Simultaneous Modeling of Multiple Endpoints in Life-Cycle Toxicity Tests

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Abstract

Standard toxicity tests do not allow extrapolations to the population level, mainly because these tests apply a short, fixed exposure time, and focus on a single endpoint only. These limitations can be overcome by (partial) life-cycle toxicity testing, although these test results are harder to analyze. DEBtox is an existing software tool for the process-based analysis of standardized bioassays, and this paper presents two extensions of this method, making it applicable to life-cycle tests: the simultaneous assessment of endpoints, and the description of ageing (senescence) of the animals. We demonstrate these adaptations by describing life-cycle tests with the springtail *Folsomia candida*, exposed to cadmium and triphenyltin in their food. The extended model is able to describe the data for all endpoints simultaneously over time with few, physiologically relevant, parameters. Further, the analysis reveals these chemicals to have distinctly different modes of action: cadmium apparently decreases the assimilation of energy from the food whereas triphenyltin increases the maintenance costs. The model fit allows calculation of the intrinsic rate of population increase, integrating effects on survival and reproduction. As the analysis is process based, population responses under food limitation can be explored, which depends critically on the selected mode of action.

Introduction

The standard protocols for laboratory toxicity testing have evolved in the seventies to address the need for consistent and comparable summary statistics for chemical risk assessment. Although this standardization has supported the establishment of risk assessment in the management of toxic chemicals, the usefulness of the test results is not beyond discussion. In general, risk assessors are interested in protecting the environment, and thus populations. However, testing population consequences is too complicated and too expensive for routine applications, and short-term testing with few selected species is used as a surrogate. Unfortunately, the current laboratory tests do not allow useful extrapolations to the population level, for several reasons. Firstly, standard tests usually focus on a single endpoint only (mortality, growth or reproduction), but population effects do not necessarily depend on the most sensitive life cycle trait (*1*), and no single endpoint seems to relate directly to population level effects (*2*). A second problem is that the standard tests apply a fixed exposure time, irrespective of the properties of the chemical under scrutiny. Already in 1969, Sprague (*3*) recognized that a single exposure time for all chemicals cannot be given, and advised to continue the acute test until mortality ceases. It is generally accepted that toxic effects are better understood from internal concentrations (see e.g. (*4, 5*)). However, internal levels are not constant in time and the toxicokinetics depend on chemical properties (especially hydrophobicity for organics (*6*)), as well as organism properties (like body size (*7, 8*)). Because the build-up of body residues is chemical- and species-specific, the test summary (e.g. the LC50 or NOEC at some exposure time) cannot be compared between chemicals and between different species, not even between adults and juveniles of the same species. As an example, differences in accumulation patterns between species were reflected in different patterns of survival in time (*9*).

Some of these problems can be overcome by life-cycle toxicity testing. In these tests, more endpoints are followed over the entire (or partial) life cycle. Although providing a wealth of information, these tests are notoriously hard to analyze. For risk assessment purposes, a summary of the results is needed, but the question arises: which summary figure integrates the test results in the most relevant manner (i.e. representing the sensitivity of the species in field populations)? The standard test summaries (EC10, EC50) can be generated for each endpoint by fitting regression equations at each exposure time, leading to an unmanageable mass of test summaries and inconsistencies. A better summary is the intrinsic rate of population increase (see e.g. (*1, 2*)), integrating all effects into a single, ecologically relevant, number for each concentration. This analysis is still purely descriptive and helps little in understanding the mechanism of toxicity (e.g. no predictions can be made for time-varying exposure or food limitation). To this end, the effects in life-cycle tests need to be described mechanistically, in terms of the underlying processes.

To understand how chemicals affect the life cycle, we first need to understand the organism's development under non-exposed conditions. The theory of dynamic energy budgets (DEB) aims to describe individual organisms on the basis of a set of simple rules for metabolic organization (*10, 11*). Exposure to toxicants can be understood as a change in energetic parameters, like an increase in the maintenance costs or a decrease of the assimilation of energy from food. This insight inspired the development of DEBtox (*12*), a suite of models to analyze toxicity tests, implemented into a user-friendly software package. However, this software was specifically tailored to deal with standard (short-term) toxicity tests like those assessing mortality, fish growth, and *Daphnia* reproduction. In this study, we present adaptations that are necessary to apply these models to life cycle tests: integrating the results for survival, growth and reproduction

in a single analysis (because endpoints share common parameters, e.g. the toxicokinetics), and effects of ageing of the animal (senescence). We demonstrate these adaptations by describing existing life-cycle toxicity tests with the springtail *Folsomia candida*, exposed to cadmium (Cd) and triphenyltin (TPT) in their food (*13*).

Methods

Experimental data set. The experiments started with 1-day old juveniles of *Folsomia candida*. Animals were fed baker's yeast with the test chemicals added, dispensed on filter paper discs. Test concentrations were 0, 64, 139, 300, 646, 1390 and 3000 mg/kg dwt. The food was freshly prepared each week, and old food was removed. Animals were kept individually in perspex containers, using 30 replicates for each treatment. For 15 animals per treatment, the fresh weight was determined, and for 15 the number of eggs produced. Full details are given in (*13*).

About DEBtox. DEBtox has been described in detail elsewhere (*12*, *14*, *15*), but we will briefly reiterate the most important principles here. The basic philosophy is that toxicants first need to be taken up by the organism before they can exert an effect (conform the concept of critical body residues (*5*)). Secondly, the toxicant, once inside, increases the probability of dying (on the basis of hazard modeling, see (*14*)), and affects a parameter of the animal model (e.g. the maintenance rate; see (*15*)). The basic structure of DEB is shown in Figure 1, as well as the modes of action that are currently implemented in DEBtox to describe effects on growth and reproduction. It should be noted that these modes of action are based on resource allocation, which differs from the more familiar use of the term, describing changes in physiology or behavior, such as narcosis or cholinesterase inhibition.

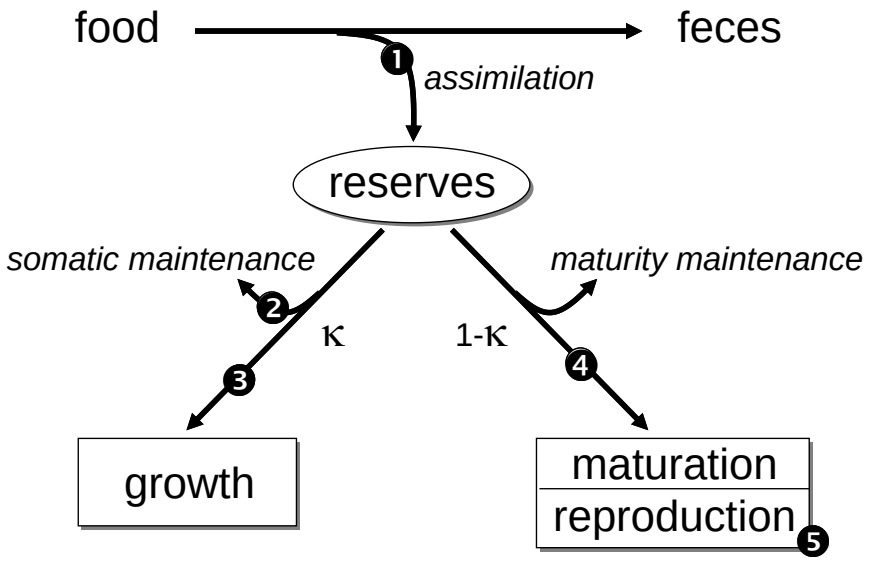

FIGURE 1. Schematic representation of DEB. Food is transformed into feces and part of the energy is assimilated and contributes to the reserves. These resources are distributed in a fixed fraction (κ) between somatic growth/maintenance and reproductive output/maturation. Numbers indicate locations where the chemical may exert a toxic effect (the modes of action): a chemical may 1) decrease assimilation, 2) increase the maintenance

costs, 3) increase costs for growth, 4) increase costs for reproduction, or 5) pose a direct hazard to the embryo.

The exact dose-response relationship between the internal concentration and the target parameter's value is unknown and cannot usually be directly established. Therefore, a pragmatic approach is to approximate the dose-response by a linear relationship between the parameter and the internal concentration, as soon as the concentration exceeds a threshold value (the No-Effect Concentration or NEC). The NEC can be interpreted as the NOEC after infinite exposure time: prolonged exposure to a concentration below the NEC causes no toxicity on the measured endpoints. In this way, the NEC is time-independent, and a model parameter (which means it can be estimated with a confidence interval), and therefore does not suffer from the well-known problems associated with the NOEC (*16, 17*). Chemical effects relate to internal concentrations, but as these are usually not measured, we can avoid this problem by expressing the NEC in external concentration units (*14*). To allow for the analysis of life-cycle tests, several adaptations are necessary, which are discussed in the following sections.

Simultaneous fitting. The current version of the DEBtox software only accepts data for a single endpoint, but in life-cycle tests, there are various endpoints that are closely related (generally, survival, growth and reproduction). For example, toxicokinetics (i.e. the elimination rate) affect the time course of the internal concentration and thereby all endpoints. Furthermore, a chemical that affects growth will automatically affect reproduction, as the size of the organism determines its feeding rate, and thus the energy available for reproduction. The close relationship between the various endpoints implies that we cannot treat each endpoint in isolation. Rather, we have to fit the model to all data simultaneously (as is shown in Figure 2).

Simultaneous assessment of all endpoints is achieved by expressing all model fits on the basis of likelihood. The analysis of survival data provides a multinomial likelihood (*14*), whereas the other endpoints are assessed by weighted least-squares regression. The weights depend on the number of animals alive at that point, and are inversely proportional to the value of the endpoint (reflecting that variation generally increases with the average). To combine this least-squares regression with the survival analysis, we have to translate the sum of squares (SSQ) to a likelihood (L). If the data (with n observations) are assumed to follow a normal distribution, we can calculate the likelihood of a parameter combination (θ) by:

$$L(\theta \mid \text{data}) \propto SSQ(\theta; \text{data})^{-n/2} \qquad (1)$$

This simple result was derived by Box and Tiao (*18*), following a Bayesian argument. However, it can also be understood from a more classical likelihood perspective as the profile likelihood where the residual standard deviation is treated as a nuisance parameter, and is profiled out (the profile likelihood can be treated as an ordinary likelihood (*19*)). Different likelihood functions can be multiplied to yield an overall likelihood that can be maximized. Furthermore, this overall likelihood can be used to derive asymptotic standard errors, as well as confidence intervals based on profile likelihoods (*20*). Simultaneous fitting of multiple endpoints using the DEBtox equations was demonstrated earlier, for mortality and internal concentrations in *Daphnia magna* (*21*). The full model is implemented in MatLab® (version 6.5), and optimized using a Nelder-Mead Simplex search.

Senescence. A further adaptation is needed to account for ageing of the animals (senescence). Standard toxicity tests are terminated before the animals show signs of old age (control mortality should be low). As life-cycle tests run longer than standard tests, we need to model the age-related survival, as well as the decrease of reproductive output. The ageing module follows the approach taken by Van Leeuwen et al. (*22*), which is based on the oxidative stress hypothesis (for reviews (*23, 24*)). The main assumption is that aerobic metabolism produces reactive oxygen species (ROS). These molecules cause damage to various macro-molecules like proteins and DNA, which in turn leads to the effects associated with senescence. This theory is, for example, supported by the fact that life span is increased by most treatments that decrease the rate of metabolism (e.g. temperature in ectotherms). The model therefore assumes that damage depends on ROS production, which in turn is proportional to the respiration rate.

The respiration rate (proportional to the catabolic rate p_C with unit energy·d^{-1}) is made up from contributions of growth (the change in structural body volume dV/dt) and maintenance (proportional to volume V) (*10*):

$$p_C \propto \left(\frac{dV}{dt} + k_M V \right) \tag{2}$$

where k_M is the maintenance rate coefficient (ratio of the costs for maintaining a unit of amount of biomass to the costs of producing it, d^{-1}), the proportionality constant (energy·m^{-3}) is not specified here. The respiration resulting as overhead from the reproductive output is implicitly included in this equation, because the energy flux allocated to reproduction is linked to growth and maintenance according to the fixed allocation rule (the κ in Figure 1).

Oxidative damage (M_Q in mol) is given by the sum of three processes: the constant production of ROS proportional to respiration, damage itself leads to more damage (amplification), and damage increases the ROS production (*22*):

$$\frac{dM_Q}{dt} = \eta_{QC} p_C + k_a M_Q + \frac{1}{E_a} M_Q p_C \tag{3}$$

where the coefficient η_{QC} links the production of damage to the respiration rate (mol·energy^{-1}), the rate constant k_a is the net result of damage amplification and repair (d^{-1}), and the energy E_a is involved in the increase in ROS production due to damage (energy).

As stated earlier, survival is analyzed using hazard modeling. The oxidative damage is considered to act on the survival probability in a similar fashion to a chemical stress, although no threshold is assumed. The hazard rate due to ageing (h_Q) is thus taken proportional to the damage density:

$$h_Q = b_Q \frac{M_Q}{V} \tag{4}$$

where b_Q is the killing rate due to damage with unit m^3·(mol·d)$^{-1}$. This additional hazard can simply be added to the hazard resulting from chemical exposure.

The damage is also affecting reproduction through an energy parameter (just like xenobiotic chemicals). As mode of action for the oxidative damage, we selected the “costs for reproduction”

(mode of action 4 in Fig. 1) because this gave the best results on the current data set. This is in accordance with fact that body size does not decrease with old age in *F. candida*, although other mechanisms cannot be ruled out. The reproduction rate without ageing (R_0 in eggs·d^{-1}) is calculated by the DEBtox method (*15*) as a function of time and concentration, but is multiplied here by a stress factor due to oxidative damage (assuming a linear relationship between damage density and costs for reproduction):

$$R = R_0\left(1+\frac{M_Q}{V}\frac{1}{c_Q}\right)^{-1} \tag{5}$$

where the constant c_Q can be interpreted as a tolerance to damage (mol·m^{-3}). Before the model equations 2-5 are compared to the data, several simplifications can be made. The unspecified proportionality constant from Eq. 2 can be combined with the constants η_{QC} and E_a in Eq. 3. Furthermore, instead of using the body volume, the model calculations are simplified by working with the scaled length of the organism (the actual length as fraction of the maximum length). Because the life-cycle test starts with very young animals, the initial amount of damage is assumed to be negligible. Due to this assumption, the number of parameters can be reduced; the constant η_{QC} is absorbed into the two proportionality constants (b_Q and c_Q). These simplifications eventually result in the model parameters with units as given in Table 1.

Despite this relatively simple model, senescence is likely to be more complex, e.g. because feeding rates tend to decline with age in *F. candida* (*25*). When the animal is fully grown, the energy allocated to growth/maintenance (Figure 1) is just enough to pay the maintenance costs. When feeding decreases, the energy balance dictates that the same body size cannot be maintained, and thus that the animal should shrink or die (which was not observed). However, in the nematode *Caenorhabditis elegans*, respiration in fully-grown adults (measured as oxygen consumption) also decreased with age (*26*). It is not clear whether the same process can be observed in *F. candida*, so the exact mechanism needs to be elucidated. For the moment, we are satisfied with the model as the description is acceptable, and because the effects on survival and reproduction are described through the same mechanism.

Population growth. The intrinsic rate of population increase (r) can be calculated from the continuous form of the Euler-Lotka equation (see e.g. (*27, 28*)):

$$1 = \int_{t=0}^{t_{max}} q(c,t)R(c,t)e^{-r(c)t}\,dt \tag{6}$$

With $q(c,t)$ as the survival probability (dimensionless) at concentration c and time t, $R(c,t)$ the reproduction rate (eggs·d^{-1}), and $r(c)$ the population growth rate (d^{-1}) at concentration c. The t_{max} is taken as the last day of exposure (after 120 days), assuming that either the survival probability or reproduction rate is zero after that point). As r is not explicit in Equation 6, it has to be found numerically. With this equation, the effects on growth, reproduction and survival are integrated into a single effect measure that is a function of the external concentration only. Because the model yields q and R, r can also be calculated for concentrations that were not tested. Furthermore, because food level has a predictable effect on growth and reproduction in the model, r can be calculated under food limitation (assuming that the intrinsic sensitivity is not affected) (see also (*27*)). However, it must be noted that any extrapolation outside the limits of the experimental data set remains speculative.

Results and discussion

General behavior. The simultaneous fits of the model are shown in Figure 2, for both compounds. For this analysis, the initial length of the organisms was fixed to the value measured in the original paper (*13*), as well as the elimination rate for TPT (as the estimation routine tended to go to extremely high values, 10 d^{-1} is a practical upper limit). Note that the measure of length that is used here is volumetric length (i.e. the cubic root of body volume, assuming a density of 1 $g \cdot cm^{-3}$). Overall, the fits are good, although for TPT, there seems to be some misfit of the reproduction data at low concentrations after 80 days exposure. However, it must be noted that at these low concentrations, most of the animals are already dead at this point (see survival plots in Figure 2). The estimate is based on the few remaining individuals, which may deviate from the average reproduction pattern. However, these data points receive less weight in the optimization (the number of live animals is used a weighing factor in the analysis). Estimates for the parameters are given in Table 1. For most of the parameters governing control performance (growth, reproduction and ageing), the estimates are quite similar for both chemicals. There are some differences (possibly because the experiments for the two compounds where done sequentially), but in all cases, the confidence intervals overlap. The best fits were obtained by assuming that Cd acts on assimilation and TPT on maintenance.

For Cd, the effect of senescence on survival and reproduction could be described by the same mechanism, as outlined in Eq. 2-5. The exposure to Cd slows down the ageing effects on both endpoints. However, for TPT, something else is happening. Although large differences are seen in the ageing pattern for survival at the different doses, there is not much difference in the ageing pattern of reproductive output. We solved this by applying the damage build-up in the control treatment to the reproduction rate for all doses. This results in a good fit, but raises questions regarding the mechanism. Another indication that the ageing model needs further elaboration is the fact that the estimate for the maintenance rate coefficient is very low for both compounds (not significantly different from zero).

TABLE 1. Maximum likelihood estimates for the toxicity parameters in the different experiments. The 95% likelihood-based confidence intervals are given in parentheses. N.e. is not estimated.

	Unit	Cadmium	Triphenyltin
Toxicological parameters			
Mode of action		Assimilation	Maintenance
Elimination rate	d^{-1}	0.12 (0.082-0.21)	10 (n.e.)
NEC growth and reproduction	mg/kg	2.0×10^{-4} (0-67)	0.11 (0-23)
Tolerance concentration for growth and reproduction	mg/kg	1.1 (1.0-1.2) $\times 10^{4}$	6.0 (5.7-6.3) $\times 10^{3}$
Physiological parameters			
Von Bertalanffy growth rate	d^{-1}	0.064 (0.061-0.067)	0.067 (0.063-0.071)
Initial length [a]	mm	0.026 (n.e.)	0.026 (n.e.)
Length at puberty [a]	mm	0.40 (0.37-0.42)	0.40 (0.38-0.41)
Maximum length [a]	mm	0.66 (0.65-0.67)	0.64 (0.63-0.65)
Maximum reproduction rate	$eggs \cdot d^{-1}$	30 (24-40)	24 (20-30)

Parameters related to ageing			
Maintenance rate coefficient (k_M)	d^{-1}	9.1×10^{-7} (0-0.0027)	1.9×10^{-7} (0-0.0020)
Damage amplification (k_a)	d^{-1}	0.024 (0.019-0.029)	0.019 (0.012-0.026)
Increase in ROS production due to damage (E_a)	[-]	0.20 (0.18-0.23)	0.17 (0.14-0.21)
Damage killing rate (b_Q)	d^{-1}	$2.1\ (1.1\text{-}3.7) \times 10^{-4}$	$1.1\ (0.50\text{-}2.4) \times 10^{-4}$
Damage tolerance on reproduction (c_Q)	[-]	26 (11-68)	$59\ (19\text{-}1.7 \times 10^{2})$

[a] The length measure presented is volumetric length (the cubic root of the volume), assuming a density of 1 $g \cdot cm^3$.

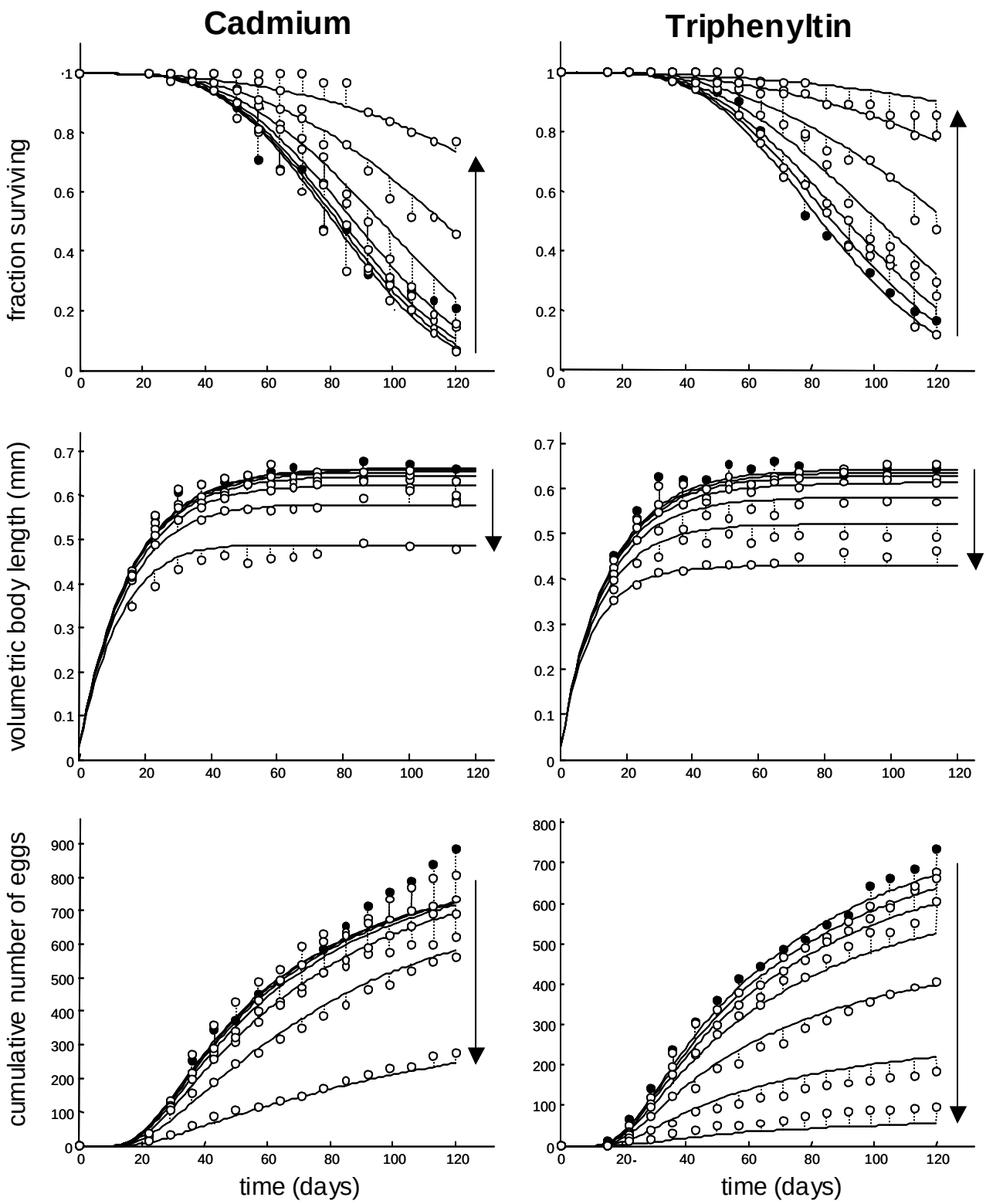

Figure 2. Simultaneous model fits for all endpoints, for exposure of *Folsomia candida* to different concentrations (0-3000 mg/kg dwt) cadmium or triphenyltin in the diet (black dots indicate control performance, arrows indicate increasing exposure concentrations). Mode

of action is decrease in assimilation for cadmium and increase in maintenance costs for triphenyltin.

Cadmium. The best fit for cadmium was obtained by assuming that this chemical acts on the assimilation of energy from food. This mode of action is able to describe the decrease in growth as well as the decrease in reproduction simultaneously (no direct effect on reproduction is assumed). This implies that we only have one mode of action for both endpoints. The estimated NEC is very close to, and not significantly different from, zero. Low levels of Cd are thus already affecting the animal's performance. There is no NEC for survival, as an increasing level of Cd in the food did not lead to mortality, but in fact increased the lifespan compared to the control. This was also observed for *Daphnia* (*29*). The ageing model explains this behavior, as cadmium reduces growth and ultimate body weight, which implies lower respiration rates, and hence less oxidative damage. In contrast, additional mortality was observed for *F. candida* when Cd was spiked to the soil (*30*), although that experiment was too short to see whether low exposures increased longevity. This difference may relate to the different mode of exposure for our data set (food vs. soil).

The mode of action "assimilation" can mean that Cd decreases the efficiency with which energy is assimilated from food, or that Cd influences the feeding rate itself. Especially the latter explanation cannot be ruled out as exposure was via food, and there are indications that this metal is avoided by *F. candida* (*31*). Although reduced feeding due to avoidance is not a toxicological effect, in the strict sense, the consequences for growth and reproduction are similar to effects on assimilation efficiency. However, a reduction in feeding may also have led to unexpected differences in toxicokinetics, which are not accounted for in the model. These potential biases can only be addressed when feeding rates and/or internal concentrations are measured.

Triphenyltin. Exposure to TPT led to a clear decrease of growth in *F. candida*. The data are best described when TPT is assumed to increase the maintenance costs. This mode of action is supported by detailed studies, indicating that TPT affects energy metabolism by inhibiting ATP synthesis (*32*). The model quite accurately describes the simultaneous effect on growth and reproduction. As with Cd, the NEC for survival cannot be estimated because TPT increases the lifespan of the springtails. The NEC for growth/reproduction is very close to, and not significantly different from, zero.

As stated earlier, the ageing pattern in survival and reproduction seems to differ, which is in marked contrast with the results for Cd. This difference may be related to the mode of action of both chemicals (maintenance vs. assimilation). The effect of assimilation is straightforward: it reduces the total energy input into the organism, and survival and reproduction are both affected to a similar degree. TPT apparently acts by increasing the maintenance costs, which could mean that the effect of decreased growth on ageing is not so straightforward. According to this mode of action, the maintenance rate coefficient (k_M in Eq. 2) is not constant anymore but increases with increasing exposure to TPT. However, this was not included in the ageing model (because of the unrealistically low k_M values). Nevertheless, it remains unclear why TPT influences the ageing process on survival in a different way than reproduction.

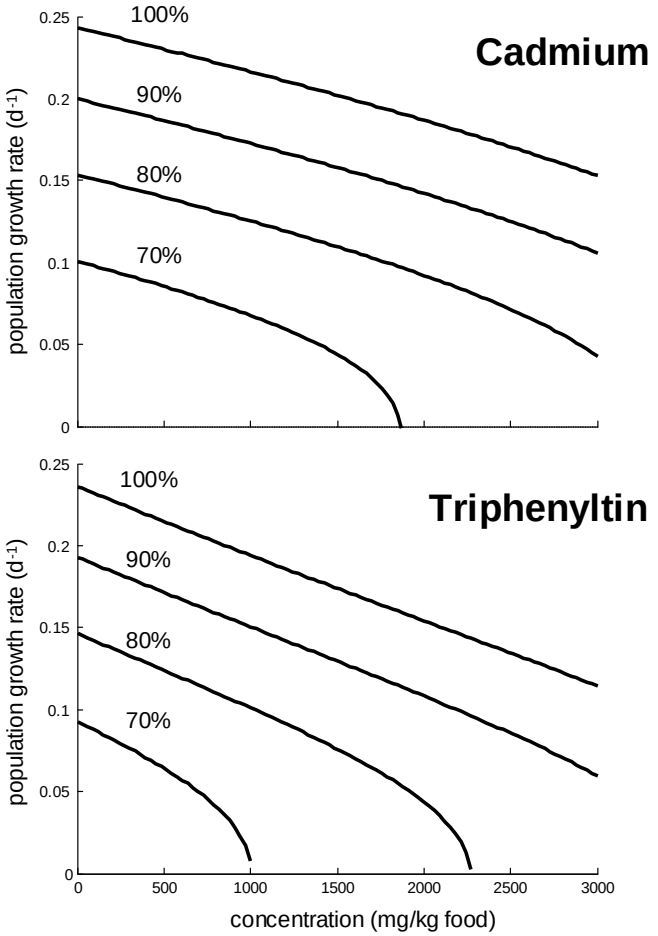

FIGURE 3. Intrinsic rate of population increase for *F. candida* exposed to cadmium and triphenyltin in the diet, for different food levels (given as percentage of the maximum ingestion rate).

Population growth. The intrinsic rate of population increase is calculated using Eq. 6 and the model fits for the entire concentration range (Figure 3). Over this range, the growth rate decreases only slowly at the highest food level. Although both chemicals have a profound effect on the number of eggs produced, this is partly counteracted by the increased longevity. At lower food levels, the population growth in the controls is also decreased (due to slower development, and decreased reproduction), but the impact of toxic chemicals may also be strongly influenced, depending on the mode of action (*27*). In the case of Cd and TPT, a population crash due to chemical stress is possible at low food availability. At the point where the growth rate becomes zero, the energy available for growth is insufficient to reach the body size where reproduction starts (the length at puberty in Table 1). This point is reached at higher ingestion rates for TPT than Cd, which may relate to the different mode of action. The fact that the NEC for both compounds is very low is also apparent in the population growth; already at low concentrations in food, growth rates starts to decrease (no effects on individuals also means no-effect on populations).

It remains questionable whether these "population effects" can be directly used to predict field effects. Eq. 6 does not account for multiple stress under field conditions (*33*), or density-dependent effects (*34*). Nevertheless, all the endpoints are integrated into an ecologically relevant

parameter, which is a huge step forward from current practice in ecotoxicological effects assessment.

Evaluation and outlook. Current protocols for toxicity testing and data analysis were developed to provide risk assessors with simple numbers like LC50s and NOECs. However, we believe that ecotoxicology needs a better understanding of how chemicals affect organisms. This is not only interesting from a scientific viewpoint, but will also, in time, lead to better risk assessment by providing the risk assessor with a result that bears more relation to the protection goal (field populations). The current study illustrates how life-cycle toxicity tests can be analyzed from a mechanistic perspective, based on the chemical's effects on the animal's energy budget. Because the DEBtox method is based on processes, it is able to deal with peculiarities of the test design. For example, the model can simply be extended to deal with time-varying exposure during the test (e.g. due to degradation or dissociation of the chemical), testing at multiple food densities, and specific details of the life cycle (e.g. (*35*)). If we have measurements of internal concentrations, they can be easily included in the analysis (*21*), as this provides direct information for the elimination rate. Measurements of respiration rates may be helpful to further test the validity of the ageing module.

To illustrate the model procedure, a very detailed life-cycle experiment for Cd and TPT was selected. However, this approach is not restricted to exposure via food, and may also be used for other compounds and smaller data sets. The current paper shows that the extended model is able to describe the life-cycle data for all endpoints simultaneously over time with few, physiologically relevant, parameters. In fact, we have used 12 parameters (Table 1) to fit 21 curves simultaneously, which is clearly very efficient. The model describes the data for Cd and TPT quite well, and indicates that these chemicals have distinctly different modes of action, even though both chemicals affect growth as well as reproduction. The ageing model that we used here is a simple one, but it is based on theoretical work and is able to describe the old-age effects on survival and reproduction with five additional parameters. For Cd, the analysis strongly suggests that ageing affects survival and reproduction through a common mechanism, although TPT apparently affects ageing in a more complex manner. The source of this discrepancy is unclear, but may relate to the different mode of action.

The model fit allows for a calculation of the intrinsic rate of population increase; a result that integrates the effects on survival and reproduction. Figure 3 provides a more solid basis for risk assessment than any of the individual endpoints by themselves. Although it is not yet clear how much decrease in population growth rate is acceptable, we support the conclusion of Forbes and Calow (*2*) that this parameter provides a more relevant measure of ecological impact than any single individual-level endpoint. However, this type of analysis requires more elaborate testing, following more endpoints over a longer exposure period (preferably the entire life cycle). The kind of experiment used in this study is too expensive for initial tiers of risk assessment, but partial life-cycle tests are not necessarily much more expensive than the current test protocols. For example, the standard *Daphnia* test can easily be extended to allow for an analysis as outlined in this paper. In the 21-day test, reproduction has to be scored every day (although only the total number of eggs is used for the NOEC), as well as the survival of the animals. In addition, measuring body size at several points in time would ensure that the mode of action (conform Figure 1) can be identified, which helps to predict the effects at limiting food densities.

Finally, we believe that it is possible to increase our understanding of toxic effects on the basis of the experiments and the model described. These methods and the knowledge gained

through them can be used to improve strategy and design in ecotoxicological testing, in order to eventually arrive at an improved and scientifically underpinned risk assessment. The software tool DEBtox for analyzing standard toxicity data, can be downloaded free of charge from http://www.bio.vu.nl/thb/deb/deblab/. The presented extensions will be placed on the same web site in the form of MatLab files, as part of the DEBtool collection.

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