC50 (dotted line). The exposure pattern in treatment 1 consists of pulses of six hours duration, the pulses in treatment 2 last four days and treatment 3 consists of very low, fluctuating concentrations. These simulations are run with the parameter set that we use for model evaluation.
Figure 4-3. Pentachlorophenol concentrations and survival of *Gammarus pulex* in experiment C.

The survival plot shows the observed survival (○) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model predicting survival on the basis of the 48hr-LC50 (dotted line). The fit of the TDM and the TWA pulses model to this experiment only result in the parameter set that we use for model evaluation in case of pentachlorophenol.
Figure 4-4. Pentachlorophenol concentrations and survival of *Gammarus pulex* in experiment D.

The survival plot shows the observed survival (○) and its standard error (n=5), the threshold damage model (solid line), the time-weighted averages model (dashed line) and the time-weighted averages model simulating survival on the basis of the 48hr-LC50 (dotted line). These simulations are run with the parameter set that we use for model evaluation.
Observed mortality from sequential pulses and fluctuating concentrations

The results of experiments A – D are shown in Figures 4-1 to 4-4. Control mortalities were 20, 26, 8 and 4% at the end of experiments A, B, C and D respectively. We compare the mortality caused by the first pulse (including recovery period, same durations in comparisons) with that of the second pulse for treatments 1, 2 and 3 of experiment A and treatments 1 and 2 of experiment B (all CPF). The mortalities resulting from the first vs. second pulse are (first pulses from treatments A1, A2 and A3 pooled): 16% vs. 33% (p=0.039), 16% vs. 51% (p<0.001), 16% vs. 53% (p<0.001), 10% vs. 9% (p=0.854), 10% vs. 27% (p=0.034) for treatments A1, A2, A3, B1 and B2 respectively. Only treatment B1 does not show a significant difference (binomial test for proportions, pooled replicates, α=0.05) between the mortalities from first and second pulses, but the combined mortality of the first and second pulse in B1 is 18% compared to 56% mortality from the third and fourth pulses combined (p<0.001). Thus all treatments show a significantly higher mortality from second or subsequent pulses of CPF. In three of these (A2, A3 and B1), the result cannot be caused by a build up of internal chlorpyrifos concentrations because there has been sufficient time for elimination between the pulses (95% of CPF is eliminated after 6.7 days; [chapter 3]). Rather internal damage, in this case inhibition of AChE, lasts even after CPF has been eliminated and damage caused by subsequent pulses leads to greater exceedance of an internal threshold, even for pulses as far apart as 14 days (treatment A3).

Treatment B3 shows no mortality within the first seven days and there is an apparent increase in mortality through time. This is further indication that chlorpyrifos-induced mortality shows up only after a threshold is exceeded. The TDM simulates that the threshold is exceeded on day 18{11} and the TDM simulation is in excellent agreement with the observed mortality in this treatment (mean error = 6% {3%}, \( r^2 = 0.97 \{0.98\} \)). Unfortunately the variability in the data prohibits a statistically sound calculation of the “hinge point” in the observed mortality data.
The comparison of the first and second pulse can only be made for two treatments (D1 and D2) in the case of PCP. No increase in toxicity from the second pulse is observed.

**Calculation of standard toxicity data with the TDM and TWA pulses models.** Besides being able to simulate survival from realistic exposure patterns, we can also use the TDM and the TWA pulses model to calculate standard toxicity data for any effect level and any test duration. Comparison of the measured 48hr-LC50s (CPF: 3.4 µg/L, PCP: 5.6 mg/L) with the modeled 48hr-LC50s (TWA pulses: 0.64 {0.78} µg/L for CPF and 12.0 {12.1} mg/L for PCP; TDM: 4.0 {7.4} µg/L for CPF and 8.4 {11.4} mg/L for PCP) shows that the values calculated with the TDM are closer to the measured values than those of the TWA pulses model.

**Comparison of the three models**

**Goodness-of-fit and simulations.** Both, the TDM and the TWA pulses model simulate the experiments much better than the TWA LC50 model. The statistics (Table 4-2) show that the TDM and the TWA pulses model explain a large part of the variation in the data (r² values between 0.77 {0.86} and 0.96 {0.96}) and the mean errors of 15% {10%} or lower mean that both models agree well with the observations (maximum error 36% {26%}). The statistical indicators do not differentiate sufficiently to support a preference for either the TDM or the TWA pulses model.

The TWA LC50 model severely underestimates mortality in the CPF experiments and overestimates mortality in the PCP experiments. For example, the model simulates 100% mortality for treatment D3 at the end of the 20-day experiment whereas in reality there is only 38% mortality. The large mean errors and the maximum error of 62% underline the inferiority of this approach.
Comparison of model structures. The weak performance of the TWA LC50 model is not surprising as the short 48hr – LC50 test does not capture either the build up of damage for CPF or the recovery between pulses of PCP. The TWA LC50 is in essence a short- to long-term extrapolation and as such struggles with all processes that change over time. A major shortcoming of both TWA methods is that they will always yield 100% mortality for sufficiently long exposure durations even at very low concentrations. Furthermore, the underlying assumption of a linear relationship between dose and response is a crude simplification. Based on its theoretical shortcomings and its weak performance in our tests the TWA LC50 method is not recommended. These theoretical shortcomings also hold for the TWA pulses model although there is a much better agreement with observed survival than for the TWA LC50 model. The TWA pulses model has practical advantages compared to the TDM. The TWA pulses model is simple and thus easily understood and it requires less data than the TDM because it does not need uptake and elimination rate constants. The latter is also a conceptual disadvantage of the TWA pulses model, because omission of process knowledge such as uptake and elimination or toxicodynamics limits our confidence in this model when we extrapolate to different scenarios. Models of more empirical character, such as the TWA pulses model, are strictly only valid within the experimental boundaries that they are calibrated on.
### Table 4-2. Indicators of model performance.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean and maximum errors [%] a</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TDM</td>
<td>TWA pulse</td>
</tr>
<tr>
<td>A: model evaluation</td>
<td>8 (18)</td>
<td>8 (18)</td>
</tr>
<tr>
<td>A: fit to all data</td>
<td>10 (23)</td>
<td>9 (26)</td>
</tr>
<tr>
<td>B: model evaluation</td>
<td>12 (36)</td>
<td>12 (26)</td>
</tr>
<tr>
<td>B: fit to all data</td>
<td>5 (19)</td>
<td>7 (19)</td>
</tr>
<tr>
<td>C: model evaluation</td>
<td>5 (18)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>C: fit to all data</td>
<td>7 (18)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>D: model evaluation</td>
<td>15 (30)</td>
<td>5 (15)</td>
</tr>
<tr>
<td>D: fit to all data</td>
<td>3 (11)</td>
<td>5 (14)</td>
</tr>
</tbody>
</table>

a Maximum error is in parentheses
The TDM simulates the larger mortality from subsequent pulses (especially treatments A2, A3 and B1, see above) in better agreement with the data than the TWA pulses model. The pronounced increase in mortality resulting from subsequent pulses of CPF in the TDM (A2: 12%{13%} vs. 39%{26%}, A3: 12%{13%} vs. 30%{21%} and B1: 37%{32%} vs. 82%{61%}; first vs. second pulse in A2 and A3, combined first and second vs. third and fourth pulses in B1) can be explained by a build up of damage and the threshold (see supporting information). In contrast, the increase in mortality from subsequent pulses in the TWA pulses model (A2: 22%{19%} vs. 28%{23%}, A3: 22%{19%} vs. 27%{22%} and B1: 38%{33%} vs. 58%{47%}; first vs. second pulse in A2 and A3, combined first and second vs. third and fourth pulses in B1) does not match the observations (A2, A3) or is partially due to the larger total dose for the third and fourth pulse (0.943 μg x day x L⁻¹) vs. the first and second pulse (0.765 μg x day x L⁻¹) in B1.

The TDM links exposure with effect through a chain of cause-effect relationships, which are representations of the underlying ecotoxicological processes. The parameter values that we estimated for the TDM are in agreement with existing knowledge about the modes of action of CPF and PCP. Hence we have more confidence in the TDM predictions when we are extrapolating to different scenarios. The disadvantage that the TDM requires uptake and elimination rates, hence more experiments, is outweighed by the fact that knowledge of the time course of the contaminant concentration in the organism is of great benefit for the risk assessment in itself, especially when dealing with repeated exposure events.

Implications for Risk Assessment
Aquatic risk assessment can be improved if we are able to quantitatively predict the effects resulting from realistic exposure patterns. We demonstrate two successful approaches for pesticides that can also be applied to other contaminants. Both models, the TDM and the TWA pulses model, simulate the absolute extent of mortality equally well. The TWA pulses model is simpler, whereas the mechanistic TDM is better at explaining the observed patterns of survival over time. Considering
the variability and inter-experimental uncertainty in the survival data, both models show a very encouraging performance (mean errors are at 15% \{10\%\} or lower).

These models enable us to use pulsed toxicity tests and toxicokinetic data (for the TDM) to predict the effects of long-term exposure patterns with sequential pulses or fluctuating concentrations. They could be used in risk assessment to interpret the output from exposure models or to estimate effects resulting from real exposure patterns obtained in monitoring studies. The additional benefits of the TDM are that its parameter values reflect chemical modes of action and can be used to calculate the times that organisms require to recover. Parameterising the TDM for different species could facilitate a better understanding of the causes for the distribution of species sensitivities towards toxicants, hence leading to new approaches for inter-species extrapolation of toxicity.
Chapter 4

References


5. Simulating toxicity of carbaryl to *Gammarus pulex* after sequential pulsed exposure

*Introduction*

Pesticides are broadly applied within the open environment and may reach water bodies via various pathways. Thus they have a relatively large potential for effects on non-target organisms. Aquatic non-target organisms are typically exposed to sequential pulses with fluctuating concentrations [1, 2], but current risk assessment generally relies on standard toxicity tests performed at constant concentrations and over fixed durations. Any extrapolation to more realistic patterns of exposure must rely on modelling and we need a theoretically sound framework for this fundamental problem in environmental risk assessment as well as appropriate mathematical models to relate fluctuating field exposures to laboratory effects data [3, 4].

The advantage of modelling is that we can extrapolate to a wide range of exposure scenarios, but when we evaluated and compared the theoretical base of available models, we found that there were no generally applicable and validated methods to link sequential or fluctuating exposure to effects [chapter 2]. Subsequently we were able to combine two approaches, one originating from the Damage Assessment Model [5] and the other from the DEBtox concept [6] to form the Threshold Damage Model (TDM) [chapter 4]. The TDM is a new process-based model and we used it to simulate survival of the aquatic invertebrate *Gammarus pulex* after fluctuating and sequential pulsed exposure to pentachlorophenol and chlorpyrifos [chapter 4].

Toxicokinetics are important for the assessment of effects because the toxicant has to enter the organism and reach the site of action to exert an effect [7, 8]. The TDM combines the toxicokinetics (uptake and elimination) with the toxicodynamics (damage accrual and recovery and exceedence of a damage threshold) in one consistent ecotoxicological model. We can simulate the processes leading from exposure to effect in a more realistic manner enabling us to assess fluctuating or sequential exposure. The differences in the toxicokinetic and toxicodynamic parameters of the models could facilitate a better understanding of the differences in sensitivity of different organisms and sensitivity to different compounds.
In this study we demonstrate the application of the TDM to simulate survival of *Gammarus pulex* after sequential pulsed exposure to the carbamate pesticide carbaryl. In addition to evaluating the TDM for a mode of action that differs from previous studies [chapter 4] we also test the toxicokinetic sub-model with measured internal concentrations in a survival experiment with repeated pulsed exposure. The toxicokinetic parameters are derived from a short uptake and elimination experiment (A). The resulting simulation of internal concentrations is evaluated with measured internal concentrations in a survival experiment (B), which is also used to estimate the toxicodynamic parameters. Then we extrapolate and simulate the effects resulting from different exposure patterns in a third experiment (C).

We compare the performance of the TDM with a much simpler model based on time-weighted averages (TWA). The TWA pulses model [chapter 4] is also calibrated using the survival data of experiment B and then simulates survival in experiment C.

**Materials and Methods**

**Organisms, exposure water and handling**

*Gammarus pulex* (mixture of males and females) were collected from a small stream, Bishop Wilton Beck, ca. 20 km northeast of York, UK. The mean weight of the *Gammarus pulex* was 28.51 mg (n = 90, standard error (SE) = 1.18 mg) in the uptake and elimination experiment (experiment A), 24.80 mg (n = 107, SE = 0.90 mg) in experiment B and 28.82 mg (n = 6, SE = 1.75 mg) in experiment C. Assuming equal proportions of male and female *Gammarus pulex* of the same average age, we can estimate the age of the organisms from their wet weight [9]. The average age was estimated to be 187 days in the uptake and elimination experiment (A), 176 days in experiment B and 188 days in experiment C. Prior to experiments, organisms were kept for three to four days in aerated streamwater under the same conditions as in the experiments and were fed in excess with re-hydrated horse chestnut leaves.

Stream water was also collected from Bishop Wilton Beck and stored at 5°C. Bishop Wilton stream water (pH 9) was used in between pulses, but during the exposure
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Pulses used buffered stream water from Keys Beck (North Yorkshire Moors, upstream catchment completely in drinking water protection area). Keys Beck water was buffered at pH 6.75 with 750 mg/L MOPS (3-N morpholino propane sulfonic acid, [10]) and NaOH. Buffering is necessary because carbaryl is not stable under alkaline conditions. The resulting changes in pH are likely to cause an additional stress on the organisms, but we assume that the effect is small compared to the toxic stress caused by carbaryl. The control group is subject to the same pH regime, hence the background mortality rate (as fitted to control mortalities) accommodates for any effects of pH on mortality.

All experiments were carried out under static conditions in 600-mL pyrex beakers filled with 500 mL exposure solution. Beakers were kept in a cooling tank with water as coolant to maintain constant temperatures (12 ± 2°C). The light regime was a cycle of 12 hours light and 12 hours dark. All beakers were sealed with parafilm and aerated with pressurised air through Pasteur pipettes. Dissolved O₂ ranged between 9.4 and 10.5 mg/L, measured with a HI 9142 dissolved oxygen meter, Hanna Instruments and pH ranged from 6.70 to 7.02 during the exposure pulses and from 8.70 to 9.10 mg/L in the elimination and recovery periods (measured with a Hanna pH 213 and HI 1131 electrode, Hanna Instruments).

Organisms were rinsed and transferred to clean water at the end of any exposure pulse or more frequently so that the maximum duration between water changes was five days (experiment C, treatment 2). The volume of methanol used in the control was equal to the largest amount of methanol used in the treatments (0.01% v/v in experiment B and 0.04% in C). We assume that the methanol evaporated very quickly and had no effect on the organisms because all beakers were aerated with pressurized air.

Carbaryl

¹⁴C-labelled carbaryl [1-naphthyl methylcarbamate] (ring-labelled, 100% purity, 503 MBq/mmol, batch # XI/39) was purchased from Institute of Isotopes, Budapest, Hungary. Unlabelled carbaryl was purchased from Sigma-Aldrich Ltd. (Gillingham,
UK, 99.8% purity). Dosing solutions were made in methanol by mixing labelled with unlabelled carbaryl.

**Design of uptake and elimination experiment (A)**

The uptake and elimination experiment followed the method of chapter 3 with slight modifications. The uptake phase, during which the organisms were exposed to carbaryl lasted 1.85 days, then the organisms were transferred to fresh streamwater and kept there for three more days (Figure 5-1). Seven replicate beakers were dosed with a nominal concentration of 6 µg/L of total carbaryl and three control beakers were used. Each beaker contained 20 *Gammarus pulex* at the start of the experiment. During the whole experiment we sampled one *Gammarus* from each replicate beaker at twelve times (after 0.13, 0.33, 0.79, 1.04, 1.31, 1.85, 2.05, 2.22, 2.41, 2.89, 3.89 and 4.85 days). Each *Gammarus* was removed from the beaker, blotted dry, weighed on a precision balance (XS205, Mettler-Toledo Inc.) and frozen until analysis.

**Design of survival experiments (B and C)**

The *Gammarus pulex* were treated with carbaryl using various exposure patterns and we observed survival over time. Experiment B (Figure 5-2) lasted for 22 days and consisted of two treatments (1 and 2) and controls. In each treatment there were six replicate beakers with ten *Gammarus* and three replicate beakers with 25 organisms at the start of the experiment. Daily counts of surviving organisms were made in the six beakers with ten *Gammarus* at the start. We sampled organisms for the measurement of internal concentrations from the three beakers with 25 *Gammarus* at the start. The two control beakers contained ten organisms at the start. Experiment C lasted for 15 days and contained three treatments and a control group. Each group consisted of five replicate beakers with ten *Gammarus* at the start of the experiment (Figure 5-3).

**Sampling and analysis**

Test solutions were sampled (1 mL) immediately after spiking and frequently thereafter. Radioactivity present in water was quantified with liquid scintillation counting (Beckman LS6000 TA Liquid Scintillation Counter, Beckman Instruments
Inc., Fullerton, USA) after adding 10 mL of Ecoscnt A scintillation cocktail (National Diagnostics, Hessle, UK). Samples were counted three times for five minutes. Sample counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method. Determination of internal concentrations of carbaryl in *Gammarus* involved extraction with Soluene-350 tissue solubiliser (Packard BioScience B.V., Groningen, The Netherlands) and analysis of radioactivity by liquid scintillation counting following the method of chapter 3.

**Data analysis and statistics**

Parameter estimation was carried out using ModelMaker version 4 (AP Benson, Wallingford, UK). The parameter values for chlorpyrifos from the robust fit in chapter 4 were used as starting values in the parameter estimation procedure for carbaryl. Simulations of internal concentration, damage and survival as well as statistical calculations were undertaken with Mathcad 2001i Professional (MathSoft Engineering & Education Inc., Cambridge, USA).

**Modelling**

*The Threshold Damage Model (TDM)*

Equations (5-1) to (5-4) constitute the TDM. Equation (5-1) is the one-compartment first-order kinetics model, which simulates the toxicokinetics i.e. the time course of the internal concentration in relation to the concentration in the water phase surrounding the organism. Internal concentration acts as a surrogate for the concentration at the target site.

\[
\frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} \times C(t) - k_{\text{out}} \times C_{\text{int}}(t) \tag{5-1}
\]

where \(C_{\text{int}}\) is the internal concentration \([\text{Amount} \times \text{Mass}^{-1}]\), \(C\) the concentration in the water \([\text{Amount} \times \text{Volume}^{-1}]\) and \(k_{\text{in}}\) and \(k_{\text{out}}\) the uptake rate constant \([\text{Volume} \times \text{Mass}^{-1} \times \text{Time}^{-1}]\) and the elimination rate constant \([\text{Time}^{-1}]\), respectively.
Equation (5-2) simulates the first part of the toxicodynamics as an accrual and, in the second term of equation (5-2), the recovery or repair of damage:

$$\frac{dD(t)}{dt} = k_k \times C_{in}(t) - k_r \times D(t)$$  \hspace{1cm} (5-2)

where $k_k$ is a killing rate constant [Mass $\times$ Amount$^{-1}$ $\times$ Time$^{-1}$], $k_r$ is the rate constant for damage recovery or repair [Time$^{-1}$] and $D(t)$ is damage [-]. The differential of $H(t)$, as used in equation (5-3) is the hazard rate, which is the probability of the organisms dying at a given time. In equation (5-3) the hazard rate rises above zero when a threshold for damage is exceeded:

$$\frac{dH(t)}{dt} = \max[D(t) - \text{threshold}, 0]$$  \hspace{1cm} (5-3)

where $\text{threshold}$ is a dimensionless threshold parameter [-].

The killing rate constant is a combined parameter, describing both damage accrual and the proportionality factor to the hazard rate and reflects the toxic potency of the compound. The recovery rate parameter lumps all processes leading to recovery, such as repair mechanisms on a cellular scale (e.g. reactivation of blocked enzymes or synthesis of new enzyme) or adaptation of the physiology and other compensating processes. The toxicodynamic parameters in equations (5-2) and (5-3), and thus the speed of recovery, depend on the mode of action of the compound.

In equation (5-4) the standard approach of linking hazard to survival is used:

$$S(t) = e^{-H(t)} \times S_{\text{background}}(t)$$  \hspace{1cm} (5-4)

where $S(t)$ is the survival probability [-] (probability of an organism surviving until time $t$) and $S_{\text{background}}(t)$ is the survival probability resulting purely from the background (or control) mortality [-].

**The TWA pulses model**

When time-weighted average (TWA) concentrations are used to predict effects from fluctuating exposures, the exposure concentration is integrated over time and then divided by the duration of the corresponding toxicity test. This method is inherently based on Haber’s law and can be modified to simulate the survival over time if we
assume a linear relationship between dose and effect [chapter 2]. The survival probability is then calculated as:

\[ S(t) = 1 - f_{\text{TWA}} \times \int_{0}^{t} C(t) \, dt \]  

(5-5)

where \( f_{\text{TWA}} \) is a scaling factor \([\text{Volume} \times \text{Mass}^{-1} \times \text{Time}^{-1}]\) and \( C \) is the concentration in the exposure solution \([\text{Mass} \times \text{Volume}^{-1}]\). Fitting equation (5-5) to the survival data in experiment B yields the scaling factor \( f_{\text{TWA}} \). When simulating experiment B or C the survival probability in the TWA model is corrected for background mortality as in the TDM (eq. 5-4) by multiplication with the survival probability resulting from background mortality.

**Estimation of toxicokinetic and toxicodynamic parameters**

The toxicokinetic model (equation 5-1) was fitted to all individual measured internal concentrations of experiment A simultaneously (Figure 5-1, middle) to estimate uptake and elimination rate constants. The best fit was found using the Levenberg-Marquardt algorithm to minimise the ordinary least squares (no weighting of data points).

Once the toxicokinetic parameters were known, the remaining toxicodynamic parameters of the TDM \( k_h \), \( k_i \), and \( \text{threshold} \) were estimated by fitting the TDM to the survival data of experiment B (Figure 5-2, top). The uptake and elimination rates were kept fixed; only \( k_h \), \( k_i \), and \( \text{threshold} \) were adjusted automatically until the best fit was found. The toxicodynamic parameters of chlorpyrifos [chapter 4] served as starting values.

The long interval between the pulses in experiment B (nine days in treatment 1 and six days in treatment 2) allows the mortality to return to background levels before the next pulse. This is important for a successful estimation of the TDM parameters, as is the requirement that the measured data are consistent between the treatments.
After calibration, the survival of *Gammarus pulex* in experiment C was simulated with both models (Figure 5-3). These simulations were driven only by the measured concentrations in the exposure solution and adjusted for background mortality.

### Table 5-1. Model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Chlorpyrifos (^d)</th>
<th>Carbaryl</th>
<th>Pentachloro-phenol (^d)</th>
<th>Units (^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake rate constant (^b)</td>
<td>(k_{\text{in}})</td>
<td>747 ± 61</td>
<td>23.4 ± 0.9</td>
<td>89 ± 7</td>
<td>L × kg(^{-1}) × day(^{-1})</td>
</tr>
<tr>
<td>Elimination rate constant (^b)</td>
<td>(k_{\text{out}})</td>
<td>0.45 ± 0.05</td>
<td>0.27 ± 0.04</td>
<td>1.76 ± 0.14</td>
<td>day(^{-1})</td>
</tr>
<tr>
<td>Killing rate constant (^c)</td>
<td>(k_k)</td>
<td>0.134 ± 0.022</td>
<td>0.42 ± 0.1</td>
<td>0.061 ± 0.003</td>
<td>g wet.w. × day(^{-1}) × (\mu g_{a.i.})(^{-1})</td>
</tr>
<tr>
<td>Recovery rate constant (^c)</td>
<td>(k_r)</td>
<td>0.169 ± 0.04</td>
<td>0.97 ± 0.24</td>
<td>66 ± 3</td>
<td>day(^{-1})</td>
</tr>
<tr>
<td>Threshold (^c)</td>
<td>threshold</td>
<td>0.022 ± 0.0045</td>
<td>0.067 ± 0.01</td>
<td>0.037 ± 0.006</td>
<td>-</td>
</tr>
<tr>
<td>Scaling factor pulses (^c)</td>
<td>(f_{\text{TWA}}) pulses</td>
<td>321 ± 9</td>
<td>7.6 ± 0.4</td>
<td>0.0206 ± 0.0004</td>
<td>L × mg(^{-1}) × day(^{-1})</td>
</tr>
</tbody>
</table>

\(^a\) Parameter value ± standard error from fit  
\(^b\) Carbaryl parameters from uptake and elimination experiment (experiment A)  
\(^c\) Carbaryl parameter set from fit to experiment B  
\(^d\) Chlorpyrifos and pentachlorophenol parameter set from fit to all data in [chapter 4]  
\(^e\) g wet.w. is g wet weight and \(\mu g_{a.i.}\) is \(\mu g\) of active ingredient (carbaryl)
Figure 5-1. The measurement of uptake and elimination of carbaryl in *Gammarus pulex* (experiment A).

Mean aqueous carbaryl concentrations (○, n=7, bottom graph), measured internal concentrations (×, middle graph) and mean internal concentrations (○, n=7, top graph, error bars show 95% confidence intervals). The solid line (middle and top graph) shows the fitted toxicokinetic model (Equation 5-1) with $k_{in} = 23.4 \text{ L} \times \text{kg}^{-1} \times \text{day}^{-1}$ and $k_{out} = 0.27 \text{ day}^{-1}$. 
Results and Discussion

Structure of the TDM

Traditional aquatic risk assessment views the organism under scrutiny as a black box where concentrations of contaminants in the water phase are directly related to effects on the organism. The Threshold Damage Model (TDM) follows a new, semi-mechanistic approach. The TDM is a compromise between a detailed description of processes (bottom-up) and top-down modelling. The level of complexity is dictated by the purpose of the model, here the simulation of survival over time after fluctuating or sequential pulsed exposure to a toxicant. Hence the TDM includes uptake and elimination as two lumped processes (eq. 5-1) and damage accrual and repair/recovery as two processes (eq. 5-2). More detailed processes such as enzyme regeneration and de novo synthesis of enzyme for repair/recovery are again lumped into one process. The hazard rate (probability of dying) increases when the damage level rises above a threshold (eq. 5-3), i.e. the whole organism only responds with increased mortality if a certain level of internal damage is exceeded. That accommodates for compensating mechanisms as well as for the change in scales: equation (5-2) describes processes on the scale of cells or sites of action, whereas the survival probability in equation (5-4) only has meaning at the scale of the whole organism.

Hence the TDM is a generic model for aquatic organisms that emulates ecotoxicologically relevant processes. Different parameter sets represent different combinations of organisms and toxicants and can be compared and interpreted with respect to the characteristics of the organism and compound. Once the parameter sets are available, the TDM should allow simulation of effects from any kind of exposure pattern for that combination of compound and organism.
Figure 5-2. Treatment 1 (left) and treatment 2 (right) of experiment B.

The graphs show aqueous concentrations of carbaryl (bottom) as well as measured (×) and predicted (solid line) concentrations of carbaryl in *Gammarus pulex* (second from bottom). Above that is the simulated time course of damage in the TDM (third graph from bottom) and the observed (○) survival with the fitted Threshold Damage Model (solid line) as well as the fitted TWA pulses model (dashed line) in the top graph.
Parameters

All parameters could be estimated successfully (Table 5-1). Simulated annealing confirmed that the toxicodynamic parameters represent a minimum of the goal function within the realistic parameter space. The toxicokinetic parameters represented a global minimum. They were \( k_{\text{in}} = 23.4 \text{ L kg}^{-1} \text{ day}^{-1} \) and \( k_{\text{out}} = 0.27 \text{ day}^{-1} \). The bioconcentration factor at steady state, calculated as \( k_{\text{in}} / k_{\text{out}} \), was 87 L kg\(^{-1}\). The elimination rate constant \( (k_{\text{out}} = 0.27 \text{ day}^{-1}) \) is smaller than that of chlorpyrifos \( (k_{\text{out}} = 0.45 \text{ day}^{-1} \) [chapter 3]), which is surprising because chlorpyrifos has a larger log Kow than carbaryl (chlorpyrifos: log Kow = 4.7, carbaryl: log Kow = 1.85; [11]). The slow elimination of carbaryl (see Figure 5-1 middle and top) leads to a build up of carbaryl in the organism in experiment B (Figure 5-2, middle). The time to depurate 95% of the carbaryl after transfer to clean water is 11 days.

The elimination rate constants measured by Landrum et al. [12] for Pontoporeia hoyi and Mysis relicta are even smaller than ours for Gammarus pulex. They measured carbaryl uptake and elimination rate constants of 90 ± 15 L kg\(^{-1}\) day\(^{-1}\) (value ± SE) and 0.0048 ± 0.0038 day\(^{-1}\) respectively in Pontoporeia hoyi (BCF = 18750 L kg\(^{-1}\)) and 3.2 L kg\(^{-1}\) day\(^{-1}\) and 0.0216 ± 0.0144 day\(^{-1}\) (value ± SE) respectively in Mysis relicta (BCF = 148 L kg\(^{-1}\)).

Since we measured radioactivity, we have also detected any metabolites present in the organism. Only metabolites that are eliminated much more slowly than the parent compound would yield a large error in our measured internal concentrations of the parent compound. Landrum et al. [12] suggested that the slow elimination of carbaryl might be due to ionization and subsequently slower transfer across membranes (ion trapping) of the major metabolite, 1-napthol (estimated log Kow: 2.7 [13]). Further research is necessary to investigate whether there is significant metabolism of carbaryl in Gammarus pulex and whether any metabolites are more hydrophobic (hence depurated slower) than the parent compound or whether indeed ion trapping occurs.
The recovery rate constant $k_r = 0.97 \text{ day}^{-1}$ results in a time to 50% damage recovery of 17 hours when assessing damage recovery separately from toxicokinetics. The time to 95% recovery of damage is 3 days. Acetylcholinesterase (AChE) inhibited by carbamates such as carbaryl shows faster reactivation than that after inhibition by organophosphates such as chlorpyrifos [14]. Hence the faster damage recovery compared to chlorpyrifos ($k_r = 0.169 \text{ day}^{-1}$, chapter 4) is plausible. Further indication of the plausibility of our estimated recovery dynamics comes from studies by Kallander et al. [15] who showed significant recovery in the number of affected *Chironomus riparius* between two 1-hour pulses of carbaryl when there were at least 6 hours for recovery between the pulses. Nevertheless comparisons and extrapolations between species have to be made with great care, because the possibility of recovery from carbaryl exposure varies between species [16]. Parsons et al. [17] did not find significant differences in toxicity of two 1-hour pulses of carbaryl to *Aedes aegypti* larvae when recovery time between the pulses was increased from zero to 24 hours.

The toxicodynamic parameters of carbaryl can be compared (Table 5-1, Figure 5-4) with those of chlorpyrifos and pentachlorophenol from chapter 4. This comparison suggests that carbaryl has a larger inherent toxic potency than chlorpyrifos as demonstrated by the larger killing rate constant, but this is compensated by the larger threshold value for carbaryl. As expected, the recovery rate constant of carbaryl is smaller than that of pentachlorophenol, an uncoupler of oxidative phosphorylation. The killing rate of carbaryl ($0.42 \text{ g wet.w.} \times \text{day}^{-1} \times \mu \text{g a.i.}^{-1}$) is nearly seven times larger than that of pentachlorophenol ($0.061 \text{ g wet.w.} \times \text{day}^{-1} \times \mu \text{g a.i.}^{-1}$) whereas the threshold for carbaryl (0.067) is only twice that of pentachlorophenol (0.037).

Interpretation of these parameter values and application of the model assumes that (i) the TDM is an appropriate model and (ii) no metabolites have significantly obscured our measured uptake and elimination rate constants. We are currently not able to quantify the uncertainty in the parameter values due to variability between experiments and different *Gammarus pulex* populations because the necessary data are not available.
If we try to estimate the toxicodynamic parameters from experiment C, the estimation fails or results in nonsensical parameter values (e.g. negative $k_r$). The time between pulses in experiment C is too short and the observed survival data are inconsistent. For example mortality in treatment 2 after nine days is similar to that in treatment 1 even though treatment 1 had received double the dose of treatment 2 at that point in time (Figure 5-3).

![Graph showing survival rates and concentrations over time for treatments 1, 2, and 3 of experiment C.]

**Figure 5-3. Treatments 1 (left), 2 (middle) and 3 (right) of experiment C.**

Aqueous concentrations of carbaryl (bottom) and observed ($\bigcirc$) survival with the predictive simulations of the Threshold Damage Model (solid line) and the TWA pulses model (dashed line) in the top graph.

We can use the TDM to calculate LC50 values (concentration at which 50% of the population dies in a test with constant exposure): 24-hr LC50 = 618 µg/L, 48-hr LC50 = 108 µg/L, 72-hr LC50 = 44 µg/L. Only the 72-hr LC50 compares favourably with the measured values (24-hr LC50 = 35 µg/L, 48-hr LC50 = 29 µg/L, 72-hr LC50 = 25 µg/L) by Bluzat et al. [18]. The discrepancy can be explained by the more sensitive organisms in the experiments of Bluzat et al [18]. For comparison, the first pulses in experiment B (one day pulses) with concentrations of 28 µg/L and 17 µg/L in treatment 1 and 2 respectively, cause 2% and 3% mortality after one day and 23% and 10% respectively on day three (in clean water after day one). Hence the discrepancy is not a modelling problem but rather one of extrapolation between experiments.
Chapter 5

Model performance

Both, the TDM and the TWA pulses model were used to simulate survival following exposure to repeated pulses. Indicators for the goodness-of-fit (experiment B) and the performance in the independent simulations (experiment C) are shown in Table 5-2. All statistical indicators show a better performance of the TDM in all treatments.

Table 5-2. Indicators of model performance.

<table>
<thead>
<tr>
<th></th>
<th>Mean and maximum errors [%] a</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TDM</td>
<td>TWA pulse</td>
</tr>
<tr>
<td>Experiment B:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>model fit</td>
<td>4 (9)</td>
<td>7 (21)</td>
</tr>
<tr>
<td>Experiment C:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>predictive simulation</td>
<td>14 (42)</td>
<td>17 (48)</td>
</tr>
</tbody>
</table>

a In \% of initial population, maximum error in parentheses

The small mean errors (4\% for TDM and 7\% for the TWA pulse model) indicate a very good fit of both models to survival data from experiment B. Use of the toxicokinetic sub-model to predict internal concentrations of carbaryl in experiment B (Figure 5-2, second graph from bottom) overestimates peak concentrations in treatment 1, but is more accurate for treatment 2. Towards the end of the experiment both treatments show a build up of internal concentrations and thus a better agreement with the model simulation. The overall performance of this sub-model of the TDM is encouraging given the possible variability between the uptake and elimination experiment (A) and experiment B.

Overall doses in experiment C were much larger than in experiment B, but the TDM simulation is in good agreement with the observed survival, especially towards the end of experiment C (Figure 5-3). This indicates that even predictions of scenarios that are very different to the calibration experiment are possible, but the model performance in experiment C (mean errors 14\% and 17\% and maximum errors 42\% and 48\% for the TDM and the TWA pulses model, respectively) is not as good as in
the calibration experiment B. The performance in experiment C may be distorted because the survival data are partly inconsistent. If we do not consider the inconsistent data of treatment 2 in experiment C, the TDM performs better (mean errors 14% and 9% for treatments 1 and 3, \( r^2 = 0.91 \) and 0.97 respectively). The TWA performance in treatments 1 and 3 of experiment C is also better (mean errors 21% and 13% for treatments 1 and 3, \( r^2 = 0.81 \) and 0.92 respectively), but still worse than that of the TDM.

The TWA pulses model simulates that all organisms die in all treatments of experiment C (as early as on day six in treatment 1), illustrating one of the main shortcomings of this model - for sufficiently long exposure or large doses it will always predict 100% mortality. This is inherent in the empirical model structure and highlights the fact that the TWA pulses model cannot extrapolate to scenarios that are very different to its calibration.

Both models performed equally well in the previous experiments with chlorpyrifos and pentachlorophenol [chapter 4]. In contrast, the TDM consistently out-performs the TWA pulses model for the current experiment. This is attributed to the process based, mechanistic character of the TDM.

**Ecotoxicological implications**

Both models could be used to simulate survival of aquatic organisms after exposure to sequential pulses or fluctuating concentrations of contaminants, but in this study the TDM performs consistently better than the TWA pulses model. The TWA pulses model is simple and thus easily understood and it requires less data than the TDM because it does not need uptake and elimination rate constants. Omission of process knowledge such as uptake and elimination or toxicodynamics limits our confidence in the TWA pulses model when we extrapolate to different scenarios. Models of more empirical character are strictly only valid within the experimental boundaries that they are calibrated on.
The disadvantage of the TDM, that it requires uptake and elimination rates and hence more experiments, is outweighed by the fact that knowledge of the time course of the contaminant concentration in the organism is of great benefit for the risk assessment in itself, especially when dealing with repeated exposure events. The TDM links exposure with effects through a chain of cause-effect relationships that are representations of the underlying ecotoxicological processes. Hence we have more confidence in the TDM predictions when we are extrapolating to different scenarios.

The TDM parameter values reflect the chemical mode of action and can be used to calculate the times that organisms require to recover. In the simulation with the TDM, as in reality, elimination and recovery occur simultaneously so the actual simulated times to recovery differ from those calculated by adding up the individual times for 95% elimination and 95% recovery when they are calculated separately. In the TDM simulation it takes 15 days for *Gammarus pulex* to completely recover (95% of maximum damage is recovered) from a one-day pulse that kills 50% of the population. This compares with 25 days for chlorpyrifos and 3 days for pentachlorophenol. For carbaryl the time is mainly needed for the slow elimination process (toxicokinetics, equation 5-1), whereas in the case of chlorpyrifos it is the actual recovery of damage that is slow (toxicodynamics, equation 5-2). For pentachlorophenol, both the toxicokinetics and the toxicodynamics, are characterized by fast rates. This information is very useful for risk assessment and it highlights the consequences of the different modes of action of the three compounds.

The picture is completed when we take the value of the threshold into consideration. The rate of mortality of the organism simulated in the TDM will return to background levels as soon as the damage level falls below the threshold. Even though there is still internal damage that might contribute to effects of a subsequent pulse, it does not result in any effects on the organism scale because levels are below the threshold. The time required for the damage level to fall below the threshold (same pulse as above) is 9 days for carbaryl, 17 days for chlorpyrifos and 3 days for pentachlorophenol.
Figure 5-4. Comparison of toxicodynamic parameters of chlorpyrifos, carbaryl and pentachlorophenol.

The recovery rate constant \( k_r \) [day\(^{-1}\), logarithmic scale] and the killing rate constant \( k_k \) [g\(_{\text{wet.w.}}\) x day\(^{-1}\) x \(\mu\text{g.a.i.}^{-1}\)] define the axes. The bubble size of each compound is proportional to the value of threshold [-] (value shown in graph). Recovery from damage caused by carbaryl (carbamate AChE inhibitor) is faster than for chlorpyrifos (organophosphate AChE inhibitor) but slower than for pentachlorophenol (uncoupling of oxidative phosphorylation). The larger toxic potency of carbaryl as seen in the larger killing rate constant is compensated by the larger threshold value.

The TDM is now parameterized for three different compounds (Figure 5-4). Further research is necessary to generate parameters for more compounds. We hypothesize that compounds with the same mode of action cluster together in the toxicodynamic parameter space (Figure 5-4). Once a sufficient database is established, the parameters of new or less well-researched compounds could be estimated based on their mode of action and properties. Furthermore, we believe that parameterising the TDM for different species could facilitate a better understanding of the causes for the distribution of species sensitivities towards toxicants, hence leading to new approaches for inter-species extrapolation of toxicity.

The TDM explicitly models toxicokinetics and toxicodynamics as separate groups of processes and combines them in one model, hence fulfilling the criteria and satisfying the need for predictive models in aquatic ecotoxicology [8] as well as facilitating assessment of risk from realistic exposure scenarios.
References


Chapter 6

6. Modelling combined effects of pulsed exposure to carbaryl and chlorpyrifos on *Gammarus pulex*

*Introduction*

It has been recognized that aquatic non-target organisms exposed to pesticides are typically exposed to sequential pulses with fluctuating concentrations [1, 2], but current risk assessment relies on standard toxicity tests performed at constant concentrations and over fixed durations. We reviewed available models to relate fluctuating field exposures to laboratory effects data and found that there was no generally applicable and validated method available [chapter 2]. Subsequently we developed, evaluated and compared two methods [chapter 4, chapter 5], the Threshold Damage Model (TDM) and a method based on time-weighted average concentrations (TWA pulses). Both models performed well when simulating survival after fluctuating or sequential pulsed exposure to either chlorpyrifos or pentachlorophenol [chapter 4], whereas simulations with carbaryl demonstrated a better performance of the TDM [chapter 5]. These models allow simulation of effects from realistic exposure patterns, but so far have only considered exposure to a single toxicant.

The next step towards more realism in environmental effects assessment of toxicants is to consider fluctuating concentrations or sequential pulses of multiple compounds. Temporally staggered environmental fate processes and spatial interaction of multiple sources of contaminants lead to sequential pulses of multiple contaminants in natural water bodies [3, 4, 5, 6]. In this study we extend the two modeling approaches (TDM and TWA pulses) to describe effects from sequential exposure to multiple compounds. The TDM predicts that the recovery of damage to *Gammarus pulex* from exposure to chlorpyrifos takes longer than that from exposure to carbaryl [chapter 5]. Lasting damage can cause increased mortality from subsequent exposures to the same compound, as shown for chlorpyrifos [chapter 4], but the TDM also predicts that lasting damage caused by one compound may increase mortality from subsequent exposure to another. Hence, if the recovery time between two exposures is long enough for sufficient recovery from damage caused by carbaryl, but not chlorpyrifos,
then the sequence of exposure should matter. This study was designed to test this hypothesis.

The detailed objectives of the study were: (i) to measure survival of the freshwater invertebrate *Gammarus pulex* after sequential pulsed exposure to carbaryl and chlorpyrifos, (ii) to test for increased mortality caused by previous exposure to the other compound and (iii) to test whether mortality is different for chlorpyrifos followed by carbaryl compared to the reverse ordering. Furthermore (iv) we evaluate the models with respect to the performance of their simulations of survival and their prediction or lack of a sequence effect.

**Materials and Methods**

**Organisms and exposure water**

The freshwater invertebrate *Gammarus pulex* is of ecological importance because it is involved in detritus processing in streams [7]. It has been used in biomonitoring [7, 8], laboratory toxicity studies [9, 10] and microcosm experiments [11, 12]. For this study, *Gammarus pulex* (mixture of males and females, length ca. 5-10 mm) were collected from a small stream, Bishop Wilton Beck, ca. 20 km north-east of York, UK. Prior to experiments, organisms were kept for four days in aerated streamwater under the same conditions as in the experiments and were fed in excess with re-hydrated horse chestnut leaves.

Stream water was also collected from Bishop Wilton Beck and stored at 5°C. Bishop Wilton stream water (pH 9) was used in between pulses, but during the exposure pulses we used buffered stream water from Keys Beck (North Yorkshire Moors, upstream catchment completely in drinking water protection area). Keys Beck water was buffered at pH 6.68 with 750 mg/L MOPS (3-N morpholino propane sulfonic acid, [13]) and NaOH. Buffering is necessary because carbaryl is not stable under alkaline conditions. The resulting changes in pH are likely to cause an additional stress on the organisms, but we assume that the effect is small compared to the toxic stress caused by the pesticides.
All experiments were carried out under static conditions in 600-mL pyrex beakers filled with 500 mL exposure solution. Beakers were kept in a cooling tank with water as coolant to maintain constant temperatures (12 ± 2°C). The light regime was a cycle of 12 hours light and 12 hours dark. All beakers were sealed with parafilm and aerated with pressurized air through Pasteur pipettes. Dissolved O₂ ranged between 9.7 and 9.9 mg/L (measured with a HI 9142 dissolved oxygen meter, Hanna Instruments) and pH ranged from 6.68 to 6.87 during the exposure pulses and from 8.91 to 9.36 mg/L in the elimination and recovery periods (measured with a Hanna pH 213 and HI 1131 electrode, Hanna Instruments).

Organisms were rinsed and transferred to clean water at the end of any exposure pulse and more frequently such that the maximum duration between water changes was five days. The largest amount of methanol used in the treatments was 0.08% v/v (chlorpyrifos dosing). We assume that the methanol evaporated very quickly and had no effect on the organisms because all beakers were aerated with pressurized air.

**Chemicals**

Chlorpyrifos and carbaryl are both insecticides that cause toxicity through inhibition of the enzyme acetylcholinesterase (AChE). Chlorpyrifos is an organophosphate and carbaryl is a carbamate. AChE inhibited by carbamates shows faster reactivation than that after inhibition by organophosphates [14]. 14C-labelled carbaryl [1-naphthyl methylcarbamate] (ring-labelled, 100% purity, 503 MBq/mmol, batch # XI/39) was purchased from Institute of Isotopes, Budapest, Hungary. Unlabelled carbaryl was purchased from Sigma-Aldrich Ltd. (Gillingham, UK, 99.8% purity). Dosing solutions were made in methanol by mixing labelled with unlabelled carbaryl. 14C-labelled chlorpyrifos [pyridine-2,6-14C] (99% purity, 32 Ci/mol, lot # 050107) was purchased from American Radiolabeled Chemical, Inc. (St. Louis, USA).

**Experiments**

Two groups of *Gammarus pulex* were exposed to a first pulse of either carbaryl or chlorpyrifos for one day and then, after a recovery period of two weeks, to a second pulse with the other compound respectively. The two treatments in our experiment
are denoted A and B (left and right parts of Figure 1). Both treatments consisted of
ten beakers with ten Gammarus in each beaker at the start of the experiment.

Treatment A was dosed with a one day pulse of carbaryl first (initial concentration
26.5 μg/L), followed by 14 days of clean water and then a one day pulse of
chlorpyrifos (initial concentration 0.497 μg/L), followed by eight days in clean water.

Treatment B was complementary to treatment A, with the same pulses of carbaryl and
chlorpyrifos, but the order of the pulses switched around (Figure 1a). Hence treatment
B was dosed with a one day pulse of chlorpyrifos first (initial concentration 0.494
μg/L), followed by 14 days of clean water and then a one day pulse of carbaryl (initial
concentration 27.5 μg/L), followed by eight days in clean water.

Daily counts of surviving organisms were made in all beakers. Test solutions were
sampled (1 mL) immediately after spiking and frequently thereafter. Radioactivity
present in water was quantified with liquid scintillation counting (Beckman LS6500
TA Liquid Scintillation Counter, Beckman Instruments Inc., Fullerton, USA) after
adding 10 mL of Ecoscint A scintillation cocktail (National Diagnostics, Hessle, UK).
Samples were counted three times for five minutes. Sample counts were corrected for
background activity by using blank controls. Counting efficiency and color
quenching were corrected using the external standard ratio method.

**Statistical analysis of mortality**

Our experiment generated data for mortality following two pulses of carbaryl and two
pulses of chlorpyrifos. The doses of both carbaryl pulses and the doses of both
chlorpyrifos pulses were comparable, but for each compound there was one pulse
where the organisms had not been previously exposed and one pulse where they had
been exposed to the other toxicant 14 days before (Figure 6-1). We compared the
mortalities following exposure to the same toxicant, thus testing for differences
caused by previous exposure to the other compound. Comparisons are made for the
time course of survival during the pulse and over the following eight days (Figure 6-
2) and for the total mortality over the full nine days (Figure 6-3).
Confidence intervals for proportions of dead organisms were calculated following [15]. Differences between mortalities were tested for significance using the binomial test for two proportions (Software MINITAB, release 14.20).

**Modelling**

*Approach.* We used the semi-mechanistic Threshold Damage Model (TDM) to simulate survival and compared it to a simpler model based on time-weighted averages (TWA pulses). Both models were first presented and discussed in [chapter 4], where we also estimated the toxicodynamic parameters for chlorpyrifos. Uptake and elimination rate parameters for chlorpyrifos in *Gammarus pulex* were measured previously [chapter 3]. The parameters for carbaryl and additional discussion of the two modeling concepts can be found in [chapter 5]. Here we extend both models to facilitate simulations with multiple toxicants (TDM<sub>mix</sub> and TWA<sub>mix</sub>) and apply the models using the previously estimated parameters (Table 6-1). Hence we are running independent, predictive simulations.

*The Threshold Damage Model for multiple toxicants (TDM<sub>mix</sub>).* The TDM combines toxicokinetics (uptake and elimination) with toxicodynamics (damage accrual and recovery and exceedence of a damage threshold) in one consistent ecotoxicological model. We can simulate the processes leading from exposure to effect in a semi-mechanistic manner enabling us to assess fluctuating or sequential exposure.

Here, the basic TDM [chapter 4] is extended to simulate the survival following exposure to multiple toxicants. In the extension, TDM<sub>mix</sub>, we calculate the internal concentrations and the damage for each toxicant individually (equations 6-1 and 6-2). Then the total damage in the organisms is calculated by summing up the individual damages (equation 6-3). As the thresholds for different toxicants differ, we calculate the mixture threshold in equation (6-4) as an average of the individual thresholds, weighted by the individual damages at any point in time. The hazard rate (probability of dying) increases when the total damage level rises above the mixture threshold (equation 6-5).
Equation (6-1) is the one-compartment first-order kinetics model,

\[
\frac{dC_{\text{int},i}(t)}{dt} = k_{\text{in},i} \times C_i(t) - k_{\text{out},i} \times C_{\text{int},i}(t)
\]

(6-1)

where \(C_{\text{int},i}\) is the internal concentration [Amount × Mass\(^{-1}\)] of compound \(i\), \(C\) the concentration in the water [Amount × Volume\(^{-1}\)] and \(k_{\text{in}}\) and \(k_{\text{out}}\) the uptake rate constant [Volume × Mass\(^{-1}\) × Time\(^{-1}\)] and the elimination rate constant [Time\(^{-1}\)] of compound \(i\), respectively. Equation (6-2) simulates the first part of the toxicodynamics as an accrual and, in the second term of equation (6-2), the recovery or repair of damage:

\[
\frac{dD_i(t)}{dt} = k_{k,i} \times C_{\text{int},i}(t) - k_{r,i} \times D_i(t)
\]

(6-2)

where \(k_{k,i}\) is a killing rate constant [Mass × Amount\(^{-1}\) × Time\(^{-1}\)], \(k_{r,i}\) is the rate constant for damage recovery or repair [Time\(^{-1}\)] and \(D_i(t)\) is damage [−] of compound \(i\), respectively. The following two equations constitute the extension of the TDM for multiple compounds. In equation (6-3) we sum up internal damage values for all toxicants:

\[
D_{\text{mix}}(t) = \sum_i D_i(t)
\]

(6-3)

where \(D_{\text{mix}}(t)\) is the total amount of damage present in the organism [−]. The damage \(D_i(t)\) of each compound is linked to its threshold value. Therefore we calculate the threshold for the total damage of the mixture as an average of the individual thresholds, weighted by the individual damages at any point in time:

\[
\text{threshold}_{\text{max}}(t) = \sum_i \left( \text{threshold}_i \times \frac{D_i(t)}{D_{\text{mix}}(t)} \right)
\]

(6-4)

where \(\text{threshold}_{\text{mix}}(t)\) is the mixture threshold [−]. The differential of \(H(t)\), as used in equation (6-5) is the hazard rate, which is the probability of the organisms dying at a given time. The hazard rate rises above zero when the mixture threshold is exceeded by the mixture damage:

\[
\frac{dH(t)}{dt} = \max[D_{\text{mix}}(t) - \text{threshold}_{\text{mix}}(t),0]
\]

(6-5)

where \(H(t)\) is the hazard [−]. The threshold is due to compensating mechanisms as well as the change in scales from processes on the scale of cells or sites of action
(equation 6-2) to the survival probability at the scale of the whole organism (equation 6-6). In equation (6-6) we use the standard approach of linking hazard to survival:
\[ S(t) = e^{-H(t)} \times S_{\text{background}}(t) \] (6-6)
where \( S(t) \) is the survival probability [-] (probability of an organism surviving until time \( t \)) and \( S_{\text{background}}(t) \) is the survival probability resulting purely from the background mortality [-].

**Comparison of the TDM with the MDAM and mixture toxicity theory.** Up to equation (6-3) some aspects of the TDM are similar to the MDAM developed by Lee at al. [16]. They developed a model that includes metabolism in the toxicokinetics and also has a damage term for the toxicodynamics but is used to predict lethal body residues. They did not use a threshold and they did not measure survival in experiments with repeated exposure pulses. Nevertheless, Lee at al. [16] showed that the assumption of damage addition, as used in the TDM and the MDAM, is equivalent to the independent action model for mixtures. However, as long as there are no toxicokinetic and toxicodynamic interactions between the compounds, the concept of damage addition is also equivalent to the concentration addition model [16]. The assumptions of no interaction in the toxicokinetics and toxicodynamics between the compounds also hold for the TDM.

The MDAM was not tested in this study because it is not designed for simulating survival after repeated pulsed exposure. Furthermore it needs to be reconsidered before general application because there is an inconsistency in the link between damage and survival [chapter 2].
The Time-Weighted Averages model for multiple toxicants (TWAmix). We compared the performance of the TDM with a modified time-weighted averages approach (TWA pulses model, [chapter 4]). The survival probability for each toxicant is calculated as:

\[
S_i(t) = 1 - f_{TWA_i} \times \int_0^t C_i(t) \, dt 
\] (6-7)

where \( f_{TWA_i} \) is the scaling factor \([\text{Volume} \times \text{Mass}^{-1} \times \text{Time}^{-1}]\) and \( C_i \) the concentration in the exposure solution \([\text{Mass} \times \text{Volume}^{-1}]\) for compound \( i \). The scaling factors for chlorpyrifos and carbaryl are taken from previous studies [chapter 4, chapter 5]. The overall survival probability for the mixture TWA model is calculated as:

\[
S(t) = S_{\text{background}}(t) \times \prod_i S_i(t) 
\] (6-8)

where \( S_{\text{background}}(t) \) is the survival probability resulting purely from the background mortality [-].

**Correction for background mortality.** The survival probabilities of both models were corrected for the background mortality in our test system. We did not have a control group in this experiment because we aimed to maximize the statistical power from our two treatments. Hence we estimated the background mortality by fitting equation (6-9) to background mortalities from experiments A, B and D in [chapter 4] consisting of 15 replicate beakers (150 *Gammarus* initially, 10 beakers until day 20 and 5 beakers until day 24).

\[
S_{\text{background}}(t) = e^{-h_b t} 
\] (6-9)

where \( h_b \) is the background hazard rate \([\text{Time}^{-1}]\) and \( t \) is time [Time].
### Table 6-1. Model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Chlorpyrifos</th>
<th>Carbaryl</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake rate constant</td>
<td>$k_{in}$</td>
<td>747&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.4</td>
<td>L $\times$ kg$^{-1}$ $\times$ day$^{-1}$</td>
</tr>
<tr>
<td>Elimination rate constant</td>
<td>$k_{out}$</td>
<td>0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27</td>
<td>day$^{-1}$</td>
</tr>
<tr>
<td>Killing rate constant</td>
<td>$k_k$</td>
<td>0.134&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42</td>
<td>g wet.w. $\times$ day$^{-1}$ $\times$ µg a.i.$^{-1}$</td>
</tr>
<tr>
<td>Recovery rate constant</td>
<td>$k_r$</td>
<td>0.169&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97</td>
<td>day$^{-1}$</td>
</tr>
<tr>
<td>Threshold</td>
<td>threshold</td>
<td>0.022&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.067</td>
<td>-</td>
</tr>
<tr>
<td>Scaling factor</td>
<td>f_TWA</td>
<td>321&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.6</td>
<td>L $\times$ mg$^{-1}$ $\times$ day$^{-1}$</td>
</tr>
<tr>
<td>Background hazard rate</td>
<td>h&lt;sub&gt;b&lt;/sub&gt;</td>
<td>0.0071</td>
<td></td>
<td>day$^{-1}$</td>
</tr>
</tbody>
</table>

<sup>a</sup> From [chapter 3]

<sup>b</sup> Parameter set from fit to all data in [chapter 4]

<sup>c</sup> Parameter set from [chapter 5]

<sup>d</sup> g wet.w. is g wet weight and µg a.i. is µg of active ingredient
Results and Discussion

Observed mortality: the sequence matters

The time between the pulses was long enough for both compounds to be depurated (Figure 6-1b). The times for depuration of 95% of chlorpyrifos and carbaryl are 7 days [chapter 3] and 11 days [chapter 5], respectively. Even though 14 days are sufficient for depuration of chlorpyrifos in treatment B, the subsequent pulse of carbaryl results in significantly higher mortality than without previous exposure to chlorpyrifos (first pulse in treatment A). The mortalities are 31% after the first pulse in treatment A vs. 55% after the second pulse in treatment B (Figures 6-2 and 6-3). The difference is significant (31/100 vs. 46/83, p=0.001). In the opposite comparison, chlorpyrifos does not show significantly increased mortality after previous exposure to carbaryl. The mortalities are 12% (12/100) after the first pulse in treatment B vs. 21% (13/61) after the second pulse in treatment A (p=0.13).

There are two implications of this result. First, it does matter whether organisms are exposed to the other toxicant, even if it is 14 days earlier, and secondly it does make a difference to which compound they were previously exposed. We also assess the combined mortalities from both pulses in each treatment by calculating the percentage killed by both subsequent pulses from the product of the respective survival probabilities. The combined mortality is 45% \(1 - (0.69 \times 0.79)\) in treatment A and 60% \(1 - (0.88 \times 0.45)\) in treatment B. The combined doses of carbaryl and chlorpyrifos were the same in both treatments. Hence the difference in the mortalities is attributed to the different sequence of exposure.
Figure 6-1. The graphs show treatment A (left) and treatment B (right).

The sequence of the pulses with carbaryl (solid line) and chlorpyrifos (dashed line) is plotted to different scales (bottom graph, (a)). The initial aqueous concentrations of the pulses are 26.5 µg/L and 27.5 µg/L (carbaryl, A and B) and 0.5 µg/L (chlorpyrifos). The next graphs (second from bottom, (b)) show simulated internal concentrations of carbaryl (solid line) and chlorpyrifos (dashed line). The third graphs (from bottom, (c)) show the time course of the mixture damage (solid line, eq. 6-3) together with the mixture threshold (dotted line, eq. 6-4). The top graphs (d) show the observed survival (X), the prediction by the TDM (solid line) and the prediction by the TWA model (dashed line).
Model performances

The predicted survival is shown for both the TDM\textsubscript{mix} and the TWA\textsubscript{mix} model in Figure 6-1d. The prediction by the TDM shows a better agreement with the observed survival than the TWA model. This is supported by the statistical indicators (Table 6-2), which show a consistently better performance of the TDM. The TDM simulation of survival results in mean errors of 4 and 8\% and maximum errors of 9 and 10\% for treatments A and B, respectively. These are independent simulations that extrapolate from previous experiments, so the errors are partly due to inter-experimental variability.

The graphs (b) and (c) in Figure 6-1 show intermediate calculation steps of the TDM, illustrating the use of the TDM for interpretation of experimental outcomes. Figure 6-1b shows the time course of the internal concentrations and illustrates that both compounds are almost completely depurated between pulses. Hence the increased mortalities from subsequent pulses in both treatments cannot be explained by residual internal concentrations of the previous compound. Figure 6-1c illustrates the time course of the damage ($D_{\text{mix}}(t)$) as simulated by the TDM. Together with the plotted threshold ($\text{threshold}_{\text{mix}}(t)$) it becomes very clear how the TDM predicts the increased mortality following the second pulse in treatment B, but not in A.

We observed a significant difference in the mortality following the two carbaryl pulses. The TDM predicts a difference for both pulses and is in good agreement with the observations (Figures 6-2 and 6-3). There was no significant difference in the mortalities following chlorpyrifos exposure and again the TDM is in good agreement with this observation (Figures 6-2 and 6-3). The individual mortalities as predicted by the TDM are 22\% vs. 23\% for chlorpyrifos (treatment B vs. A, observed: 12\% vs. 21\%, not significant) and 33\% vs. 47\% for carbaryl (treatment A vs. B, observed: 31\% vs. 55\%, significant difference).

The TWA model also predicts no difference in the mortalities following exposure to chlorpyrifos (treatment B vs. A; predicted: 22\% vs. 22\%; observed: 12\% vs. 21\%, not significant). In contrast to the TDM and the observations, the TWA model also
predicts only a minor difference in the mortalities following exposure to carbaryl (treatment A vs. B; predicted: 26% vs. 28%; observed: 31% vs. 55%, significant difference). The minor increase is attributed to the slightly larger initial concentration of carbaryl in the second pulse (26.5 μg/L in treatment A vs. 27.5 μg/L in B).

The TDM outperforms the TWA model with respect to the prediction of the time course of survival (Figure 6-1, Table 6-2) and the prediction of the significant difference in mortalities following exposure to carbaryl, i.e. the TWA model does not simulate the effect of the sequence of exposure.

**Figure 6-2.** The graph to the left shows survival following the first (no pre-exposure, treatment B, ×) and second (14 days after pre-exposure, treatment A, ○) pulse to chlorpyrifos.

There is no significant difference between the mortalities from the two chlorpyrifos pulses, because organisms were able to recover from the previous exposure to carbaryl in treatment A. The graph to the right shows survival (and 95% confidence intervals of proportions) following the first (no pre-exposure, treatment A, ×) and second (14 days after pre-exposure, treatment B, ○) pulse to carbaryl. The second pulse of carbaryl (treatment B) causes significantly more mortality than the first pulse (treatment A), because the organisms in treatment B have not yet recovered from the previous exposure to chlorpyrifos.
Figure 6-3. Comparison of the mortalities caused by chlorpyrifos and carbaryl with model predictions.

The bars show observed (white, ± 95% CI) and predicted mortality (TDM: chequered, TWA: striped). From left to right: first pulse in treatment B, second pulse in A, first pulse in A and second pulse in B.

Table 6-2. Indicators of model performance.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TDM</th>
<th>TWA pulse</th>
<th>TDM</th>
<th>TWA pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment A</td>
<td>4 (9)</td>
<td>7 (20)</td>
<td>0.94</td>
<td>0.85</td>
</tr>
<tr>
<td>Treatment B</td>
<td>8 (16)</td>
<td>10 (25)</td>
<td>0.92</td>
<td>0.81</td>
</tr>
</tbody>
</table>

a In % of initial population, maximum error in parentheses.
Process based interpretation: toxicodynamics

The TDM facilitates explanation of how previous exposure to chlorpyrifos leads to increased mortality from subsequent pulses of carbaryl, and why there is no such effect when the sequence is reversed. The damage following exposure to carbaryl (Figure 6-1c, treatment A) falls below threshold levels on day 8 due to fairly quick recovery processes, whereas the slower recovery for damage caused by chlorpyrifos results in damage levels above the threshold until day 15 when the second pulse starts (treatment B). Hence our results are explained by the different toxicodynamic characteristics of chlorpyrifos and carbaryl. The difference in their toxicokinetics cannot explain our observations.

In the TDM concept, the parameters killing rate constant, recovery rate constant and threshold are estimated by inverse modeling from survival data. Hence the term damage is not directly associated with a specific mechanism of action and its attributed measure of toxic action (e.g. inhibition of acetylcholinesterase). Rather the term damage represents the overall reduction in fitness of the organisms. The fitness of the organisms is determined by various mechanisms, but measured biochemical transformation rates such as the rate of recovery from inhibition of acetylcholinesterase (AChE) show faster reactivation after inhibition by carbamates (e.g. carbaryl) than that after inhibition by organophosphates (e.g. chlorpyrifos) [14]. Hence the biochemical evidence, i.e. the different speed of recovery from inhibition of AChE, supports the different toxicodynamics of carbaryl and chlorpyrifos in the TDM.

In some cases, such as the two compounds used in this study, damage is likely to be linked to the same target, e.g. inhibition of AChE, and summing up of individual damage values is clearly well justified. Here we would also expect that toxicity from sequential pulsed exposure can be sensitive to the sequence of exposure if the compounds show different speed of recovery. Nevertheless the TDM is not restricted to toxicants that act through one mechanism only. Damage in the TDM is a generic measure for the reductions in fitness of the organism, so the model might be applicable to independently acting toxicants as well. Further research is necessary to
test whether the sequence of exposure also matters for compounds that act on different target sites.

**Implications for Risk assessment**

It is important to establish the duration, following an initial pulse, over which effects from subsequent pulses are still affected by the previous exposure. This applies whether subsequent pulses are from the same or different compounds. The TDM could serve as a tool to establish this duration. When the internal damage has declined sufficiently it will not contribute to the effects of subsequent pulses anymore. If we define that it is sufficient for damage to fall below 5% of its maximum, then the relevant durations would be 3 days following a pulse of pentachlorophenol, 15 days after exposure to carbaryl and 25 days after exposure to chlorpyrifos [chapter 5].

Aquatic risk assessment could be improved by quantitatively predicting the effects resulting from realistic exposure patterns including sequential pulsed exposure to multiple compounds. Here, two approaches were compared in an independent model test, with the semi-mechanistic TDM performing best. The experimental results of this study can be predicted by the TDM when parameterized using independent experiments and the simulations reveal how slow recovery from internal damage caused by chlorpyrifos and faster recovery for carbaryl have consequences for sequential exposure to two different toxicants. This study demonstrates interactions between chemical pulses even after depuration, but more research is necessary to investigate whether similar interactions as well as an effect of the sequence of exposure also exist for other organisms, compounds and exposure patterns.
References


Chapter 6


7. Overall discussion and conclusions

Progress towards a unified model

In chapter 2 various models and ecotoxicological concepts were analyzed and classified. Two concepts were highlighted as the most promising approaches and it was indicated that some of the other major models were special instances of the two. Since the TDM combines both approaches selected in chapter 2 it is a unifying model. It can be demonstrated that the TDM includes all the other major concepts as special cases. For that purpose the four equations of the TDM are repeated below. Detailed explanation is given in chapter 4.

The TDM

\[
\frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} \times C(t) - k_{\text{out}} \times C_{\text{int}}(t) \tag{7-1}
\]

Where \( C_{\text{int}} \) is the internal concentration \([\text{Amount} \times \text{Mass}^{-1}]\), \( C \) the concentration in the water \([\text{Amount} \times \text{Volume}^{-1}]\) and \( k_{\text{in}} \) and \( k_{\text{out}} \) the uptake rate constant \([\text{Volume} \times \text{Mass}^{-1} \times \text{Time}^{-1}]\) and the elimination rate constant \([\text{Time}^{-1}]\), respectively.

\[
\frac{dD(t)}{dt} = k_{\text{k}} \times C_{\text{int}}(t) - k_{\text{r}} \times D(t) \tag{7-2}
\]

Where \( k_{\text{k}} \) is a killing rate constant \([\text{Mass} \times \text{Amount}^{-1} \times \text{Time}^{-1}]\), \( k_{\text{r}} \) is the rate constant for damage recovery or repair \([\text{Time}^{-1}]\) and \( D(t) \) is damage \([-]\). The differential of \( H(t) \), as used in equation (7-3) is the hazard rate, which is the probability of the organisms dying at a given time and threshold is a dimensionless threshold parameter \([-]\).

\[
\frac{dH(t)}{dt} = \max[D(t) - \text{threshold}, 0] \tag{7-3}
\]

\[
S(t) = e^{-H(t)} \tag{7-4}
\]

\( S(t) \) is the survival probability \([-]\), i.e. the probability of an organism surviving until time \( t \).
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The other models

There are only four major toxicodynamic concepts in ecotoxicology prior to the TDM: the DAM, the THM or DEBtox approach, the CAUC/CTO models and the CBR concept [see chapter 2 for details]. Most of the other available models are more or less related to one of those four or are not considered further for some other reason [chapter 2]. The CAUC/CTO and CBR models have not been formulated to yield survival over time. If the most popular concept, the CBR approach, were reformulated as a dynamic model it would take the form of the simple hazard model [chapter 2]. The simple hazard model assumes that the hazard is proportional to the internal concentration, which is the equivalent of the assumption that the effect is proportional to the internal concentration (CBR) after toxicokinetic steady state is obtained. The simple hazard model is:

\[
\frac{dH(t)}{dt} = \theta \times C_{\text{int}}(t) \tag{7-5}
\]

with \( \theta \) being a proportionality constant. The assumption underlying the CTO/CAUC models, i.e. that the effect is proportional to the integral of the internal concentration (area under the curve), can be translated into a hazard model for the purpose of dynamic simulation as follows:

\[
\frac{dH(t)}{dt} = \theta \times \int C_{\text{int}}(t)dt \tag{7-6}
\]

or with a threshold in terms of CTO as:

\[
\frac{dH(t)}{dt} = \theta \times \max[\int C_{\text{int}}(t)dt - \text{threshold}_{\text{CTO}}, 0] \tag{7-7}
\]
Demonstrating the relationships between models

**TDM vs. DAM model**

Now, using the TDM as a starting point, the relationships between the models can be demonstrated. The first instance is the extreme case of \( \text{threshold} = 0 \), i.e. there is no threshold. Then equation (7-3) changes into:

\[
\frac{dH(t)}{dt} = D(t)
\]  

(7-8)

With equations (7-1), (7-2) and (7-4) being the same as in the TDM this is the DAM model, here reformulated to eliminate the inconsistency in its original formulation when simulating survival [chapter 2].

**TDM vs. CTO/CAUC models**

The next extreme case is \( k_r = 0 \), corresponding to no recovery or irreversible damage. In that case equation (7-2) is rewritten as:

\[
\frac{dD(t)}{dt} = k_k \times C_{in}(t)
\]

(7-9)

And consequently, after integration and substitution, equation (7-3) is

\[
\frac{dH(t)}{dt} = k_k \times \max[\int C_{in}(t)dt - \frac{\text{threshold}}{k_k}, 0]
\]

(7-10)

Equation (7-10) becomes identical with equation (7-7) by defining

\[\text{threshold}_{CTO} := \frac{\text{threshold}}{k_k} \quad \text{and} \quad \theta := k_k.\]

Hence the CTO/CAUC models with a threshold are a special case of the TDM. If, in addition to \( k_r \) being zero, the threshold is zero (\( k_r = 0 \wedge \text{threshold} = 0 \)), then equation (7-10) becomes:

\[
\frac{dH(t)}{dt} = k_k \times \int C_{in}(t)dt
\]

(7-11)

Equation (7-11) is identical to equation (7-6), the formulation of the CAUC/CTO models without a threshold.
**TDM vs. THM (DEBtox)**

If $k_r = \infty$ (or very large), corresponding to very fast, instant recovery then toxicodynamic steady state is achieved instantly and the time course of $D(t)$ mimics that of $C_{int}(t)$. At toxicodynamic steady state, $\frac{dD(t)}{dt} = 0$, and equation (7-2) can be rearranged to $k_r \times D(t) = k_i \times C_{int}(t) \iff D(t) = \frac{k_i}{k_r} \times C_{int}(t)$. Replacing $k_i / k_r$ with $k_{k2}$ by defining $k_{k2} = k_i / k_r$ yields $D(t) = k_{k2} \times C_{int}(t)$ and substitution into equation (7-3) leads to

$$\frac{dH(t)}{dt} = k_{k2} \times \max[C_{int}(t) - \frac{\text{threshold}}{k_{k2}}, 0]$$

(7-12)

If we define the threshold for internal concentration in the THM [chapter 2] as $C_{\text{internal \ threshold}} = \frac{\text{threshold}}{k_{k2}}$ then equation (7-12) becomes

$$\frac{dH(t)}{dt} = k_{k2} \times \max[C_{int}(t) - C_{\text{internal \ threshold}}, 0]$$

(7-13)

Equation (7-13) is identical to the toxicodynamics of the THM [chapter 2], which is based on the same assumptions as the toxic action part of DEBtox.

**TDM vs. CBR**

If $k_r = \infty$ (or very large) and $\text{threshold} = 0$, then substitution of the rearranged form of equation (7-2) $D(t) = \frac{k_i}{k_r} \times C_{int}(t)$ into equation (7-3) yields:

$$\frac{dH(t)}{dt} = \frac{k_i}{k_r} \times C_{int}(t)$$

(7-14)

If $\theta$ is defined as $\theta = k_i / k_r$ then equation (7-14) is identical with the simple hazard model (eq. 7-5) which is the CBR concept translated into a dynamic simulation model.
Table 7-1. Toxicodynamic assumptions in ecotoxicological models.

<table>
<thead>
<tr>
<th>Model</th>
<th>A priori restrictions (assumptions)</th>
<th>$k_r$</th>
<th>threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDM</td>
<td>-</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>DAM</td>
<td>No threshold</td>
<td>variable</td>
<td>= 0</td>
</tr>
<tr>
<td>CTO / CAUC With threshold</td>
<td>Irreversible damage</td>
<td>= 0</td>
<td>variable</td>
</tr>
<tr>
<td>CTO / CAUC Without threshold</td>
<td>Irreversible damage, no threshold</td>
<td>= 0</td>
<td>= 0</td>
</tr>
<tr>
<td>THM (DEBtox)</td>
<td>Instant recovery</td>
<td>$\infty$</td>
<td>variable</td>
</tr>
<tr>
<td>Simple hazard model (CBR)</td>
<td>Instant recovery, no threshold</td>
<td>$\infty$</td>
<td>= 0</td>
</tr>
</tbody>
</table>

The four main ecotoxicological models are special instances of the TDM (Table 7-1). Application of each of these models requires \textit{a priori} assumptions about the reversibility of the toxic action or the existence of a threshold. No \textit{a priori} restrictions are made in the TDM; rather the parameters are found by inverse modeling. Hence, successful parameter estimation is possible for compounds with unknown modes of action and would even result in some initial information about their modes of action.
Limitations and requirements for future improvement

Having said that in theory the TDM does not need a priori knowledge about the mode of action it must be noted that the parameter estimation of the toxicodynamic parameters is the most critical step where it is still necessary to restrict parameter values within “plausible” boundaries. This is necessary due to non-uniqueness in the surface of the optimized goal function. The parameter estimation as described in chapters 4 and 5 minimizes the squared difference between observed and simulated survival by adjusting the three toxicodynamic parameters. If the parameters are unrestricted it can happen that no sensible solution is found, e.g. with negative recovery rate constants or that multiple solutions are found that produce an equally good fit.

Survival experiments with sequential pulses yield information on the speed of recovery. From the shape of the survival curves following the pulses, e.g. in chapter 4, it is inferred that recovery from pentachlorophenol must be very fast because survival returns to background levels immediately after the pulses, i.e. recovery was completed within the resolution of the measurement, which was one day. For chlorpyrifos, however it emerges that recovery takes several days. Together, toxicokinetics and observations of delayed effects can limit the possible range of \( k_r \) and thus facilitate parameter estimation. Furthermore, extremely fast recovery, e.g. recovery from lethal stress within a few minutes, can be excluded as unrealistic for an organism such as *Gammarus pulex*. Such information can be used to restrict the parameter space and ensure “plausible” parameter sets.

Since fitting of the toxicodynamics is the most critical step in the TDM modelling process, improvements to reduce the need for external knowledge and parameter restriction are highly desirable. There are several possibilities. First the design of the survival experiments should include a wide range of contrasting treatments that allow for differing degrees of recovery between pulses. The toxicokinetics also need consideration by allowing near complete depuration between pulses. Secondly, it could be possible to reduce non-uniqueness by including the dimension of time in the goal function. So far, only the differences in survival, plotted on the y-axis, are
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minimised. Nevertheless the differences in the timing of the mortality, plotted on the x-axis, could also be included in the goal function. Thirdly, biochemical stress indicators or the time-course of affected target sites, when measured in parallel to survival, could help characterizing the dynamics of recovery and the nature of the threshold. Furthermore, the time-step of measurements needs to be adjusted according to the speed of recovery and the organism. Generally, smaller organisms and faster recovery mechanisms need a higher resolution in the measurements; thus daily measurement intervals might not capture the recovery dynamics but hourly measurements might. And finally reducing the variability in the measured endpoints will enable more robust parameter estimation. To this end, the test organism should be standardised and cultured in the laboratory.

Extending the TDM approach to non-lethal endpoints such as reproduction and population growth in model organisms such as *Daphnia magna* should be possible, but requires further research. It needs to be investigated how the toxicodynamics of different life stages differ and how toxic effects on life-cycle dependent endpoints such as reproduction can be modelled.

**Potential problems when measuring total radioactivity**

Since total radioactivity was measured in the uptake and elimination experiments any metabolite present in the organisms was also detected and counted as parent because all three compounds were ring-labelled. Consequently, the formation of metabolites could potentially lead to errors in the parameters of the TDM. Generally non-polar organic compounds are metabolised to more polar, hence more water-soluble conjugates [1], which are depurated faster than the parent compound. For example pentachlorophenol can be conjugated with uridine diphosphate glucuronic acid and then excreted rapidly [1]. In that case no error in the measurement of internal concentrations and subsequent toxicodynamic parameter estimation is made.

If the elimination of the metabolite is slower than that of the parent then the elimination rate constant of the parent will be underestimated. The error made in the estimation of the toxicodynamic parameters of the TDM depends on whether the
metabolite is more toxic than the parent or less (intrinsic toxicity). If the slowly depurated metabolite is less toxic than the parent, then the killing rate parameter for the parent compound will be under- and the recovery rate parameter overestimated. If a slowly depurated metabolite is more toxic than the parent, then the killing rate parameter of the parent is over- and the recovery rate parameter underestimated. In case the metabolite is eliminated slower than the parent, but has similar toxicity, there will be no error in the toxicodynamic parameters, but an underestimation of the elimination rate constant of the parent.

Transformation products of pesticides are usually less toxic than their parent compounds [2]. The metabolites of chlorpyrifos either have a lower $K_{ow}$, indicating faster elimination, or are less toxic to *Daphnia* than chlorpyrifos [2]. The possible metabolites of carbaryl include three dihydroxybenzenes, all three less toxic to *Daphnia* and possessing lower $K_{ow}$ values than carbaryl, as well as 5-hydroxy-1,4-naphtoquinone which has a lower $K_{ow}$ and unknown toxicity to *Daphnia* [2]. The most likely metabolite of carbaryl is 1-naphtol, which has a larger $K_{ow}$ than carbaryl [2, 3] and might be subject to ion trapping [4, see also chapter 5]. There is no toxicity data for *Daphnia*, but data for fish show a similar toxicity of 1-naphtol and carbaryl [2].

There are no data about metabolism of chlorpyrifos, carbaryl and pentachlorophenol in *Gammarus pulex*. Hence it is not possible to assess the extent of the error that might be introduced into this work by unintentional measurement of metabolites instead of parent compounds. Nevertheless the available data for pentachlorophenol and chlorpyrifos suggests that metabolism is unlikely to cause significant error in the parameters of the TDM. If there is significant metabolism of carbaryl in *Gammarus pulex*, the most likely error is an underestimation of the elimination rate constant of carbaryl.

In future research, transformation products in the organism under investigation should be measured wherever feasible. The measurement of parent compound and metabolites instead of total radioactivity would not only allow for more reliable modelling, but also further refine the understanding of the mechanism of action. It is
also anticipated that measuring and simulating the concentration of the compound at
the target site or in the target organ could further reduce uncertainty in the
toxicokinetic simulations and provide a better starting point for toxicodynamic
modelling.

**Applications in risk assessment**
The TDM can be used in risk assessment to simulate effects on aquatic organisms
following fluctuating or sequential pulsed exposure. Once the parameters are
determined, effects caused by any exposure pattern can be simulated. This includes
those traditionally used for standard toxicity tests, i.e. standard toxicity data can be
calculated with the TDM if desired.

![Diagram](image)

**Figure 7-1. Scheme for modelling approach with the TDM as an example.**

The general approach would follow the scheme in Figure 7-1. First of all an
appropriate model needs to be selected. In this thesis the only ecotoxicological
endpoint that was simulated was survival or mortality. For this purpose *Gammarus
pulex* is an ideal model organism because its growth is very slow compared to the
toxicokinetics and does not need to be modelled. The life span of *Gammarus pulex*
exceeds the test durations by far and their size allows fairly easy measurement of
internal concentrations. If the aim is to simulate other endpoints, then the model and the organism need to be reconsidered. Some non-lethal endpoints, such as feeding rate, would just require some careful model revision to replace survival with feeding rate. Others, such as reproduction and population growth need to be measured on different organisms, e.g. *Daphnia magna*. If the TDM concept is transferred to *Daphnia magna* all parts of the model need to be checked and modified where necessary. Growth dilution of internal concentrations by rapidly growing organisms, such as *Daphnia*, needs to be simulated. The relationship between internal damage and endpoints like reproduction and how to model them requires further research.

Despite these limitations the work in this thesis demonstrates that risk assessment could benefit if toxicokinetic and toxicodynamic modelling replaced risk quotients consisting of time-weighted average concentrations and standard toxicity data. First of all, toxicokinetics provide a useful tool on their own, because they predict whether depuration between subsequent pulses can be achieved. Total recovery times (chapter 5 and Figure 7-2) allow for differentiated assessment of different compounds. The example of chlorpyrifos, carbaryl and pentachlorophenol illustrates that the duration of effects varies greatly between compounds.

![Figure 7-2](image)

**Figure 7-2. Total recovery times for chlorpyrifos, carbaryl and pentachlorophenol (25, 15 and 3 days, from left to right).**

In risk assessment one generally calculates the risk for the maximum time-weighted average concentration over a fixed duration, for example the duration of the *Daphnia magna* NOEC test [5-7]. This approach is also followed in risk indicators [8-10]. The exposure period with the largest time-weighted average concentration does not necessarily correspond to the period that causes the largest mortality. Figure 7-3
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illustrates a constructed example with chlorpyrifos and survival simulated by the TDM. The two pulses on the left represent a larger time-weighted average concentration than the two pulses to the right, because the first pulse on the left has a higher initial concentration than all the other pulses. Following the time-weighted average assumptions the scenario on the left would pose the greatest risk, but the TDM simulates recovery between the pulses and predicts 26% mortality on the left and 36% on the right. This indicates the possibility that current risk assessment and risk indicators might underestimate risk for some exposure patterns.

![Graphs showing concentration and survival over time](image)

**Figure 7-3. Comparison of two exposure scenarios**

One of the major challenges in using risk indicators is the aggregation of risk events. Different compounds are assessed individually first and then their risks are aggregated. This approach does not take into account the possible interactions between different chemicals such as those demonstrated in chapter 6. Hence, risk indicators could be improved by modelling these interactions. A possible framework using the TDM is described in chapter 6, but further research is necessary to investigate the prevalence of such interactions for a wide range of organisms, compounds and exposure patterns.
Opportunities for ecotoxicology

After establishment of a sufficient database with parameter sets for different combinations of compounds and species there could be benefits for ecotoxicology in two directions. First, relationships of compounds with similar structure or mode of action can be identified and those relationships can then be used to extrapolate to new compounds. Also, generalisations for the recovery dynamics of whole classes of compounds could be possible. Secondly, it is anticipated that both sets of parameters, those for toxicokinetics and those for the toxicodynamics, are related to species traits in a meaningful way. Whether those relationships can be identified, used for extrapolation and possibly applied to help to explain differences in species sensitivity needs to be investigated.
**General conclusions**

1. It is possible to simulate survival of aquatic organisms following exposure to fluctuating or sequential pulsed exposure to pesticides.

2. The TDM can be parameterized with a limited number of laboratory studies and then extrapolated to any type of exposure pattern.

3. The TDM has been identified as a general model that includes other ecotoxicological models as special cases.

4. Extrapolation from short-term standard toxicity tests using a time-weighted averages approach cannot predict survival following sequential pulsed exposure.

5. A simple time-weighted averages model can predict survival following sequential pulsed exposure in some instances if it is calibrated on pulsed exposure tests itself. Overall the TDM outperforms the TWA pulses model.

6. The TDM can be extended to suit multiple compounds or mixtures.

7. Interactions between chemical pulses of multiple compounds have been predicted by the TDM and confirmed experimentally. Toxicokinetics cannot explain the sequence effect of pulsed exposure to carbaryl and chlorpyrifos (chapter 6), but the simulations of the different recovery dynamics in the TDM can.

8. Toxicokinetic experiments and sequential pulsed exposure tests accompanied by process oriented modelling yield much more useful information than standard toxicity tests.
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8. Full list of references


Full list of references


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9. Appendices

Appendix A: Table of parameters and variables in chapter 2 with description and dimensions

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<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Dimensions $^{(1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{in}$</td>
<td>uptake rate constant</td>
<td>Volume $\times$ Mass$^{-1}$ $\times$ Time$^{-1}$</td>
</tr>
<tr>
<td>$k_{out}$</td>
<td>elimination rate constant</td>
<td>Time$^{-1}$</td>
</tr>
<tr>
<td>$C$</td>
<td>concentration</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$C_{int}$</td>
<td>internal concentration</td>
<td>Amount $\times$ Mass$^{-1}$</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
<td>Time</td>
</tr>
<tr>
<td>$y$</td>
<td>power term</td>
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<tr>
<td>$a, b$</td>
<td>variables</td>
<td>-</td>
</tr>
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<td>$Z$</td>
<td>probit of the response</td>
<td>-</td>
</tr>
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<td>$b_0, b_1, b_2$</td>
<td>regression constants</td>
<td>-</td>
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</tr>
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<td>$k_{reality}$</td>
<td>slope in the log $C$ vs. log $t$ plot</td>
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<tr>
<td>$\mu$</td>
<td>mortality rate</td>
<td>Time$^{-1}$</td>
</tr>
<tr>
<td>$b$</td>
<td>slope parameter</td>
<td>-</td>
</tr>
<tr>
<td>$\beta$</td>
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<td>-</td>
</tr>
<tr>
<td>$n$</td>
<td>number of organisms</td>
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<tr>
<td>$S$</td>
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<td>-</td>
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<tr>
<td>$h$</td>
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<tr>
<td>$A$</td>
<td>species specific constant</td>
<td>Amount $\times$ Volume$^{-1}$ $\times$ Time</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
<td>Unit</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>$t_d$</td>
<td>time to death</td>
<td>Time</td>
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<tr>
<td>$CEC$</td>
<td>cumulative exposure concentration</td>
<td>Amount $\times$ Volume$^{-1}$</td>
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<td>$ESC$</td>
<td>estimated safe concentration</td>
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<td>total time</td>
<td>Time</td>
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<td>$t_a$</td>
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<td>$I_{tot}$</td>
<td>intervals</td>
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<tr>
<td>$LC_{50}$</td>
<td>lethal concentration for 50% of individuals</td>
<td>Amount $\times$ Volume$^{-1}$</td>
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<td>$t_{LC50test}$</td>
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<td>duration of toxicity test</td>
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<tr>
<td></td>
<td>constant</td>
<td></td>
</tr>
<tr>
<td>$NLT_{50}$</td>
<td>average normal lethal time (life expectancy)</td>
<td>Time</td>
</tr>
<tr>
<td>$LT_{50}$</td>
<td>lethal time (exposure time in a toxicity test with 50% mortality)</td>
<td>Time</td>
</tr>
<tr>
<td>$CBR$</td>
<td>critical body residue</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$c_0$</td>
<td>concentration in water at no-effect level</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$EC_{50}$</td>
<td>concentration at 50% effect</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$CAUC$</td>
<td>critical area under the curve</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$CTO$</td>
<td>critical target occupation</td>
<td>Amount $\times$ Volume$^{-1}$</td>
</tr>
<tr>
<td>$BCF$</td>
<td>bioconcentration factor</td>
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### Appendixes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_i$</td>
<td>first order rate constant</td>
<td>$\text{Time}^{-1}$</td>
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<tr>
<td>$\theta$</td>
<td>proportionality constant</td>
<td>$\text{Time}^{-1} \times \text{Volume} \times \text{Amount}$</td>
</tr>
<tr>
<td>$k_{k1}$</td>
<td>killing rate constant in modified damage assessment model</td>
<td>$\text{Mass} \times \text{Amount}^{-1} \times \text{Time}^{-1}$</td>
</tr>
<tr>
<td>$k_{k2}$</td>
<td>killing rate constant in threshold hazard model</td>
<td>$\text{Mass} \times \text{Amount}^{-1} \times \text{Time}^{-1}$</td>
</tr>
<tr>
<td>$C_{\text{internal}}$</td>
<td>internal threshold concentration</td>
<td>$\text{Amount} \times \text{Volume}^{-1}$</td>
</tr>
<tr>
<td>$k_a$</td>
<td>damage accrual rate</td>
<td>$\text{Mass} \times \text{Amount}^{-1} \times \text{Time}^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>coefficient</td>
<td>-</td>
</tr>
<tr>
<td>$D$</td>
<td>damage</td>
<td>-</td>
</tr>
<tr>
<td>$k_r$</td>
<td>repair/recovery rate constant</td>
<td>$\text{Time}^{-1}$</td>
</tr>
</tbody>
</table>

*1) The unit for [Amount] is mol. [Mass] refers to the wet weight of the aquatic organisms.
### Appendix B: Published data on effects of pulsed exposure to pesticides

<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Specie(s)</th>
<th>Type of pulses</th>
<th>Effect endpoints</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>bluegill sunfish ((Lepomis macrochirus))</td>
<td>repeated with recovery time</td>
<td>survival, length, weight</td>
<td>[1]</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td><em>Daphnia magna</em></td>
<td>repeated with various recovery times + durations</td>
<td>survival, mobility</td>
<td>[2, 3]</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>fathead minnows, bluegills, others</td>
<td>one continuous, one repeated pulse scenario</td>
<td>survival, growth, reproduction</td>
<td>[4]</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>guppies ((Poecilia reticulata))</td>
<td>one pulse (14 d exposure) + 14 d recovery period</td>
<td>daily AChE activity</td>
<td>[5]</td>
</tr>
<tr>
<td>chlorpyrifos, endrin, fenvalerate</td>
<td>fathead minnow larvae ((Pimephales promelas))</td>
<td>1h, 3h, 5h, 30d exposure, each 5 concentrations, 96h-LC50 for 7 exp. durations</td>
<td>survival after 30d, deformities after 30d</td>
<td>[6]</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>caddisfly larvae ((Limnephilus lunatus))</td>
<td>various durations</td>
<td>chronic effects</td>
<td>[7]</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>steelhead trout ((Salmo gairdneri))</td>
<td>daily pulses + constant exp.</td>
<td>survival</td>
<td>[8]</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td><em>Daphnia magna</em></td>
<td>24 h, 21 days</td>
<td>survival, reproduction</td>
<td>[9]</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td><em>Daphnia magna</em></td>
<td>24 h pulses, varying food conditions</td>
<td>survival, growth, reproduction</td>
<td>[10]</td>
</tr>
<tr>
<td>Esfenvalerate</td>
<td>Gammarus pulex</td>
<td>1 h pulses</td>
<td>survival, reproduction</td>
<td>[11]</td>
</tr>
<tr>
<td>Esfenvalerate</td>
<td><em>Chironomus riparius</em></td>
<td>1 h pulses, varying sediment conditions and population density</td>
<td>survival, development, reproduction</td>
<td>[12]</td>
</tr>
<tr>
<td>Esfenvalerate</td>
<td>bluegill ((Lepomis macrochirus)), zooplankton, phytoplankton</td>
<td>repeated with recovery time</td>
<td>survival, growth, reproduction</td>
<td>[13]</td>
</tr>
<tr>
<td>Chemical</td>
<td>Organism / Species</td>
<td>Exposure Duration</td>
<td>Response / Measurement</td>
<td>References</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------------------</td>
<td>-------------------</td>
<td>-------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>esfenvalerate</td>
<td>bluegill (Lepomis macrochirus)</td>
<td>repeated with recovery time</td>
<td>survival, behaviour</td>
<td>[14]</td>
</tr>
<tr>
<td>carbaryl</td>
<td>stream invertebrates (Calineuria californica and Cinygma sp.)</td>
<td>various durations</td>
<td>survival</td>
<td>[15]</td>
</tr>
<tr>
<td>carbaryl, aldicarb, malathion, parathion, propoxur, carbofuran</td>
<td>midge (Chironimus riparius)</td>
<td>repeated with various recovery times</td>
<td>intoxication symptoms, AChE activity</td>
<td>[16, 17]</td>
</tr>
<tr>
<td>carbaryl, permethrin, fenitrothion, carbofuran</td>
<td>mosquito larvae (Aedes aegypti)</td>
<td>repeated with various recovery times</td>
<td>survival</td>
<td>[18]</td>
</tr>
<tr>
<td>carbofuran</td>
<td>goldfish (Carassius auratus)</td>
<td>2, 4, 6, 8 hrs exposure</td>
<td>swimming activity</td>
<td>[19]</td>
</tr>
<tr>
<td>azadirachtin</td>
<td>Chironomus riparius</td>
<td>repeated with recovery time</td>
<td>emergence of larvae</td>
<td>[20]</td>
</tr>
<tr>
<td>dichlorvos</td>
<td>common lobster (Homarus gammarus)</td>
<td>various durations, repeated with recovery time</td>
<td>survival, moults, AChE activity</td>
<td>[21]</td>
</tr>
<tr>
<td>fenitrothion, parathon-methyl</td>
<td>Gammarus pulex, Gammarus spec.</td>
<td>5 days exposure, 16 days recovery</td>
<td>AChE inhibition and recovery</td>
<td>[22, 23]</td>
</tr>
<tr>
<td>fenoxycarb</td>
<td>Daphnia magna</td>
<td>1 pulse with decline, 5 conc.</td>
<td>21d survival, others</td>
<td>[24]</td>
</tr>
<tr>
<td>irgarol 1051</td>
<td>seagrass (Zostera capricorni)</td>
<td>repeated with recovery time</td>
<td>quantum yield of PS II</td>
<td>[25]</td>
</tr>
<tr>
<td>lambda-cyhalothrin</td>
<td>Gammarus pulex, macroinvertebrates</td>
<td>30 min pulse at different concentrations</td>
<td>drift response, structural change in community</td>
<td>[26]</td>
</tr>
<tr>
<td>DDE</td>
<td>Hyalella azteca</td>
<td>single pulses of various durations</td>
<td>survival</td>
<td>[27]</td>
</tr>
<tr>
<td>parathion</td>
<td>Chironimus riparius</td>
<td>various durations + concentration</td>
<td>symptoms of toxicity, AChE inhibition</td>
<td>[52]</td>
</tr>
</tbody>
</table>
References


(17) Kallander DB. 1993. Quantitative structure activity relationships to predict the fate and effects of selected organophosphorus and carbamate insecticides in aquatic systems. PhD thesis. The Ohio State University. Columbus. Ohio. USA.


## Appendix C: Published data on pesticide uptake rate constants ($k_{in}$) and elimination rate constants ($k_{out}$)

<table>
<thead>
<tr>
<th>$k_{in}$</th>
<th>$k_{out}$</th>
<th>BCF</th>
<th>Compounds</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>DDT, DDE, DDD</td>
<td><em>Hyalella azteca + Diporeia spec.</em></td>
<td>[1, 2]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>PCP, methyl-parathion, fluoranthene, HCBP</td>
<td><em>Hyalella azteca</em></td>
<td>[3]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>dieldrin</td>
<td><em>Hyalella azteca</em></td>
<td>[4]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>PCP, carbaryl</td>
<td><em>Pontoporeia hoyi + Mysis relicta</em></td>
<td>[5]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>cypermethrin, deltamethrin, fenvalerate, permethrin</td>
<td><em>Chironomus tentans</em> larvae</td>
<td>[6]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>chlorthion</td>
<td><em>Lymnea stagnalis</em> (pond snail)</td>
<td>[7]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>chlorpyrifos, chlorfenviphos, methidathion</td>
<td><em>Mytilus galloprovincialis</em> (mussel)</td>
<td>[8]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>chlordane, endosulfan, allethrin, fenvalerate</td>
<td><em>Anodonta piscinalis</em> (mussels)</td>
<td>[9]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>fluridone, terbutryn</td>
<td><em>Chironomus tentans + rainbow trout</em></td>
<td>[10]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>cypermethrin, deltamethrin, fenvalerate, permethrin, DDT</td>
<td>rainbow trout</td>
<td>[11]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>paraoxon</td>
<td>rainbow trout</td>
<td>[12]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>cyanophos, SV5, methyl-parathion, fenitrothion, methylisocyanothion, chlorothion, dicapthon, fenthion-S2145, fenthion, fenchlorphos, bromophos, iodofenphos, 1,2,3,5-TECB-I, 1,2,3,5-TECB-II</td>
<td><em>Poecilia reticulata</em> (guppy)</td>
<td>[13]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>diazinon</td>
<td><em>Poecilia reticulata</em> (guppy) + <em>Brachydanio rerio</em> (zebra fish)</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>chlorpyrifos-methyl, vamidothion, edifenphos, ethoprophos, bendiocarb, pirimicarb, methyl-parathion</td>
<td><em>Gnathopogon caerulescens</em> (willow shiner)</td>
<td>[16]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>dichlorvos, salithion, methidathion, pyridaphenthion, fenthion, phosmet, phenthoate, EPN</td>
<td><em>Gnathopogon caerulescens</em> (willow shiner)</td>
<td>[17]</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>butachlor, thiobencarb, chlomethoxyfen</td>
<td><em>Aristichthys nobilis</em> (black silver carp)</td>
<td>[18]</td>
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<tr>
<td>x</td>
<td>x</td>
<td></td>
<td>fenitrothion</td>
<td><em>Anguilla anguilla</em> (European eel)</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>----------</td>
<td>--------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>diazinon</td>
<td>Anguilla anguilla (European eel)</td>
<td>[20, 21]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>diazinon, malathion, fenithrothion, EPN, fenithrothion oxon, EPN oxon</td>
<td>Oryzias latipes (killifish)</td>
<td>[22]</td>
<td></td>
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<tr>
<td>x</td>
<td></td>
<td>toxaphene</td>
<td>lake trout + white suckers</td>
<td>[23]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>chlorpyrifos</td>
<td>three-spined stickleback</td>
<td>[24]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td></td>
<td>39 pesticides</td>
<td>various fish species</td>
<td>[25]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>PCP</td>
<td>sea urchin</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>diazinon</td>
<td>Porcellio scaber (terrestrial isopod)</td>
<td>[27]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>x</td>
<td>chlorpyrifos</td>
<td>Poecilia reticulata (guppy)</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td></td>
<td>diquat</td>
<td>channel catfish</td>
<td>[29]</td>
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</tr>
<tr>
<td>x</td>
<td>x</td>
<td>x</td>
<td>PCP, 2,4,5-TCP</td>
<td>Lumbricus variegatus</td>
<td>[30]</td>
</tr>
</tbody>
</table>
References


organophosphorus pesticide chlorthion in the pond snail *Lymnaea stagnalis* -a comparison with the guppy *Poecilia reticulata*. *Aquat Toxicol* 41:301-323.


Appendices


Appendix D: Supporting information to chapter 4

Experimental Details

General experimental conditions and procedures

Beakers were kept in a cooling tank with water as coolant to maintain constant temperatures (12°C ± 2°C). The light regime was a cycle of 12 hours light and 12 hours dark. All beakers were sealed with parafilm and aerated with pressurised air through Pasteur pipettes. Dissolved O₂ ranged between 9.7 and 10.3 mg/L, measured with a HI 9142 dissolved oxygen meter, Hanna Instruments and pH ranged from 8.3 to 9.2, measured with a Hanna pH 213 and HI 1131 electrode, Hanna Instruments.

Organisms were rinsed and transferred to clean water at the end of any exposure pulse or more frequently so that the maximum duration between water changes was six days. Transfer to clean water was achieved by pouring the test solution with the organisms through a sieve, thus retaining the organisms without damaging them. Then the organisms were rinsed while still in the sieve and subsequently the sieve was submerged in the fresh test solution thus releasing the *Gammarus pulex*. The organisms in the control beakers were subject to exactly the same handling procedures and were dosed with methanol only. The volume of methanol used in the control was equal to the largest amount of methanol used in the treatments. The maximum concentrations (volume/volume) of methanol were 0.08% in experiment A, 0.16% in B1, 0.11% in C1 and 0.18% in D2 and D3. We assume that the methanol evaporated very quickly because all beakers were aerated with pressurized air and had no effect on the organisms. Even if there were small effects on the organisms the background mortality rate (as fitted to the control mortalities) would accommodate
for that, thus further reducing any possible error in our model parameters caused by
the solvent.

Both compounds were dissolved in methanol prior to addition to the beakers,
followed immediately by stirring. The dosing solutions of PCP were prepared by
mixing labelled and unlabelled PCP. *Gammarus* were counted as dead when they did
not show any movement of the appendages after gentle prodding with a spatula. Any
dead *Gammarus* were removed from the beakers.

Cannibalism amongst the *Gammarus* might contribute to the observed mortality,
especially under stress from a toxicant. This would lead to increased mortality,
particularly at the beginning of experiments and at lower concentrations. We
refrained from using less animals per beaker, and thus reducing the risk of
cannibalism, because the benefits of having large numbers of organisms tested
outweigh the risk of cannibalism.

**Chemicals**

$^{14}$C-labelled chemicals [pyridine-2,6-$^{14}$C] chlorpyrifos (99% purity, 32 Ci/mol, lot #
050107) and [$^{14}$C(U)] pentachlorophenol (99% purity, 11.9 Ci/mol, lot # 050112)
were purchased from American Radiolabeled Chemical, Inc. (St. Louis, US).
Unlabelled pentachlorophenol was purchased from Sigma-Aldrich Ltd. (Gillingham,
UK, 99.5% purity, lot # 330-97A).
Sampling and analysis of test solutions

Solutions were sampled immediately after spiking and after every 24 hours by taking 5 and 1 mL of the test solution for CPF and PCP, respectively. Radioactivity was quantified with liquid scintillation counting (Beckman LS6000 TA Liquid Scintillation Counter, Beckman Instruments Inc., Fullerton, USA) after adding 15 mL of Ecoscint A scintillation cocktail (National Diagnostics, Hessle, UK) to the 5 mL CPF samples and 10 mL Ecoscint A to the 1 mL PCP samples. Samples were counted three times for 5 minutes. Sample counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

Analysis of standard toxicity tests

Probit analysis (PROBIT version 7.0a, Central Science Laboratories, York, UK) was used to calculate 48hr-LC50 values from standard toxicity tests.

Statistical indicators of model performance

The goodness of fit was assessed using the mean error and $r^2$ (Table 4-2). The mean error (ME) was calculated as:

$$ ME = \frac{1}{n} \sum_{i=1}^{n} |Model_i - Observed_i | $$

where $n$ is the number of observations and $Model$ and $Observed$ are the modeled survival probability and the observed fraction surviving respectively (both expressed as % of initial population). This statistic has most practical value because it tells us about the average deviation of the model from reality across all observation times.
Appendices

Plots of intermediate calculations in the TDM (model evaluation)

The following plots (Fig. A-1 to A-4) illustrate the intermediate steps of the TDM simulations with the parameter set that we use for model evaluation. The time course of the internal concentration (equation 4-1), the damage (equation 4-2), the hazard rate (equation 4-3) and the cumulative hazard \(H(t)\) (appears in equation 4-4) are plotted for all treatments of experiments A to D. The plot of the damage (second from left) also indicates the value of the *threshold* (dotted line and printed value).

![Intermediate plots](image)

**Figure A-1: Intermediate steps as simulated by the TDM for experiment A.**

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (\(C_{\text{int}}(t)\), eq. 4-1), damage (\(D(t)\), eq. 4-2), hazard rate (\(dH(t)/dt\), eq. 4-3) and cumulative Hazard (\(H(t)\)).
Figure A-2: Intermediate steps as simulated by the TDM for experiment B.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (C\text{int}(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate (dH(t)/dt, eq. 4-3) and cumulative Hazard (H(t)).
Figure A-3: Intermediate steps as simulated by the TDM for experiment C.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (Cint(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate (dH(t)/dt, eq. 4-3) and cumulative Hazard (H(t)).
Figure A-4: Intermediate steps as simulated by the TDM for experiment D.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (Cint(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate (dH(t)/dt, eq. 4-3) and cumulative Hazard (H(t)).
Plots of residuals and correlation (model evaluation)

These plots (Fig. A-5 to A-8) are graphical representations of the goodness of fit corresponding to the statistical indicators for the goodness of fit (Table 4-2). The correlation plots correspond to the $r^2$ value and the residual plots show the same residual that is used to calculate the mean and maximum errors (see above).

Figure A-5: Correlation plots (top) and plots of the residuals (bottom) for experiment A.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 ($\times$), 2 (○) and 3 (□).
Figure A-6: Correlation plots (top) and plots of the residuals (bottom) for experiment B.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Figure A-7: Correlation plots (top) and plots of the residuals (bottom) for experiment C.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Figure A-8: Correlation plots (top) and plots of the residuals (bottom) for experiment D.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Optimization parameter settings in ModelMaker 4

Convergence change: 0.1
Convergence steps: 5
Retry count: 5
Initial lambda: 0.01
Minimum change: $10^{-5}$
Fractional change: 0.01

Starting values for the calibration of the TDM

Starting values for the parameters, as required by the fitting algorithm, were obtained from independent knowledge. Assuming an approximate recovery half-life of 7 days for CPF and 0.2 days for PCP yields approximate values for $k_r$ and by fixing the threshold at 0 we could fit the model to our measured 48hr-LC50 values and thus obtain an initial guess value for $k_k$. The initial value of threshold was set to the value of $D(0.5)$ in the simulation of the 48hr-LC50 experiment (after 0.5 days mortality starts in the simulation). Hence the initial values for the parameter estimation were:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Chlorpyrifos</th>
<th>Pentachlorophenol</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killing rate constant</td>
<td>$k_k$</td>
<td>0.257</td>
<td>0.047</td>
<td>$g_{\text{wet. w.}} \times \text{day}^{-1} \times \mu g_{\text{a.i.}}^{-1}$</td>
</tr>
<tr>
<td>Recovery rate constant</td>
<td>$k_r$</td>
<td>0.1</td>
<td>3</td>
<td>day$^{-1}$</td>
</tr>
<tr>
<td>Threshold</td>
<td>threshold</td>
<td>0.07</td>
<td>0.138</td>
<td>-</td>
</tr>
</tbody>
</table>
Experiments A to D simulated with parameter set from fit to all experiments

FIGURE A-9. Chlorpyrifos concentrations and survival of *Gammarus pulex* in experiment A.

The survival plot shows the observed survival (○) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model predicting survival on the basis of the 48hr-LC50 (dotted line). These simulations use the parameter set from the fit to all data.
FIGURE A-10. Chlorpyrifos concentrations and survival of *Gammarus pulex* in experiment B.

The survival plot shows the observed survival (O) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model simulating survival on the basis of the 48hr-LC50 (dotted line). The exposure pattern in treatment 1 consists of pulses of six hours duration, the pulses in treatment 2 last four days and treatment 3 consists of very low, fluctuating concentrations. These simulations use the parameter set from the fit to all data.
FIGURE A-11. Pentachlorophenol concentrations and survival of *Gammarus pulex* in experiment C.

The survival plot shows the observed survival (○) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model predicting survival on the basis of the 48hr-LC50 (dotted line). These simulations use the parameter set from the fit to all data.
FIGURE A-12. Pentachlorophenol concentrations and survival of *Gammarus pulex* in experiment D.

The survival plot shows the observed survival (•) and its standard error (n=5), the fitted threshold damage model (solid line), the fitted time-weighted averages model (dashed line) and the time-weighted averages model simulating survival on the basis of the 48hr-LC50 (dotted line). These simulations use the parameter set from the fit to all data.
Appendices

Plots of intermediate calculations (TDM) with parameter set from fit to all experiments

The following plots (Fig. A-13 to A-16) illustrate the intermediate steps of the TDM simulations using the robust parameter set. The time course of the internal concentration (equation 4-1), the damage (equation 4-2), the hazard rate (equation 4-3) and the cumulative hazard $H(t)$ (appears in equation 4-4) are plotted for all treatments of experiments A to D. The plot of the damage (second from left) also indicates the value of the threshold (dotted line and printed value).

![Figure A-13: Intermediate steps as simulated by the TDM for experiment A.](image)

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (Cint(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate ($\frac{dH(t)}{dt}$, eq. 4-3) and cumulative Hazard ($H(t)$). 

Figure A-14: Intermediate steps as simulated by the TDM for experiment B.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (Cint(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate (dH(t)/dt, eq. 4-3) and cumulative Hazard (H(t)).
Figure A-15: Intermediate steps as simulated by the TDM for experiment C.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration (Cint(t), eq. 4-1), damage (D(t), eq. 4-2), hazard rate (dH(t)/dt, eq. 4-3) and cumulative Hazard (H(t)).
Figure A-16: Intermediate steps as simulated by the TDM for experiment D.

Treatments 1 to 3 from top to bottom and from left to right: internal concentration \((\text{Cint}(t), \text{eq. 4-1})\), damage \((\text{D}(t), \text{eq. 4-2})\), hazard rate \((\text{dH}(t)/\text{dt}, \text{eq. 4-3})\) and cumulative Hazard \((\text{H}(t))\).
Plots of residuals and correlation (with parameter set from fit to all data)

These plots (Fig. A-17 to A-20) are graphical representations of the goodness of fit corresponding to the statistical indicators for the goodness of fit (Table 4-2). The correlation plots correspond to the $r^2$ value and the residual plots show the same residual that is used to calculate the mean and maximum errors (see above).

Figure A-17: Correlation plots (top) and plots of the residuals (bottom) for experiment A.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Figure A-18: Correlation plots (top) and plots of the residuals (bottom) for experiment B.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Figure A-19: Correlation plots (top) and plots of the residuals (bottom) for experiment C.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).
Figure A-20: Correlation plots (top) and plots of the residuals (bottom) for experiment D.

From left to right: the fitted Threshold Damage Model (TDM), the fitted time-weighted averages model (TWA pulses) and the simulation of the time-weighted averages model based on the 48hrs-LC50 (TWA LC50). All plots show treatments 1 (×), 2 (○) and 3 (□).