Modeling receptor kinetics in the analysis of survival data for organophosphorus pesticides

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Abstract - Acute ecotoxicological tests usually focus on survival at a standardized exposure time. However, LC50s decrease in time in a manner that depends both on the chemical and the organism. DEBtox is an existing approach to analyze toxicity data in time, based on hazard modeling (the internal concentration increases the probability to die). However, certain chemicals elicit their response through (irreversible) interaction with a specific receptor, such as inhibition of acetylcholinesterase (AChE). Effects therefore do not solely depend on the actual internal concentration, but also on its (recent) past. In this paper, the DEBtox method is extended with a simple mechanistic model to deal with receptor interactions. We analyzed data from the literature for organophosphorus pesticides in guppies, fathead minnows and springtails. Overall, the observed survival patterns do not clearly differ from those of chemicals with a less-specific mode of action. However, using the receptor model, resulting parameter estimates are easier to interpret in terms of underlying mechanisms, and reveal similarities between the various pesticides. We observed that the no-effect concentration estimated from the receptor model is basically identical to the value from standard DEBtox, illustrating the robustness of this summary statistic.

Introduction

The initial tiers of a chemical risk assessment usually rely on acute survival experiments with standard test species such as daphnids, fish, earthworms or springtails. The current test protocols have been optimized to produce simple numbers such as the LC50 at a rather arbitrarily standardized exposure time (e.g. two days for an acute Daphnia test and four days for fish). Such standardization may be helpful to streamline the risk assessment process, but is by no means scientifically defensible. It is generally accepted that internal concentrations are better predictors for effects than external ones (see e.g. *1, 2*). As a result, the development of toxic effects in time will depend upon the build-up of body residues in time (toxico-kinetics). Toxico-kinetics depend on properties of the exposed organisms such as body size and lipid content (*3, 4*) or biotransformation capacity, and on properties of the compound such as hydrophobicity for organic chemicals (*5*). From this argument, it is clear that a single representative exposure time for all chemicals cannot be given; the test duration should depend on the organism selected (species, as well as body size), and on chemical properties. This was already recognized by Sprague (*6*) in 1969, who advised to continue the acute test until mortality ceases (to yield an incipient LC50). Clearly, exposure time must be explicitly included as a variable in the analysis.

First steps towards a description of survival in time were based on the concept of Critical Body Residues (CBR, see *1, 7*). Basically, this approach assumes that an organism dies (instantly) when an internal threshold concentration is exceeded (usually expressed as whole body concentration). Because animals differ in sensitivity, not all animals die at the same time; the slope of the dose-response is thus a measure of the variation in sensitivity of the tested population. However, these assumptions can be questioned, for example, the large difference in sensitivity needed to explain the dose-response curves is not supported by the variation observed in sub-lethal toxicity tests (*8*). It seems therefore more likely that mortality is stochastic at the level of the individual, and not deterministic. An alternative stochastic description of mortality is given by the DEBtox model (*9*), which is based on hazard modeling (the actual internal concentration increases the probability to die).

Both the CBR and DEBtox approaches assume that the time course of the body residues determines the dynamics of toxicity. However, this may not be true for all chemicals, especially when a compound interacts irreversibly with a specific receptor. Organophosporus (OP) esters form a large group of broad-spectrum insecticides. Even though OPs have been around for a long time, they are still the most widely used insecticides today (in 2001, this group made up 70% of the insecticide sales in the United States (*10*)). These compounds bind to acetylcholinesterase (AChE), inhibiting the action of this enzyme, causing a buildup of the neurotransmitter acetylcholine, and thereby killing the organism (*11*). This inhibition is often considered to be irreversible, and it is therefore not the actual internal concentration that is important in describing the effects, but also the exposure history. An extension of the CBR model was presented by Legierse et al. (*12*), assuming that instant death occurs when a critical number of targets is irreversibly occupied: the Critical Target Occupation (CTO) model. Another extension, allowing for a more flexible link between internal concentrations and toxic effects, the Damage Assessment Model (DAM), was developed by Lee et al. (*13*). These authors extended the CBR model with an additional first-order accumulation of “damage”, which is able to accommodate reversible as well as irreversible effects. In this paper, we present an extension of the DEBtox model to deal with receptor kinetics, and re-analyze the data sets for guppies presented by Legierse et al. (*12*), as well as data for fathead minnows (*14*), and springtails (*15*).

Methods

The DEBtox model. The theory of Dynamic Energy Budgets (DEB) (*16*) describes how individuals acquire and utilize energy, based on a set of simple rules for metabolic organization. As a spin-off, DEBtox was developed to provide a process-based description of results from toxicity tests (*4*, *9*). DEBtox explicitly considers exposure time, and effects data are fitted in the time-concentration plane. In the CBR, CTO and DAM models, mortality is deterministic whereas the animals differ in sensitivity; in the DEBtox approach, the animals have equal sensitivity, but mortality itself is a chance process. Instead of instant death when exceeding a threshold, it is assumed that death is an inherently stochastic process. The truth is probably somewhere in between these two views, although dedicated fish studies showed that the stochastic component dominated for mortality (*17*). The stochastic DEBtox approach also has more intuitive appeal, given the painstaking effort of ecotoxicologists to standardize their test organisms (e.g. by using clones), and the fact that sub-lethal effects, such as reproduction, do not reveal the substantial differences in sensitivity among individuals that would be necessary to explain the survival patterns (*8*).

To cause a toxic effect, the chemical first needs to be taken up into the organisms (Figure 1). Subsequently, the internal concentration increases the probability to die, when the internal concentration exceeds a threshold: the no-effect concentration or NEC (*9*). This stochastic view of mortality results in an LC50 that decreases in time, and a slope that increases. Eventually, all animals exposed above their NEC will die, as even a small hazard rate will lead to a large probability to die after a long waiting time (thus eventually resulting in a very steep dose-response curve). This corresponds with the classical insight from toxicology that effects should be related to the product of concentration and exposure time. The standard DEBtox approach assumes that the internal concentration directly affects mortality (and thus reversible interactions with the target), but includes a form of history (an organism that has died will not come alive). Mortality can be directly linked to internal concentrations with this approach, as was shown for cadmium in *Daphnia magna* (*18*).

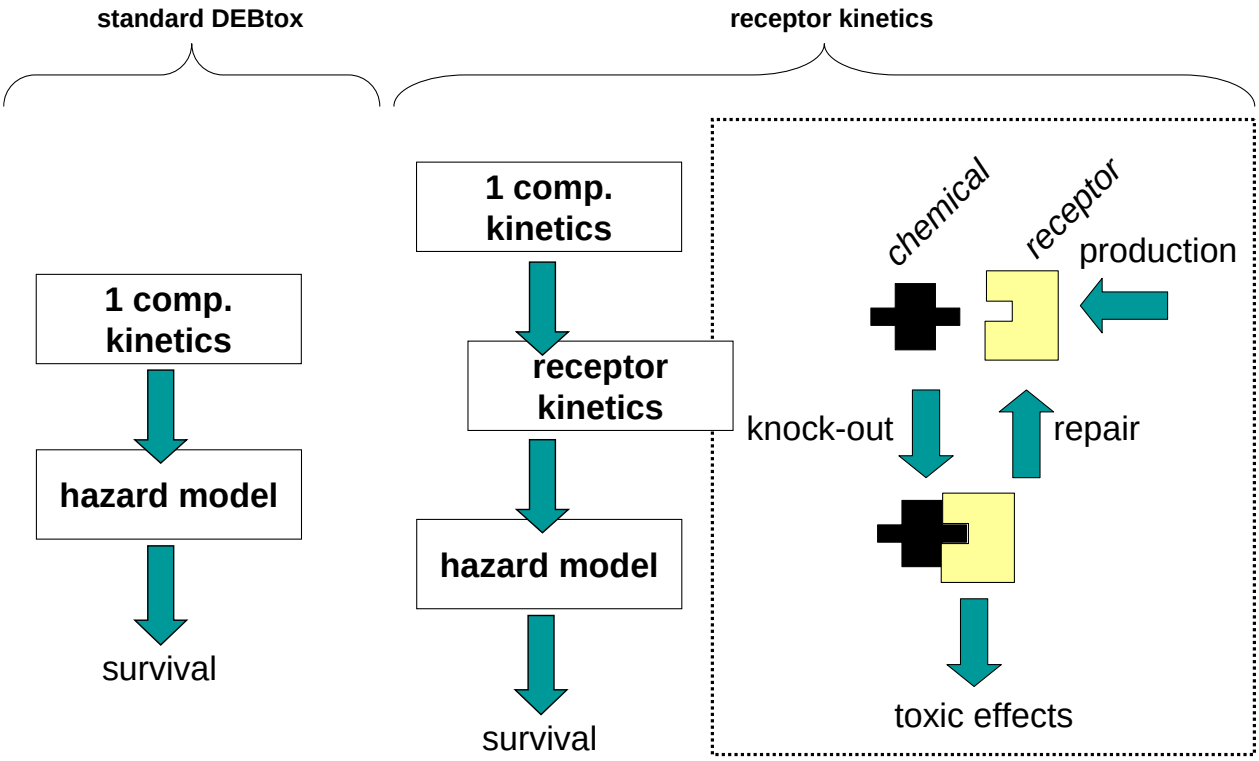

FIGURE 1. Basic structure of DEBtox and the extended model with receptor kinetics.

DEBtox receptor model. The receptor model is based on the model proposed earlier (*16*), but is here extended to deal with growing organisms. Growth must be accounted for in chronic experiments, but can usually be ignored for acute studies (because the animals are not fed). A consequence of the DEB theory is that animals grow according to a Von Bertalanffy curve as long as the environment remains constant:

$$\frac{d}{dt} l = r_B (1 - l) \qquad (1)$$

where r_B is the growth rate constant (d^{-1}) and l is the scaled length (dimensionless), i.e. the actual length divided by the maximum length. Any length measure can be used, as long as the animal does not change in shape, such as whole body length, length of a specific organ, or the third root of body weight (assuming a constant density). This curve was found to describe body size data for a huge range of organisms, from yeast to mammals (*16*).

The internal concentration is assumed to follow simple one-compartment kinetics, but accounting for the effects of body growth. Growth acts to dilute the internal concentration, but also affects the elimination rate constant (k in d^{-1}) via the ratio of surface area to body volume (*16*):

$$\frac{d}{dt} c_v = \frac{k}{l} (c_d - c_v) - c_v \frac{d}{dt} \ln l^3 \qquad (2)$$

where c_d is the external concentration (e.g. µM), and c_v is the scaled tissue concentration (internal concentration divided by the bioconcentration factor, to yield external concentration equivalents (e.g. µM), see (*4*)). The last term stands for growth dilution (note that $d/dt \ln l^3$ is the relative growth rate on volume basis).

Functional receptors in the organism are knocked out by the chemical, which produces non-functional ones. The total number of receptors is a constant fraction of the body volume (thus implying a net production of new functional receptors when the animal grows):

$$N_+ = N_n + N_f = n_+ l^3 \tag{3}$$

Where N_+ is the total number of receptors, and n_+ is the total number of receptors in a fully grown animal (thus when l = 1), which is assumed constant. The number of functional and non-functional receptors is given by N_f and N_n, respectively. Functional receptors are turned into non-functional ones by reaction with the chemical. Thus, the production rate of non-functional receptors depends on the meeting frequency between functional receptors and the molecules of the compound inside the organism. Non-functional receptors may be repaired, either by removing the inhibitor from the receptor, or by general receptor turnover (replacing "old" receptors by new ones). This leads to the following differential equation for the number of non-functional receptors:

$$\frac{d}{dt} N_n = b_{fn} c_v N_f - r_{nf} N_n \tag{4}$$

where b_{fn} is the affinity for the receptor, or the "knock-out" rate (e.g. $\mu M^{-1} \cdot d^{-1}$), and r_{nf} the specific recovery rate (d^{-1}). Using Eq. 3, the differential equation can be written in terms of N_+:

$$\frac{d}{dt} N_n = b_{fn} c_v N_+ - \left(r_{nf} + b_{fn} c_v \right) N_n \tag{5}$$

Dividing both sides by N_+ gives an equation for the number of non-functional receptors, as fraction of the total number of receptors ($f_n = N_n/N_+$):

$$\frac{d}{dt} f_n = b_{fn} c_v - \left(r_{nf} + b_{fn} c_v + \frac{d}{dt} \ln l^3 \right) f_n \tag{6}$$

The last term between brackets accounts for dilution of non-functional receptors by body growth, which enters the equation because N_+ is not a constant, but depends on body size (Eq. 3).

Effects on endpoints such as survival can be related to the fraction of receptors that is occupied. In this way, receptor occupancy can increase the probability of dying through the hazard rate (h_n in d^{-1}):

$$h_n = b_n \left(f_n - c_0 \right)_+ \tag{7}$$

where c_0 is the no-effect concentration (as occupied fraction of receptors, dimensionless), the + indicates that the maximum of the expression between brackets and zero must be taken; in other

words, the hazard rate h_n is zero when receptor occupation is below the NEC ($f_n < c_0$), and proportional to $f_n - c_0$ when receptor occupation exceeds the NEC ($f_n > c_0$). The proportionality constant is called the killing rate (b_n in d^{-1}). This relationship is directly comparable to the effects of non-receptor chemicals in DEBtox, where the scaled internal concentration (c_v) is applied instead of f_n (see *9*). The hazard rate due to receptor occupation can easily be added to the hazard rate due to other independent causes of death (e.g. senescence or other compounds). The hazard rate relates to the survival probability (S, as function of concentration and time) as:

$$S(c,t) = \exp\left[-\int_0^t h_n(c,\tau) d\tau \right] \tag{8}$$

Equations 1, 2 and 6-8 were implemented in MatLab® (version 7.0), and solved with an ODE solver, the scripts used to calculate the model are part of the DEBtool collection that can be obtained from http://www.bio.vu.nl/thb/deb/deblab/. Parameters are fitted using maximum likelihood estimation (see *9*), confidence intervals were generated using profile likelihoods (see *19*), and the performance of the standard DEBtox was compared to the receptor model using the likelihood ratio test.

Difference with the CTO and DAM models. In contrast to the CTO model, the presented receptor model can deal with irreversible as well as reversible interactions, because it includes a repair rate (which can be set to zero for irreversible interactions). Truly irreversible binding in a static receptor pool would mean that the incipient LC50 is zero, because even low internal concentrations would eventually lead to the binding of all available receptors. Because an incipient LC50 is usually observed in experiments, an LC50(∞) was added in the CTO model, in a rather ad hoc fashion. The DEBtox receptor model yields a non-zero incipient LC50 when the repair rate is larger than zero.

The dynamic receptor stage in our model is comparable to the state of "damage" as used in the DAM. However, we applied a model based on the meeting frequency between internal compound and receptor (Eq. 4), where the DAM assumes a first-order process (at low receptor occupation, this yields similar results). Furthermore, we link receptor occupation to survival using hazard modeling, where the CTO and DAM assume death occurs when the cumulated damage or receptor occupation exceeds a certain threshold. Lee et al. (*13*) also present a combination of the DAM model with hazard modeling, but we do not agree with their approach. These authors take the *cumulative* hazard proportional to the damage (instead of the hazard rate, compare with Eq. 7). This implies that hazard is proportional to the *change* in damage with time, which has the physically impossible consequence that the hazard rate will become negative when damage decreases in time. Therefore, the DAM should be seen as a more general case of the CBR and CTO models, and not as a variation of the DEBtox approach.

Finally, both the CTO and DAM require fitting empirical dose-response curves to the survival data at each individual time step to estimate LC50s. Apart from the inefficiency of this approach (in terms of numer of estimated parameters) it is also prone to error, as will be discussed later in this paper. In the DEBtox method, the survival data themselves are used; the dose-response curves result from the underlying models, thereby making full use of all the information in the data set.

Results and discussion

Re-analysis of Guppy data. The data set for the guppy (*Poecilia reticulata*) as used by Legierse et al. (*12*) originates from De Bruijn & Hermens (*20*). We were able to recover the original survival data for five compounds: azinophos-methyl, malathion, methidathion, phentoate and phosmet. The dataset comprises the results for ten animals per concentration, and survival is reported on each day for a total exposure period of 14 days. As the mode of action of these compounds involves receptor interactions, we would expect that the standard DEBtox model would not be able to describe the results. However, the fit of the standard model without receptor kinetics is already very good (Fig. 2 for azinophos-methyl, and supporting information), with only three parameters: elimination rate, no-effect concentration, and killing rate. Apparently, the pattern of mortality in time for OP pesticides is not essentially different from that of chemicals with a less specific mode of action. Therefore, the survival pattern itself will not necessarily reveal a mode of action that differs from simple reversible interactions.

Legierse et al (*12*) state that the pattern of LC50s versus time clearly favors the CTO model above the CBR model, a conclusion that solely rests upon the choice to fix the elimination rate in the CBR and CTO model to the value dictated by a QSAR. The estimate for the elimination rate derived from the DEBtox analysis is approximately a factor of 10 below this estimate. Even though the QSAR may not be representative (derived for chlorobenzenes, and perhaps different fish species and/or size), we would expect to see higher elimination rates for these compounds (as they tend to be biotransformed (*21*)), and not lower ones. Thus, despite the excellent fit, the DEBtox elimination rate appears to be unrealistically low.

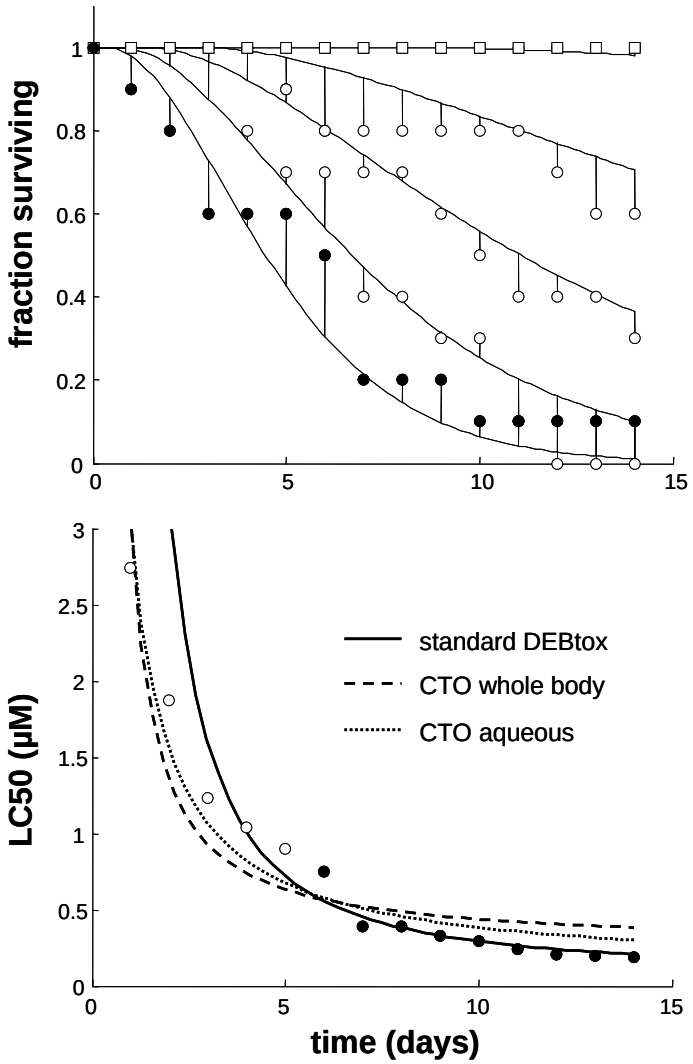

FIGURE 2. Effects on survival of guppies exposed to azinophos-methyl. Top: data and standard DEBtox fit, lines are the various exposure levels; lowest concentration indicated by open squares, highest concentration by black circles. Bottom: iso-effect lines with LC50s (symbols) and estimated curves from standard DEBtox and the CTO model. LC50s represented by open symbols are extrapolated (there is less than 50% mortality at days 1-5).

Model performance is often demonstrated by plotting LC50s versus time, although this procedure is prone to errors. In Figure 2, the DEBtox LC50-time relationship is compared to the CTO model for azinophos-methyl (both the "whole-body" and the "aqueous" models, the latter assuming instantaneous equilibrium between organism and medium). Clearly, the DEBtox model performs better for the LC50s after day 6, but shows a prominent misfit for the earlier time points. However, the LC50s for day 1-5 are based on extrapolation of effects (there is less than 50% effect at the highest tested concentration at those time points), and are therefore unreliable (plotting the confidence intervals with the LC50s would clarify this). In fact, at the first 3 days of exposure, there is only one dose that shows effects, which is clearly insufficient to estimate an LC50. This analysis also requires a large number of parameters to be estimated from the data (30 in total; two for the dose-response at each time point, and two for the CTO model), because

individual dose-response curves are fitted to the data. Such a model analysis makes inefficient use of the information in the data sets, and the parameters estimates (and their confidence intervals) should be questioned as the uncertainty in the LC50 is ignored for the estimation of the CBR, CTO or DAM parameters.

Compared to the standard DEBtox model (as applied for Fig. 2), the receptor model requires two additional parameters to be estimated from the data (the knock-out and repair rates), which bring the total number of parameters to five. When only short-term mortality data are available, this number of parameters is too high to obtain accurate estimates. However, we have reason to believe that several of the parameter values must be equal for all compounds. To start with, we may take the elimination rate to be infinitely large, to mimic the situation where the aqueous phase inside the organism is important for receptor binding (as was done in the aqueous CTO model (*12*)). Furthermore, the no-effect concentration (c_0) and the killing rate (b_n) relate the fraction of occupied receptors to the toxic effects. When we assume that it does not matter whether a receptor is occupied by compound A or B, it follows that, for a given species, these two parameters must be the same for all AChE inhibitors that share the same active site. Similarly, it is realistic to assume that also the receptor repair rate will be equal for all compounds (e.g., representing reactivation and *de novo* synthesis of the enzyme). In effect, the only parameter that will be chemical dependent is the knock-out rate (e.g. representing differences in metabolic activation, or different affinities to the receptor). Therefore, the five compounds need to be analyzed simultaneously, with a reduced set of parameters.

The simultaneous analysis resulted in a reasonable fit (Fig. 3), although additional assumptions were needed for malathion and phosmet, which show a peculiar deviation from the expected pattern. This pattern of mortality in the low concentrations halfway in the experiment, stopping after a couple of days, is an indication of a decrease in the hazard rate. This can either be caused by degradation of the compound in the exposure medium (which is unlikely since the experimental set up included renewal of the test medium), or induced biotransformation. Biotransformation of OP-pesticides in guppies is likely (*22*), and may even be concentration dependent (*23*) (which would fit even better with the observed mortality patterns of malathion and phosmet). Since both processes have a similar effect, we included a first-order degradation rate constant in the model for these two compounds, resulting in an adequate fit, specifically for the second half of the data (t > 7 days) at low concentrations (Fig. 3, Table 1). The resulting fit is slightly poorer than the standard DEBtox fit, but not significantly so, as less parameters have been estimated (p>0.10, likelihood-ratio test, see supporting information). The repair rate is greater than zero, indicating a reversibility of the receptor binding. However, the corresponding half life is approximately 4 days, and could result from *de novo* synthesis of AChE. The NEC, killing rate, and knock-out rate are still poorly defined by the data, indicated by the broad confidence intervals. A large confidence interval implies that the model fit is not sensitive to the value of that parameter. However, this does not mean that the values of these parameters are unimportant, because the confidence intervals will be highly correlated. To understand this, it must be clarified that the shape of the curve for occupied receptors in time depends solely on the repair rate, at least for low values of occupation (when N_f in Eq. 4 is nearly constant). A high value for the knock-out rate (b_{fn}) leads to higher levels of receptor occupation, but with the same shape in time. To fit the data, a high value of the knock-out rate can therefore easily be compensated by a higher NEC and a lower killing rate (see Eq. 7). Survival data alone will therefore not generally be sufficient to accurately determine the NEC, killing rate and knock-out rate individually.

The simultaneous assessment shows that these chemicals can indeed be assumed to share the same receptor mechanism, and differ only in their affinity for the receptor. The intrinsic toxicity increases in the following order malathion<phosmet<azinophos-methyl<methidation<phentoate, but differs less than a factor of 6. The parameter estimates show that no effects of these compounds on survival are expected as long as the receptor occupation is below 10.8%. Nevertheless, the inclusion of the "degradation" rate is speculative and deserves further attention.

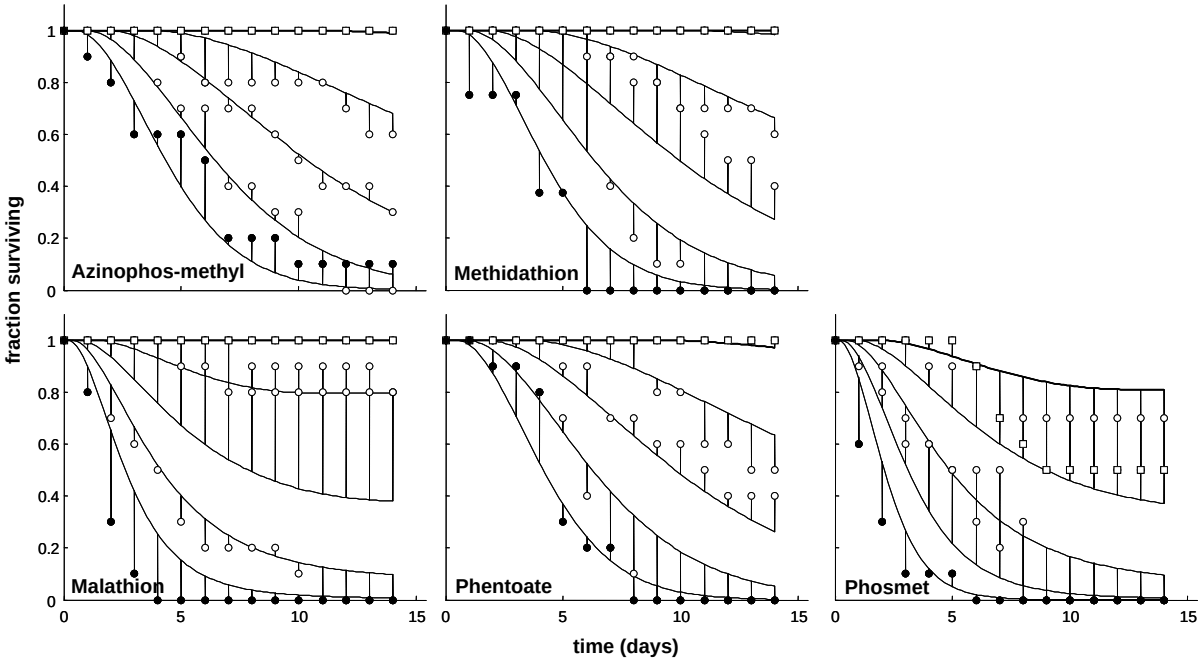

FIGURE 3. Data and fit for the DEBtox receptor model (parameters in Table 1), for the effects on survival of guppies exposed to five organophosphorus pesticides. Lines are the various exposure levels; lowest concentration is indicated by open squares, highest concentration by black circles.

TABLE 1. Parameter estimates for the model fits on the dataset for organophosphorus pesticides in guppies and fathead minnows, using the DEBtox receptor model (Fig. 3 and 4). Values between brackets are the 95% likelihood-based confidence intervals (a < sign implies that the interval extends to, but does not include, zero; a > sign means that the interval extends to infinity), n.e. is not estimated.

Guppies	**Azinophos-methyl**	**Malathion**	**Methidathion**	**Phentoate**	**Phosmet**
Elimination rate (k), d^{-1}	∞ (n.e.)				
No-effect conc. (c_0), (-)	0.00160 (<0.108)				
Killing rate (b_n), d^{-1}	36.7 (>0.72)				
Knock-out rate (b_{fn}), $\mu M^{-1}.d^{-1}$	0.00404 (<0.321)	0.00143 (<0.101)	0.00736 (<0.526)	0.00785 (<0.623)	0.00226 (<0.164)
Repair rate (r_{nf}), d^{-1}	0.170 (0.0693-0.291)				
"Degradation" rate, d^{-1}	0 (n.e.)	0.306 (0.133-1.78)	0 (n.e.)	0 (n.e.)	0.193 (0.0547-0.518)
Blank hazard, d^{-1}	0 (n.e.)				

Fathead minnows	**Terbufos**	**Disulfoton**	**Chlorpyrifos**	**Diazinon**	**Malathion**
Elimination rate (k), d^{-1}	∞ (n.e.)				
No-effect conc. (c_0), (-)	0.0113 (<0.0248)				
Killing rate (b_n), d^{-1}	73.7 (>33.5)				
Knock-out rate (b_{fn}), $\mu M^{-1}.d^{-1}$	25.5 (<56.1)	0.0663 (<0.146)	1.00 (<2.21)	0.0228 (<0.0501)	0.000542 (<0.00119)
Repair rate (r_{nf}), d^{-1}	42.8 (>17.2)				1.37 (0.712-2.42)
"Degradation" rate, d^{-1}	0.763 (0.319-1.23)	0.302 (0.233-0.445)	0 (n.e.)	0 (n.e.)	0 (n.e.)
Blank hazard, d^{-1}	0.00380 (0.00224-0.00611)				

Data for fathead minnows. Survival data for five OP-pesticides in fathead minnows (*Pimephales promelas*) where obtained from Geiger et al. (*14*). Sub-chronic data were also available for this species, but they only covered body weight at a single exposure time, which is insufficient to fit dynamic models such as ours. The fish (approx. 30 days old, and 20 mm in length) were exposed for four days, and mortality was reported daily. Water concentrations were also measured daily, and the average values were used in the analysis. Firstly, it must be noted that this data set is not as strong as the set for guppies, because survival is followed for four days only. A constant background hazard rate was added (Table 1), mainly because some mortality is observed at low doses for malathion and chlorpyrifos that may not be related to the compound, which leads to a reasonable description of the data (Figure 4). The leveling off of mortality, as was observed for malathion and phosmet (Figure 3), was also prominent for terbufos and disulfoton; little increase in mortality is observed after two days. Again, a "degradation" rate constant is assumed for these compounds. Overall, the fit for these five chemicals is much poorer than the standard DEBtox fit (p<0.01, likelihood-ratio test, see supporting information), which is mainly caused by the particularly poor fit of disulfoton. The fit is such that is can be speculated that this compound exhibits an additional toxic effect, different from AChE inhibition (although stronger data are required). As was the case for the guppy data set, the NEC, killing rate and knock-out rate are not well defined by the data (half-open confidence intervals in Table 1).

Malathion shows a different pattern of mortality from the other compounds. The reason may lie in the fact that the active site of this compound is slightly different. Malathion, as well as all

the compounds tested on guppies, are dimethyl phosphorylated (bearing two methoxy groups on the phosphorus atom), whereas the other compounds are diethyl phosphorylated. This should not affect the NEC or the killing rate (as these are based on receptor occupation), but may affect the rate at which the receptor is reactivated. Therefore, this compound is allowed to have a different repair rate. The repair rate for the diethyl compounds is very high, indicating highly reversible receptor interactions. On the other hand, the repair rate for malathion is much lower, and more in line with guppy set (which were all dimethyl compounds). Apparently, AChE inhibited by the diethyl compounds is more easily reactivated. Interestingly enough, dedicated studies indicate that this should be the other way around (*24, 25*), although diethyl phosphorylated AChE was more readily reactivated by oximes (*26*). However, in our model, assuming irreversible binding for diethyl OPs leads to very poor fits indeed (see supporting information). The reason for this discrepancy is yet unclear, although it is clear that the diethyl OPs show a quite different pattern of mortality from the monoethyl OPs.

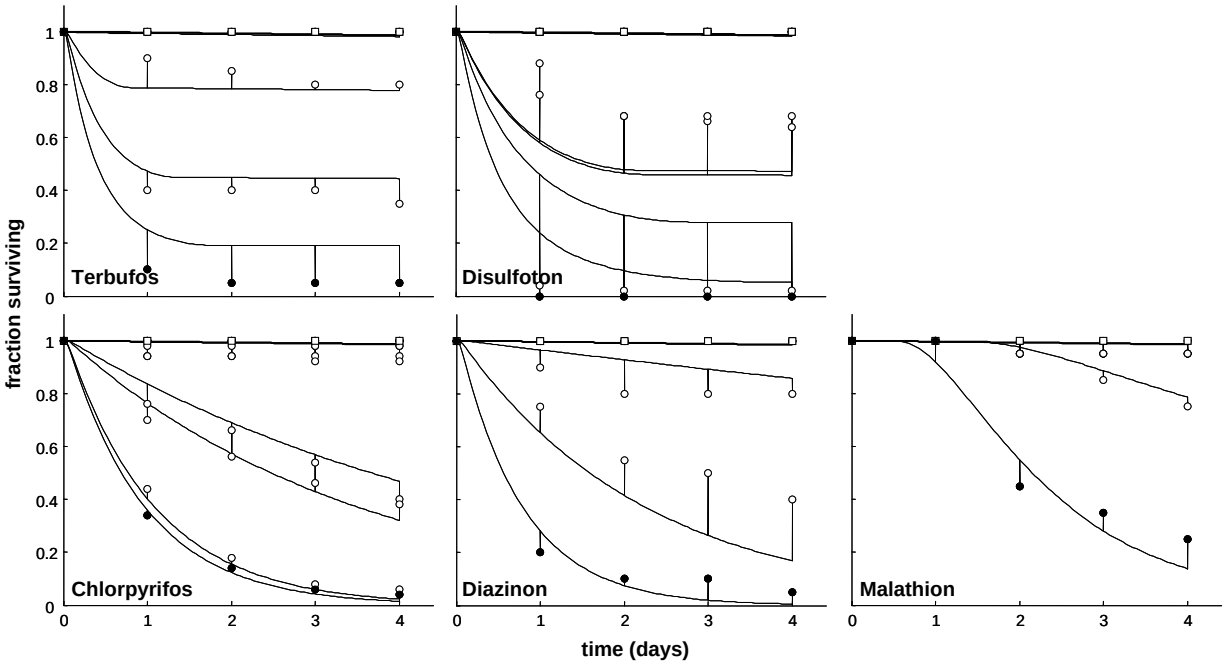

FIGURE 4. Data and fit for the DEBtox receptor model (parameters in Table 1), for the effects on survival of fathead minnows exposed to five organophosphorus pesticides. Lines are the various exposure levels; lowest concentration is indicated by open squares, highest concentration by black circles.

Data for chlorpyrifos in springtails. The dataset for chlorpyrifos (CPF) in the springtail *Folsomia candida* is taken from Crommentuijn et al. (*15*). The experiments start with juveniles, and the animals are provided with CPF-spiked food. In contrast with the guppies and minnows (that were not fed), the springtails grew rapidly during the experiment (see supporting information). Therefore, the effect of growth on toxico-kinetics (Eq. 2) and receptor dynamics (Eq. 6) needs to be considered. Although the dataset comprises the full life cycle, we will only use survival in the first 45 days to focus on the performance of the receptor model. CPF had no effect on the growth pattern, not even at concentrations that seriously affect survival and reproduction (*15*). Therefore, we can use a single growth curve for all exposure concentrations (see Eq. 1, curve and parameter estimates in supporting information). The survival data are

shown in Figure 5, together with the model fits for the standard hazard model, as well as the receptor model (parameter estimates shown in Table 2). At the cost of two additional parameters, the receptor model provides a better description of the data (significant, $p<0.01$ using a likelihood-ratio test), especially for the initial part of the curves. The data show no effect in the first 10 days of exposure, which could not be properly covered by the standard hazard model. Nevertheless, the visual improvement of the receptor model is very limited. Again, the fact that CPF likely acts through a receptor mechanism is not directly obvious from the survival pattern. The only argument against the standard DEBtox model is the (probably unrealistically) low elimination rate.

For the guppy data set, we assumed an infinitely large elimination rate. For the *Folsomia* data, elimination is a free parameter, but the estimated value is very high and not significantly different from infinite (instantaneous equilibrium between organism and medium). Despite the fact that the receptor model does not lead to a radically different fit, we can obtain some support for this interpretation of the toxic mechanism from the overlapping confidence intervals of the NEC for springtails (Table 2), guppies and fathead minnows (Table 1). However, the killing rate based on receptor occupation is much higher for the fish species. Interestingly, the repair rate for the springtail AChE is more in line with the results for the dimethyl OPs in fish than for the di-ethyl OPs (such as CPF tested on the fathead minnows).

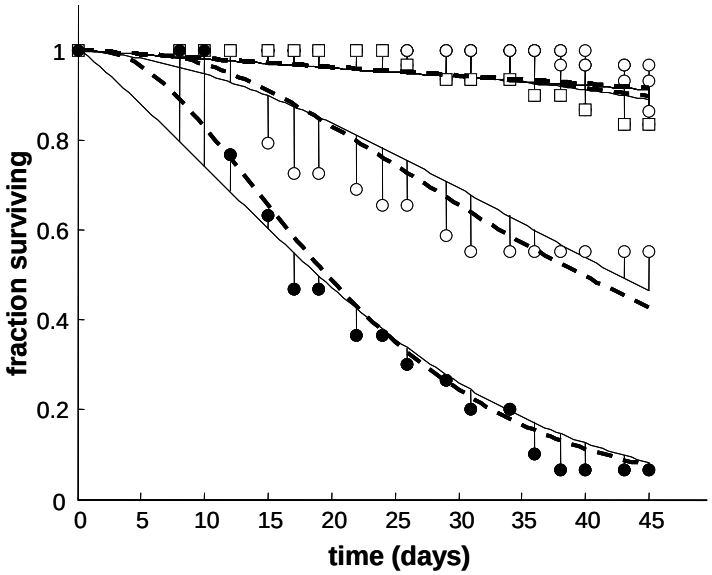

FIGURE 5. Data and DEBtox fits for effects of chlorpyrifos on survival of growing springtails (*Folsomia candida*). Control response is shown as squares, highest concentration as black circles. Solid lines are the standard DEBtox fits, broken line is fit using receptor kinetics.

TABLE 2. Parameter estimates with likelihood-based 95% confidence intervals for the survival data of chlorpyrifos in growing springtails (*Folsomia candida*).

	Standard model	**Receptor model**
Elimination rate (k)	0.0732 (0.0104-0.309) d^{-1}	2.48 (0.120-∞) d^{-1}
Blank hazard rate	2.03 (1.02-3.51)·10^{-3} d^{-1}	2.02 (1.00-3.46)·10^{-3} d^{-1}
No-effect concentration (c_0)	2.77 (0.651-5.90) mg·kg_{food}^{-1}	0.0216 (0.0119-0.0497) [-]
Killing rate (b_n)	6.50 (3.07-27.1) ·10^{-3} kg_{food}·(mg d)$^{-1}$	1.00 (0.120-1.19) d^{-1}
Receptor knock-out (b_{fn})	-	1.89 (0.996-5.46)·10^{-3} kg_{food}·(mg d)$^{-1}$
Receptor repair (r_{nf})	-	0.318 (0.154-0.888) d^{-1}

Relation to measured AChE inhibition. The estimates for AChE inhibition associated with mortality are usually much larger than the values that are reached in our model. The estimate for the NEC is quite low, and in fact, receptor occupation does not exceed 10% in the model for any exposure concentration (using the maximum likelihood estimates of Table 1 and 2). This is in sharp contrast with the percentage AChE inhibition related to mortality, generally exceeding 70% in brain tissue of fish (*27*). On whole-body basis, guppies survived at 80-90% AChE inhibition (*28*), and in *Daphnia magna*, 50% inhibition was generally associated with immobility (*29, 30*). Such high inhibition values are not consistent with our receptor model, and would lead to poor fits (see supporting information). However, the experimental evidence for the relationship between AChE inhibition and mortality is not very clear. This is especially true for invertebrates (*27, 29, 31*), but also for fish, uncertainty remains (see e.g. *32, 33*). It is possible that AChE in specific organs has different kinetics or is more sensitive than AChE in the brain or measured in the whole body (*28*), and different esterases may differ in sensitivity (*30*). Furthermore, it has been suggested than another target than AChE may be responsible for the toxic effects (*29, 32*), and it was also speculated that high acute exposures may lead to narcotic effects, whereas more time is needed for the AChE effects to emerge (*23*). To make things even more complicated, OPs have also been shown to bind directly to the ACh receptor (see *24*).

Basically, the mechanism for toxicity of OP-esters can be divided into two steps: from external concentrations to AChE inhibition, and from AChE inhibition to mortality. The receptor mechanism that we propose may fail to describe either of these processes. We model the first step quite simplistically (infinitely high elimination rate, direct interaction with receptor in a well-mixed environment), whereas the situation is more complex in reality (e.g. metabolic activation is required for these compounds, and the phosphorylated AChE may also be subject to aging, rendering it irreversibly inhibited). However, a quick comparison between model predictions and literature data for AChE inhibition in coho salmon, exposed for four days to CPF, showed that the model simplifications can provide an adequate description (see supporting information). The most likely candidate for model revision is the second step: from AChE inhibition to mortality. In the current receptor model, the hazard rate is directly related to the fraction of occupied receptor, which is the enzyme AChE. However, effects are likely related to occupation of the ACh-receptor, which is not explicitly modeled. Occupation of the ACh-receptor is likely related to the fraction of functional enzyme, but this relationship probably requires an additional dynamic step.

Evaluation of receptor models. Even though OP pesticides are likely causing acute mortality by inhibiting AChE, the survival pattern in time is not radically different from other compounds with a less specific mode of action. The standard DEBtox model provides an excellent fit to the

data (see supporting information), although the elimination rates estimated from the toxicity data for guppies, minnows and springtails do not appear to be realistic for the whole-body toxicokinetics. This probably means that a more complicated mechanism mimics a simple behavior. From a scientific viewpoint, it is important to try to understand the processes that cause mortality, but for risk assessment purposes, the standard DEBtox model may still be useful. The standard model is able to describe the time course of mortality, and results in a very similar value for the NEC (when receptor occupation is translated back to external concentration). So the NEC is a robust summary statistic, even if the true mechanism is more complicated. However, it must be realized that the estimated elimination rate will not represent the actual toxico-kinetics, but will be a compound parameter, governed by more complex dynamics at the site of action. Therefore, measured internal concentrations cannot be combined with survival data (e.g. as was done in (*18*)).

In this paper, we present a simple mechanistic model, describing the kinetics of receptor occupation and repair that can be included in hazard-based survival models such as DEBtox. The receptor model is also able to describe the survival data for guppies and fathead minnows. Although the goodness of fit is somewhat poorer than for the standard model, less parameters have been used because the survival data for five different compounds were analyzed simultaneously. A single value for the NEC, killing rate, and receptor repair rate (at least for the guppy data) could be used, indicating a similarity in the mode of action between the tested OP-pesticides, and a possible influence of biotransformation for several compounds. For CPF in growing springtails, the receptor model does fit the survival pattern better than the standard hazard model, although the visual improvement is only marginal. The receptor model of DEBtox has several advantages. Firstly, the parameters are directly linked to physical quantities, and the DEBtox approach thereby offers more starting points for dedicated experiments (e.g. when internal concentrations and/or AChE inhibition is measured). Secondly, the receptor model allows for a simultaneous assessment of different chemicals, revealing similarities in the toxic mechanism of various OP pesticides, and revealing differences between diethyl and monoethyl OPs. And finally, the DEBtox receptor model makes more efficient use of the data. For the guppy data set, for example, our analysis requires ten parameters to be estimated for five compounds, an average of two parameters per chemical. In contrast, the CTO model requires 30 parameters per chemical, because individual dose-response curves are fit on every time point. This large efficiency can be obtained because our model directly describes the survival data themselves (without the need for empirical dose-response curves to estimate LC50s), and because the data for five compounds are analyzed simultaneously. By combining different data sets that share parameters, more elaborate models can be fitted to limited experimental results.

In any case, the exact mechanism will be more complicated than the model of Figure 1, and may include e.g. metabolic activation of the compounds, biotransformation, dynamics of the ACh receptor, and aging of the phosphorylated AChE. As already evident from the poor identification of several parameters, the current datasets are insufficient to fit more complicated mechanistic models. However, in combination with other dynamic measurements, such as internal concentrations, AChE inhibition in various organs, and the reversibility of inhibition and effects, it may be possible to make further progress towards a mechanistic model for receptor-mediated toxicity. The presented receptor model contains enough handles to act as a starting point for such investigations. However, even though scientific research is better served by a more complex model, the standard DEBtox method can still be safely applied for risk assessment purposes.

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Supporting information available
Standard DEBtox fits for the guppy and fathead minnow data, and additional fits of the receptor model assuming a NEC of 70% receptor inhibition, and fits assuming truly irreversible binding. Growth curve for *Folsomia candida* exposed to chlorpyrifos, and the description of measured AChE inhibition (equations and model fit on coho salmon data).

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