Dynamic modeling of sub-lethal mixture toxicity in the nematode *Caenorhabditis elegans*

Tjalling Jager,*[,†] Eva María Gudmundsdóttir,[‡] Nina Cedergreen [‡]

[†] Dept. of Theoretical Biology, VU University Amsterdam, de Boelelaan 1085, NL-1081 HV, Amsterdam, the Netherlands
[‡] Dept. of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

This document is the Accepted Manuscript version of a Published Work that appeared in final form in Environmental Science & Technology:

Jager T, Gudmundsdóttir EM, Cedergreen N. 2014. Dynamic modeling of sub-lethal mixture toxicity in the nematode *Caenorhabditis elegans*. Environmental Science & Technology 48:7026-7033.

ABSTRACT: Dynamic models for toxic effects (TKTD models) are increasingly used in the analysis of toxicity data for single-chemical exposure. However, these models also offer a natural extension to the effects of chemical mixtures. Here, we demonstrate how a simple model for the energy budget (DEBkiss) can be used to interpret the effects of cadmium and fluoranthene, both in single and mixed exposure, on the nematode *Caenorhabditis elegans*. The data for all time points and all endpoints (growth and reproduction) are combined into a single coherent framework. These modeling results are compared to a more traditional independent-action approach, based on the dose-response curves for a single endpoint at a single time point. The analysis with DEBkiss does not lead to a radically different interpretation of the mixture effects; both indicating an antagonistic interaction in the mixture. The DEBkiss analysis does, however, provide much more insight into the relevant dynamic processes underlying the toxic effect on the organism, and allows the generation of mechanistic hypotheses that can be used to guide further research.

INTRODUCTION

In the environment, organisms never encounter a single chemical in isolation; simultaneous exposure to multiple stressors is the norm. Even though there is still a strong single-chemical focus in ecotoxicology, mixture toxicity has become a well-established discipline over the last decades.[1] Effects data from mixture experiments are traditionally analyzed in a descriptive manner; describing the combined effect of a mixture on one endpoint (e.g., growth, survival or reproduction), at a single time point, under constant external exposure. Such an approach obscures the fact that mixture effects depend on the endpoint,[2,3] the exposure time,[2,4] as well as on the timing of exposure events.[5] The response of an organism to a mixture is the response of a dynamic system, in which the different endpoints of an individual are causally linked. If we are to further our understanding of mixture toxicity, we need to consider these aspects explicitly.

Process-based models for toxic effects are gaining popularity,[6] and some have been extended to include mixture effects. Most of the work has focused on survival,[4,5,7] as analyses for this endpoint can rely on relatively simple toxicokinetic-toxicodynamic (TKTD) models. For sub-lethal endpoints, we are aware of only one published example of a TKTD approach for mixtures in ecotoxicology.[8] In that study, the effects of two polycyclic aromatic hydrocarbons (PAHs) on growth, reproduction and survival of *Daphnia magna* were modeled simultaneously over time. The TKTD model was based on Dynamic Energy Budget (DEB) theory,[9] a theory that is commonly used for the analysis of toxicity data from single-chemical exposure.[10] Even though the experimental data set was not optimal, this study provided important proof-of-concept for a mixture of two very similar chemicals. Here, we build upon this earlier work by focusing on a mixture of two distinctly dissimilar chemicals. In the context of energy budgets, we can talk about 'similar' and 'dissimilar' chemicals in terms of their physiological or metabolic mode of action (mMoA).[11] The mMoA represents the large-scale metabolic process that is affected by the chemical (e.g., assimilation or maintenance), leading to a specific suite of effects on all endpoints over the life cycle. The mMoA thus differs from the more commonly used mechanism of action (dealing with molecular targets) and mode of action (more general physiological/behavioral responses), although there are certainly links between these concepts. In practice, the most likely mMoA for a toxicant in a species can be deduced from the observed effects on growth and reproduction over time.

In this study, we use the nematode *Caenorhabditis elegans* for its short life cycle, ease of handling, and because it has been subjected to DEB-based analyses in the past.[12] The chemicals selected are the heavy metal cadmium (Cd) and the PAH fluoranthene (FA), which demonstrated distinctly different metabolic modes of action in an earlier study: Cd affecting assimilation, whereas FA increased costs for growth and reproduction.[13] As these chemicals have a different mMoA, we can logically assume that they affect different molecular target sites in the organism. Therefore, our starting hypothesis is that the combined effect of Cd and FA can be accurately predicted by modeling an independent action on both target sites (without the need to specify the details of this target site). It should be stressed that this assumption of independence differs from the traditional approach for independent action (IA). In the classical IA, independence is applied to the percentage effect on an endpoint (at one time point), whereas in the TKTD model this assumption is applied at the level of the processes in the energy budget (and hence specifies the mixture effects on all endpoints over the entire life cycle). Assuming independent action without interaction at the target sites may easily lead to apparent interactions in the dose-response curve as the TKTD processes inevitably interact.[8] Here, we compare the results of the TKTD analysis

to a traditional IA prediction (for both growth and reproduction at a single time point) to illustrate the difference in philosophy between both approaches.

MATERIAL AND METHODS

Experimental setup. *Caenorhabditis elegans* of the N2 Bristol strain (obtained from the *C. elegans* Genetics Centre, Minnesota, USA) were cultivated at 20˚C, in darkness on plates of a modified bacteriological agar (nematode growth medium, NGM[14]) and fed *Escherichia coli*, of the uracil deficient strain OP50.

Three experiments were conducted with *C. elegans*, in all cases following growth, reproduction and survival, testing the effects of cadmium (Cd), fluoranthene (FA), and the mixture of the two chemicals at equitoxic ratios. Concentration ranges of cadmium and fluoranthene were estimated based on pilot experiments. Subsequently, the effects of the individual chemical exposures on reproduction were used to calculate equitoxic concentration ranges for the mixture toxicity experiment. Equitoxicity is not essential for the model analysis but it is a pragmatic choice to ensure that both chemicals attribute to the mixture effect, and it facilitates plotting and interpretation of the results.

Cadmium was prepared in stock solution of 500 mg/L $CdCl_2$ (Sigma-Aldrich, purity >99%) in demineralized water and added to the warm (60-70°C) agar to obtain concentrations of 0, 0.625, 1.25, 2.5, 5 and 10 mg Cd/L agar. Fluoranthene (Sigma-Aldrich, purity >99.9%) was dissolved in 99.9% ethanol resulting in a range of stock solutions with the concentrations 0.125, 0.250, 0.5, 1, 2 g/L. The dilutions were made in ethanol in order to have equal solvent concentrations in all treatments. The fluoranthene stocks were kept in a freezer at -18°C. To dose the agar, 1 mL/L was added to warm agar to obtain fluoranthene concentrations of: 0 (blank and solvent control), 0.125, 0.25, 0.5, 1 and 2 mg/L agar. The mixture ratio of Cd:FA giving a 50:50% effect was determined to be 2:1, based on EC50-values for cumulated reproduction of 2.02±0.39 mg Cd/L and 1.17±0.19 mg FA/L (EC50±s.e., derived from pilot experiments). This gave the following Cd:FA concentrations in the mixtures: 0:0, 0.125:0.0625, 0.25:0.125, 0.5:0.25, 1:0.5, and 2:1 mg/L agar (no solvent control was included).

After mixing the chemicals with agar, the agar was distributed either into Petri dishes, used for synchronization of the worms, or into 12-well Tissue Culture (TC) plates. One 12-well plate was used per treatment, with a single individual nematode per well. New plates were made every 3-5 days. Plates older than five days were not used because of potential changes of the chemical concentrations in the agar. One day before introducing nematodes into the agar, the *E. coli* strain OP50, cultivated in lysogeny broth at approximately 20°C, was inoculated onto the agar and left to cultivate overnight.

Synchronization of the nematodes for toxicity testing was achieved by leaving 10 fertile adult individuals (3-4 days old) on a Petri dish for each concentration for approximately three hours to lay eggs, after which the adults were removed. When the individuals, hatched from these eggs, had reached a size where they could be transferred without being damaged (≥400 µm), they were individually placed into wells of a TC plate at the same exposure concentration they were hatched on. The nematodes were transferred to a new well every day, and the number of fertilized and unfertilized eggs was counted daily until death of the individual. Incubation conditions were as described above.

During the experiment, the length of the nematodes was regularly determined using a Nikon DS-Fi1 camera connected to a Nikon SMZ 800 stereomicroscope. Pictures were taken the first

days after synchronization on twelve random individuals on the synchronization plates and thereafter on each individual transferred to the 12-well plates. Body length was measured using Nikon NIS Elements Imaging Software 3.2. Length measurements where stopped in the Cd experiment at day 5, but for the subsequent experiments, these observations were continued for longer to obtain a better estimate for the ultimate size.

Basic DEBkiss model. The model we used is a simplified DEB model, known as DEBkiss.[15] A detailed model derivation is supplied elsewhere,[15] but we added the complete set of model equations in the Supporting Information (SI). In the model, food is taken up by the animal from the environment, a fraction κ of the assimilated energy is used for the soma (growth and somatic maintenance), whereas the remainder (1-κ) is used for maturation (in juveniles), maturity maintenance and reproduction (in adults). In contrast to other simplified DEB approaches,[10] DEBkiss builds upon an explicit mass balance, includes the embryonic stage, and excludes the distinction of biomass in a structure and reserve compartment (all biomass is treated as 'structure', which is an acceptable simplification for small invertebrates).

The extensions for toxicokinetics and toxicodynamics follow the principles laid down in other DEB-based studies.[8,10] Uptake of chemicals is modeled using a first-order, one-compartment, toxicokinetics model, accounting for the effects of changes in body size and losses due to reproduction (see SI). For the egg, we assume either no uptake at all (Cd), or instantaneous steady state with the external concentration (FA). This is consistent with the observed effects and avoids the complications of modeling TK in eggs. The chemical, once taken up, affects one or more of the metabolic processes (the mass fluxes) in the organism; the physiological or metabolic mode of action or mMoA.[11] The use of DEBkiss in a complete TKTD framework was first demonstrated by Barsi and coworkers.[16]

To analyze mixture toxicity data, very little changes to the model are required. According to previous analyses, cadmium affects assimilation of energy from food,[13,17] whereas fluoranthene affects the costs for growth and the costs for reproduction simultaneously.[13] Since these chemicals have a different mMoA, they affect different target sites, and therefore we depart from the assumption that the combined effects of the two chemicals can be modeled as independent effects on each target process.[8]

Specific extensions for nematodes. Total body length of the nematodes was measured, but the model requires the body size as volume and dry weight. For the conversion from total length (L_w) to volume (V), a shape correction (δ_M) is used:

$$L_w = \frac{V^{1/3}}{\delta_M}$$

For a fully-grown animal (1.4 mm long), we found an estimate for the fresh weight of 4.3 µg,[18] and an estimated volume of 4.5 nL.[19] These estimates support a wet density of 1 mg/mm^3, and result in δ_M = 0.12. We compared this value to the estimated volume of several worms in our control experiments, calculated by approximating the body with a cylinder. The diameter of the cylinder is taken as the mean of seven measurements at different parts of the worm (see SI). The resulting δ_M = 0.12 (s.d. 0.0079, n = 22) corresponds very well to the value derived above. The dry weight density was taken as d_V = 0.25 mg/mm^3, which is a representative value for benthic nematodes.[20]

Growth in *C. elegans* deviates from the von Bertalanffy pattern, which is the expected curve from standard DEB models for organism that do not change shape over ontogeny. Initially, the juveniles grow slower, which likely reflects their difficulty feeding on the supplied bacterial cells.[21] As in earlier work,[12] we applied a hyperbolic relationship for the scaled functional response (f) with body size. The reduced feeding in early juveniles is also assumed to reduce the exchange rate of the chemical between the medium and the animal (see SI), as chemical uptake in *C. elegans* appears to be closely related to the feeding process.[22]

Here, we must explicitly consider embryonic development as the experiments were initiated by allowing adults to deposit eggs into a petri dish for three hours (with toxicants, in the exposure treatments). For the model calculations, we define $t = 0$ half-way into the synchronization (after 1.5 hours). Embryonic development is sometimes slower than expected from the juvenile/adult parameters, which can be corrected by assuming a slower use of assimilates from the egg buffer.[15,16] This introduces an additional parameter f_B, the apparent scaled functional response of the embryo.

The exact moment of hatching was not observed, and the first observation was 20 hours after egg deposition. To obtain a reasonable representation of the embryonic development, we decided to constrain the fit by forcing the model to make a realistic prediction for the hatching time in the control fit. In each iteration of the optimization, the predicted hatching time is provided with a probability density, given a normal distribution for the hatching time with a mean of 11 hours (at 20°C),[18] and an arbitrarily small s.d. of 0.1 hours. This probability density is then included into the overall likelihood function (which is very similar to the Bayesian use of priors).

The dry weight of a single egg was estimated as 8.8 ng, using a literature value for the fresh weight[18] and assuming a dwt/wwt ratio of 0.25. This estimate can be compared to the measured size of the eggs in our experiment. Length and width were determined for a number of eggs, and translated into volume by approximating the egg as a prolate spheroid: V_{B0} = 0.024 nL (s.d. 0.0068, n=31). By assuming a wet weight density of 1, and a dwt/wwt ratio of 0.25, we obtain an egg weight of 6.1 ng; quite comparable though slightly smaller than the first estimate.

An additional aspect of the reproductive behavior of *C. elegans* (as hermaphrodite) is that reproduction stops rather abruptly when the storage of sperm cells runs out (sperm production stops when egg production commences). In earlier work, we addressed this with a new parameter (maximum total number of eggs), which was apparently also affected by toxicant exposure.[13,17] Here we avoid these additional complexities by only using the reproduction data up to t = 6 days. For a similar reason, we also did not use the information about mortality in the analysis. Almost all of the mortality occurred after 6 days, and that is also where control mortality was affected. A DEB-based explanation of mortality patterns should thus involve an interaction between ageing and the toxicant,[17] which we decided would be unnecessarily speculative for the current analysis.

Model fitting. The model was implemented in Matlab 2011a. Parameter optimization was performed by maximizing the overall likelihood function for all endpoints. For the distribution of the residuals, we assumed independent normal distributions after square-root transformation.[10] Confidence intervals were calculated by profiling the likelihood function.

We first estimated the physiological parameters by fitting the DEBkiss model to the data for the controls in the three experiments (for FA, the solvent control and the blank were combined as there was no significant difference, see SI). Next, we fitted the model to the data for the single-chemical exposures, fixing the basic physiological parameters on the data for the control treatments. This procedure ensures that the model does not compensate for a poor representation

of the treatment data by changing control behavior. For the mixture data, we predicted the effects patterns on growth and reproduction by fixing the toxicological parameters to the values obtained from the single chemicals, and the physiological parameters to the values obtained from the mixture controls. This step involves no fitting to the mixture data, and thus represents a validation of the model assumptions.

As the model parameters inevitably contain uncertainties, an additional analysis was performed, fitting all data simultaneously (growth and reproduction for the two single-exposure treatments as well as the mixture). Forcing the same toxicological parameters in the single treatments and in the mixture provides the best possible fit to all data assuming no interaction between the chemicals. This fit can be compared to a fit in which some toxicological parameters in the mixture are allowed to differ from the values in the single-chemical exposures, hence representing an interaction in the mixture. The difference in goodness-of-fit with and without interaction was judged using a likelihood-ratio test.

Independent Action model. The toxicity data were also analyzed in a more traditional way by describing growth and reproduction at a fixed time point with a log-logistic two-parameter dose-response model,[23] making an Independent Action (IA) prediction for the mixture and then comparing that to the data from the mixture experiment. The IA prediction was made according to Cedergreen et al,[24] where the response (given as length or produced eggs relative to the untreated control) of the mixture (R_{mix}) is equal to the product of the response of Cd (R_{Cd}), at the concentration used in the mixture, and FA (R_{FA}), at the concentration used in the mixture. R_{Cd} and R_{FA} are predicted from the dose-response relationship of the individual chemicals, and are given as proportions of the control average. Day 4 was chosen, as this is the time where large deviations in growth and reproduction can be distinguished between treatments, and as it is the recommended test time of the *C. elegans* reproduction tests.[25,26] Other times were also tested, but showed very similar results regarding the mixture interaction. The analyses were done in R version 3.0.2, and more details of the procedure are supplied in the SI.

RESULTS AND DISCUSSION

Table 1. Parameters values used in this study. For fitted parameters, maximum likelihood values with 95% confidence intervals are provided. Elimination rates are constrained between 0.001 and 100 d^{-1}.

Symbol	Description	Value (C.I.)	Unit
Fixed parameters and conversion factors			
δ_M	Shape-correction coefficient	0.12	[-]
d_V	Dry weight density of structure	0.25	mg/mm^3
y_{VA}	Yield of structure on assimilates	0.8	mg/mg
y_{BA}	Yield of egg buffer on assimilates	0.95	mg/mg
f	Scaled functional response (mixture)	1	[-]
κ	Allocation fraction to soma (embryo)	1	[-]
W_{B0}	Dry weight of a single egg	$8.8\ 10^{-6}$	mg
Physiological parameters estimated from the data in controls			
$J_{Am}{}^a$	Specific maximum assimilation rate	0.21 (0.21-0.22)	$mg/mm^2/d$
$J_M{}^v$	Specific somatic maintenance rate	0.80 (0.76-0.85)	$mg/mm^3/d$
κ	Allocation fraction to soma	0.65 (0.62-0.67)	[-]
L_{wf}	Physical length at half-max. feeding	0.36 (0.36-0.36)	mm
L_{wp}	Physical length at puberty	0.87 (0.84-0.90)	mm
f	Scaled functional response (single chemicals)	0.96 (0.95-0.97)	[-]
f_B	Scaled functional response embryo	0.32 (0.31-0.33)	[-]
Toxicological parameters for Cd (c_0 fixed to 0), mMoA assimilation			
k_e	Elimination rate constant	0.0010 (<0.025)	d^{-1}
c_A	Addition to external concentration	9.1 (8.1-10)	mg/L
c_T	Tolerance concentration	0.12 (0.11-2.9)	mg/L
Toxicological parameters for FA, mMoA costs for growth and reproduction			
k_e	Elimination rate constant	63 (10-100)	d^{-1}
c_0	No-effect threshold	$3.3\ (3.3\text{-}3.5)\ 10^{-3}$	mg/L
c_T	Tolerance concentration	2.3 (2.3-2.3)	mg/L

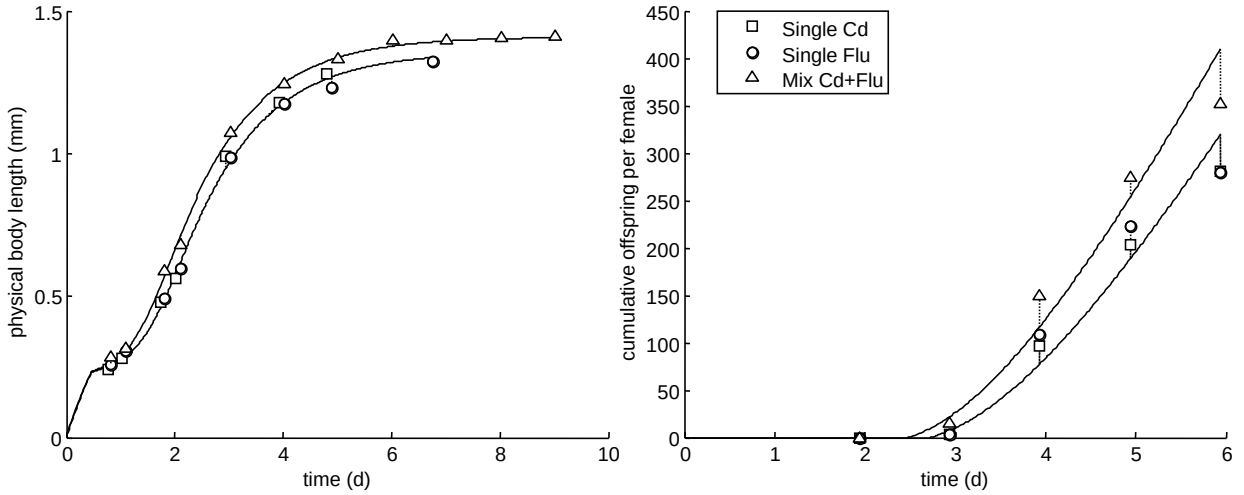

Figure 1. Fit for the control treatment of the three experiments. All data were described by the same parameters except for the food availability, which was assumed to be lower in the two single-chemical experiments. Parameter estimates are provided in Table 1. Body length before hatching (around t = 0.5 d) is a hypothetical size for the structural component of the embryo.

Embryonic growth. As the experiments start with eggs, we need to consider the embryonic phase in the model analysis. In earlier work,[17] a fixed hatching time was assumed and no uptake or effect of the toxicant on the egg. Due to the observed dose-related decrease in size at the first observations in the FA experiment (see Figure 3), we need to consider effects on the embryo as well. As we have no direct observations on the first 20 hours of development, we constrained the optimization to yield a realistic estimate for the hatching time in the control (see Material and Methods).

Initial fits (not shown) revealed that the estimated egg weight (W_{B0}) is too small to accurately predict the first size measurements in juveniles. In DEBkiss, embryos follow the same rules as juveniles, which implies losses of mass due to maintenance costs, overhead costs for growth, and maturation (the 1-κ flux is burned to increase maturity). However, to achieve a hatchling length of some 0.27 mm,[18] almost the total egg mass would need to be converted into a hatchling (assuming that the hatchlings have the same shape coefficient δ_M as adults). In DEBkiss, the fresh egg starts with a small amount of structure and a considerable amount of egg buffer (i.e., yolk) fueling development.[15] This does not reflect the developmental process of nematodes, where the complete egg follows a series of cleavage divisions until the final number of somatic cells is achieved. The body length of the embryo, as plotted in Figure 1 (the almost straight line in the first 11 hours), is thus a hypothetical structural size. The energetics of *C. elegans* embryos requires further study, but for the purpose of this study we can get a good correspondence to the initial part of the growth curve by fixing κ = 1 for the embryo.

Size and reproduction in controls. The DEBkiss model was fitted to the control data for growth and reproduction from the three experiments simultaneously (Figure 1). Reproduction data for t > 6 days were discarded as we did not attempt to provide a mechanism for the stop of egg production. The data for the two single chemical experiments were very close, and could easily be described with the same set of parameters. In the mixture experiments, the control showed a slightly enhanced growth and reproduction. The nature of this discrepancy is unclear, but we could capture it by assuming a slightly lower scaled functional response (f) in the single chemical

analyses (in the mixture experiment, we fix f = 1). A lower specific maintenance rate in the mixture experiment can also cover these differences, but seems less plausible. From the fit, the embryonic phase can be clearly distinguished: at hatching, the individual switches from feeding on the buffer of assimilates in the egg to feeding from the external environment. Growth slows down immediately after hatching because we included a size-dependent food limitation for the juveniles.

The number of estimated parameters is quite large (seven), given the information content in the data (three growth and reproduction curves). This is due to the extra parameter for the deviating growth curve (L_{wf}), and the apparent food availability for the embryo (f_B) needed to match the observed hatching time. With the additional constraint on the hatching time, and fixing the egg weight, the data allow for a precise estimate of all model parameters (as evidenced from the tight confidence intervals in Table 1). We fixed the physiological parameters to the values in the controls (Table 1) for the subsequent fitting of the treatments.

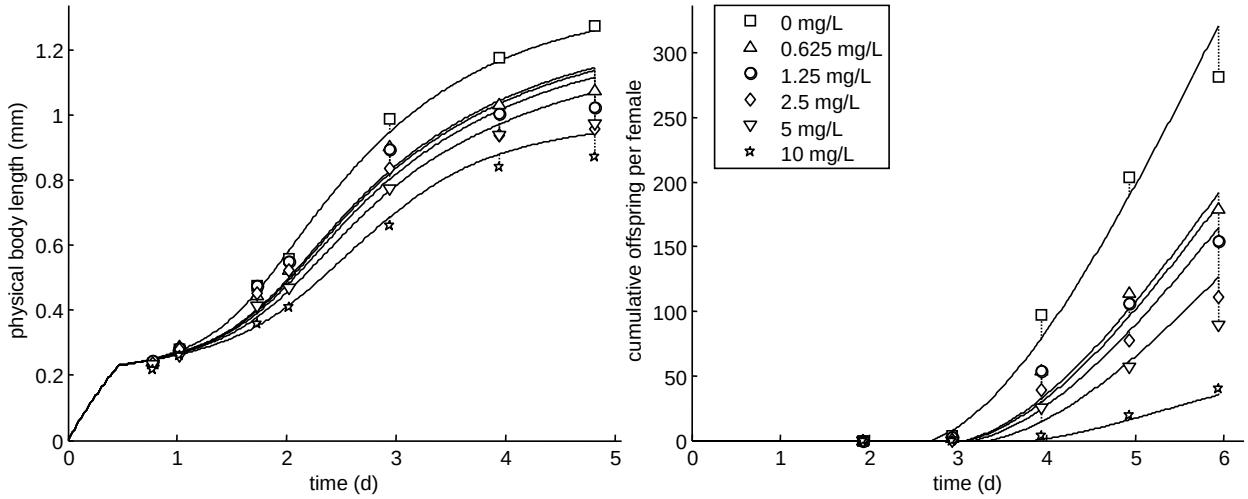

Figure 2. Simultaneous fit to growth and reproduction data for *C. elegans* exposed to cadmium. Parameters for the controls were fixed (Table 1).

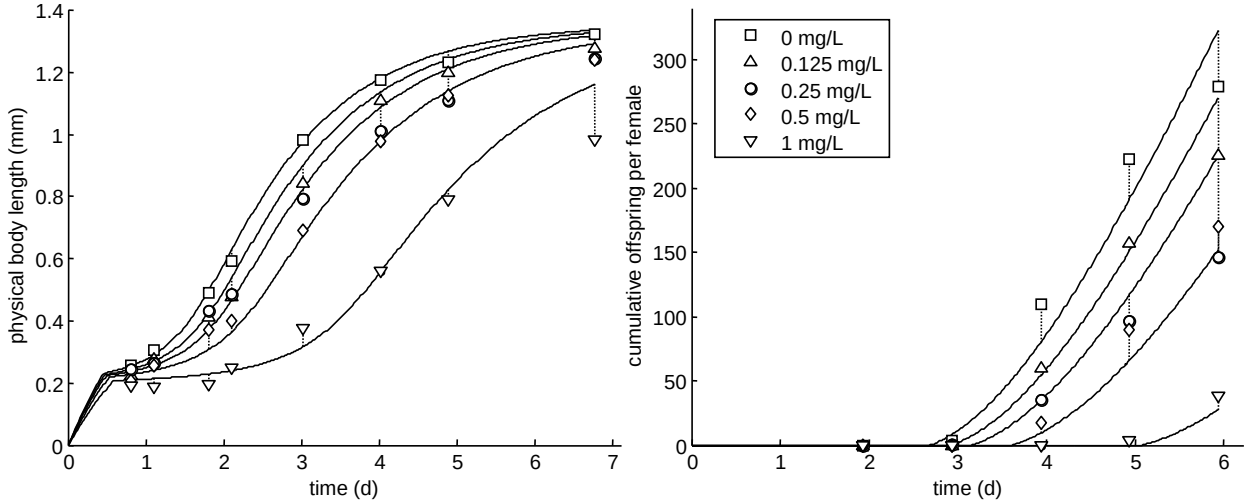

Figure 3. Simultaneous fit to growth and reproduction data for *C. elegans* exposed to fluoranthene. Parameters for the controls were fixed (Table 1).

Single chemicals. The best-fitting mMoA for Cd was a decrease in the assimilation of energy from food, and for FA an increase in the costs for both growth and reproduction. This is consistent with previous analyses,[13,17] and confirms that the mMoA is a robust property of a chemical in a particular species. However, we made a few different assumptions in comparison to the earlier analyses. Firstly, in contrast to the previous work, the current data did not reveal a clear decrease of the length at puberty (L_{wp}) under stress, for both chemicals. This saves us from using an additional parameter, but it is unclear why there is a difference with the earlier studies.

For Cd, the observed difference in effect between treatments is surprising (Figure 2): the first dose already gives a strong effect, but higher doses produce less than the expected proportional increase in effect with increasing exposure. This pattern cannot be captured by the basic first-order toxicokinetics. Here, we can provide a good explanation of the dose response by adding a fixed amount (c_A) to the external concentration, and setting the no-effect threshold (c_0) to zero. In the earlier analyses, a non-zero threshold and saturation of the uptake kinetics was assumed, without an additional c_A. This also provides a reasonable, but less convincing, explanation of the data, and requires an additional parameter (see SI). For Cd, simple first-order toxicokinetics is apparently insufficient to provide an accurate description of the complexities of bioavailability, uptake, speciation (internal and external), internal redistribution, and possibly inactivation of this heavy metal. For the purpose of this study, we therefore stick to the addition of a fixed amount to the external concentration.

For FA, the basic model fits the data well (Figure 3). The effect on the growth costs implies less growth but the same ultimate size. The initial food limitation exaggerates the effect of the chemical: the slower growth due to the toxicant forces the juvenile to stay longer in the size class where feeding is inhibited. This interaction between food limitation and toxicity was already predicted,[27] and strengthens the case for this explanation of the deviating growth pattern. The confidence intervals on c_0 and c_T are very tight, which may represent numerical problems with the optimization algorithm or with peculiarities of this particular data set (the profile likelihood suggest that for c_0 an interval of 0-0.006 mg/L may be more realistic, see SI). The 2 mg/L treatment is not shown as there was very little hatching, and the few worms that hatched died within hours.

For Cd, there is no effect on the embryo in the model, as we assumed that the eggs do not take up Cd from the medium. It is possible that metal ions are not able to cross the cell wall, but in any case, this assumption matches the size data at the first measurement (assuming a steady state between egg and medium would have led to large effects on the embryo). Furthermore, this assumption is consistent with the low estimated elimination rate (Table 1) for juveniles/adults. The elimination rate determines the time needed to reach steady state between organism and environment, and thus reflects the rate of chemical exchange in both directions. In contrast, for FA we assumed instantaneous steady state for the body residues, which is consistent with the very high estimated elimination rate (Table 1). The effects of the chemical on the growth costs leads to a slower development of the embryo, and delayed hatching at a smaller size. This prediction cannot be corroborated from direct observations but matches the initial size measurements for the juveniles quite well. Furthermore, the strong effect on hatching success at 2 mg/L supports the hypothesis that FA is taken up in the egg and affects the embryonic development.

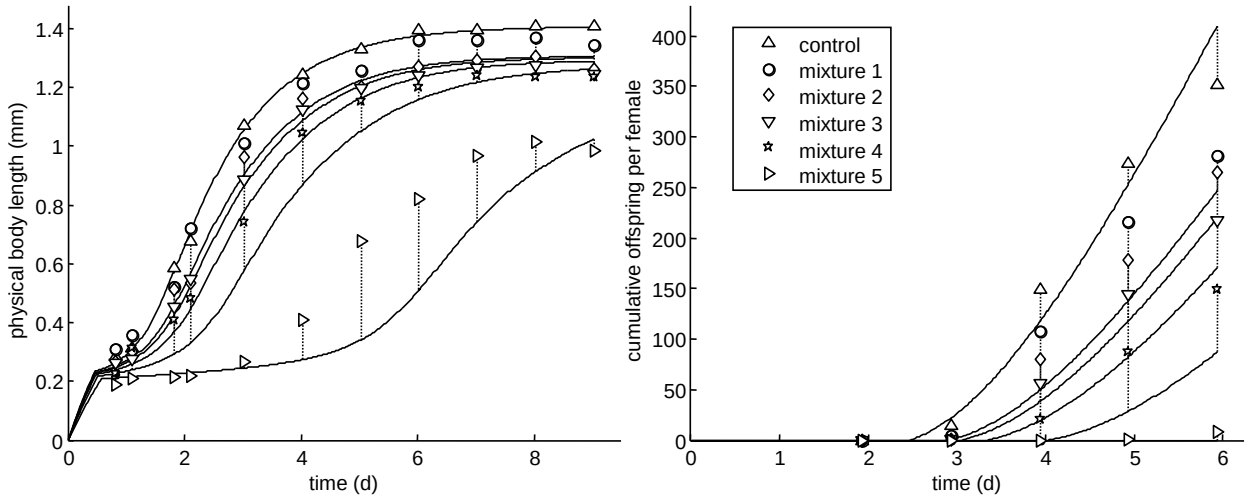

Figure 4. Prediction (no parameters fitted) of growth and reproduction data for *C. elegans* exposed to five mixture treatments of cadmium and fluoranthene (same ratio, different strengths). All parameters were fixed to the values for the controls and the single-chemical fits (Table 1). Note that for the highest treatment, the model predicts no reproduction, so the model line overlaps with the x-axis.

Mixture. Using the toxicological parameters from the single-chemical experiments, and the physiological parameters from the control treatments, we obtain a reasonable match to the data for the mixture of the two chemicals, without any fitting (Figure 4). This indicates that the assumption of total independent action of cadmium and fluoranthene is not unrealistic, and that we can reasonably predict the behavior of a binary mixture from the single components, for growth and reproduction over time. However, it is clear that the effect of the mixture is consistently overestimated by the model. This indicates that these two chemicals sport an antagonistic interaction in mixed exposure: less effects on the measured endpoints than predicted from the model.

To indicate the statistical significance of the interaction, we fitted the data for all three experiments simultaneously, and compared the fit for 'no interaction' (same toxicological parameters in the single exposure and in the mixture) to the fit for 'interactions' (allowing some or all of the parameters in the mixture to deviate from their values in single exposure). The antagonistic interaction is significant, in that a better fit can be achieved by allowing the elimination rate (k_e) or the tolerance concentration (c_T) of the two chemicals to be different in the mixture (details provided in the SI). Allowing for an interaction on the elimination rate (and thereby the rate of chemical exchange in both directions) provides the greatest improvement in the fit (see SI), and interestingly, all of this improvement comes from assuming a lower elimination rate for FA in the mixture than in single exposure. This analysis therefore suggests that Cd decreases the exchange of FA between nematode and medium. A possible explanation for this interaction is that Cd decreases assimilation by decreasing the feeding rate. As feeding constitutes an important route of chemical exchange for nematodes,[22] a decrease in feeding by Cd is likely to affect the elimination rate for FA.

Regarding the statistical testing, a few warnings are in order. Firstly, the initial food limitation interacts with toxicity, and hence, small uncertainties in parameter values will lead to large differences in effects later in life.[27] Furthermore, the statistical model for the deviations from the model predictions is a poor match to the problem;[10] for example, we assume independence of the

observations over time even though they were taken on the same animals. An additional uncertainty is introduced due to the selection of the exposure concentrations in the mixture, which includes lower concentrations than were tested in the single exposures. This is valid if the assumptions underlying the model are realistic, but that is in this case questionable given the rather descriptive assumptions for Cd (adding c_A to the external concentration).

IA model predictions. Figure 5 shows the log-logistic dose-response relationships for the single chemicals at a single time point (4 days) for the two endpoints (body length and reproduction). Clearly, the effects on reproduction are larger than on body length. This makes sense as a small reduction in size is usually associated with a more severe reduction in reproduction (which can be reduced to zero, in contrast to body size). For the mixture effect, two curves are shown: one fitted to the mixture effects (thin broken line) and one predicted from the effects of the single chemicals (thick broken line). The fitted line is to the right of the predicted line for IA, which indicates an antagonistic interaction. The antagonism was statistically significant both at day 4, as shown in the figure, and for day 6, which was also tested (see SI).

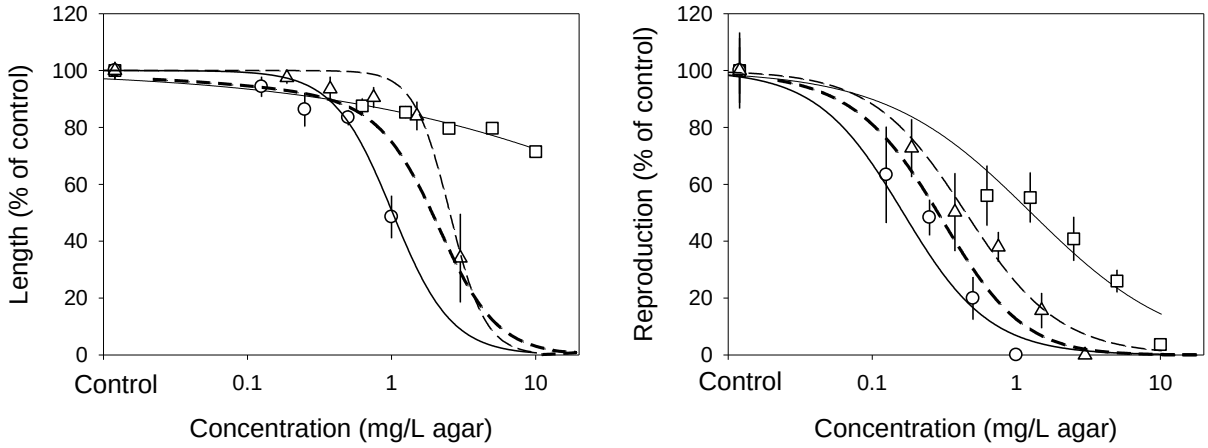

Figure 5. Nematode length and reproduction after 4 days, as percent of the untreated control average for nematodes treated with Cd (Squares), FA (circles), and the mixture of the two (triangles). Data for the individual chemicals (mean±95% conf. int., n = 12) are described with a two parameter log-logistic dose-response model (curve), as is the mixture data (thin broken curve). The IA prediction based on the dose-response model of the individual chemicals is given by the thick broken curve.

Evaluation and outlook. In conjunction with the earlier work on *Daphnia*,[8] we can conclude that DEB-based models provide a natural framework for investigating the simultaneous effects of mixtures on different endpoints over the life cycle. Even though this analysis does not yield a radically different conclusion from the more traditional dose-response analysis, it provides far more insight into the underlying dynamic mechanisms. This insight allows the generation of mechanistic hypotheses for observed mixture interactions. In the present case study, the testable hypothesis that we put forward was that Cd induced a reduction of food intake by the nematodes, which in turn affected the rates of uptake and excretion for FA. Such a specific hypothesis would not have been obvious from analyzing the data using traditional descriptive methodologies.

Mechanistic modeling not only allows studying the nature of mixture interaction, but also facilitates extrapolations beyond the conditions in the experimental test (vital for predicting

population consequences under realistic scenarios). Particularly, time-varying exposures cannot be dealt with using the traditional mixture concepts based on dose-response modelling, but are amenable to TKTD modeling.[5] Furthermore, these mechanistic insights at the level of the energy budget are indispensable to eventually link changes at the biochemical level to changes in life-history traits.[13] We also hope that focusing on common mechanisms in animals will provide a better basis for extrapolation between chemicals and between species, and for identifying the intrinsic reasons for differences in toxicity and sensitivity. Plenty of work remains to be done in the area of TKTD modeling for sub-lethal effects,[28] but models of this type are absolutely essential for a more mechanistic understanding of mixture ecotoxicology.

ASSOCIATED CONTENT

Supporting information

Detailed model description and additional model analyses are provided. This information is available free of charge via the Internet at http://pubs.acs.org/.

AUTHOR INFORMATION

Corresponding author

Phone: +31-20-59 87134; fax: +31-20-59 87123; e-mail: tjalling.jager@vu.nl.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

We wish to thank A. P. Møllers fund for Icelandic students in Denmark and the Memorial grant fund of Anna Claessen la Cour for graduate studies and research in Denmark for making this study possible.

REFERENCES

(1) Altenburger, R.; Backhaus, T.; Boedeker, W.; Faust, M.; Scholze, M. Simplifying complexity: Mixture toxicity assessment in the last 20 years. *Environ. Toxicol. Chem.* **2013**, *32*, 1685-1687.

(2) Van Gestel, C. A. M.; Hensbergen, P. J. Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. *Environ. Toxicol. Chem.* **1997**, *16*, 1177-1186.

(3) Cedergreen, N.; Streibig, J. C. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? *Environ. Toxicol. Chem.* **2005**, *24*, 1676-1683.

(4) Baas, J.; Van Houte, B. P. P.; Van Gestel, C. A. M.; Kooijman, S. A. L. M. Modelling the effects of binary mixtures on survival in time. *Environ. Toxicol. Chem.* **2007**, *26*, 1320-1327.

(5) Ashauer, R.; Boxall, A. B. A.; Brown, C. D. Modeling combined effects of pulsed exposure to carbaryl and chlorpyrifos on *Gammarus pulex*. *Environ. Sci. Technol.* **2007**, *41*, 5535-5541.

(6) Ashauer, R.; Escher, B. I. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. *J. Environ. Monit.* **2010**, *12*, 2056-2061.

(7) Lee, J. H.; Landrum, P. F. Development of a multi-component damage assessment model (MDAM) for time-dependent mixture toxicity with toxicokinetic interactions. *Environ. Sci. Technol.* **2006**, *40*, 1341-1349.
(8) Jager, T.; Vandenbrouck, T.; Baas, J.; De Coen, W. M.; Kooijman, S. A. L. M. A biology-based approach for mixture toxicity of multiple endpoints over the life cycle. *Ecotoxicology* **2010**, *19*, 351-361.
(9) Nisbet, R. M.; Muller, E. B.; Lika, K.; Kooijman, S. A. L. M. From molecules to ecosystems through dynamic energy budget models. *J. Anim. Ecol.* **2000**, *69*, 913-926.
(10) Jager, T.; Zimmer, E. I. Simplified Dynamic Energy Budget model for analysing ecotoxicity data. *Ecol. Modell.* **2012**, *225*, 74-81.
(11) Alda Álvarez, O.; Jager, T.; Marco Redondo, E.; Kammenga, J. E. Physiological modes of action of toxic chemicals in the nematode *Acrobeloides nanus*. *Environ. Toxicol. Chem.* **2006**, *25*, 3230-3237.
(12) Jager, T.; Alda Álvarez, O.; Kammenga, J. E.; Kooijman, S. A. L. M. Modelling nematode life cycles using dynamic energy budgets. *Funct. Ecol.* **2005**, *19*, 136-144.
(13) Swain, S.; Wren, J. F.; Stürzenbaum, S. R.; Kille, P.; Morgan, A. J.; Jager, T.; Jonker, M. J.; Hankard, P. K.; Svendsen, C.; Owen, J.; Hedley, B. A.; Blaxter, M.; Spurgeon, D. J. Linking toxicant physiological mode of action with induced gene expression changes in *Caenorhabditis elegans*. *BMC Systems Biology* **2010**, *4*, 32.
(14) Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **1974**, *77*, 71-94.
(15) Jager, T.; Martin, B. T.; Zimmer, E. I. DEBkiss or the quest for the simplest generic model of animal life history. *J. Theor. Biol.* **2013**, *328*, 9-18.
(16) Barsi, A.; Jager, T.; Collinet, M.; Lagadic, L.; Ducrot, V. Considerations for test design to accommodate energy-budget models in ecotoxicology: a case study for acetone in the pond snail *Lymnaea stagnalis*. *Environ. Toxicol. Chem.* **2014**, *Acc.*, DOI 10.1002/etc.2399.
(17) Alda Álvarez, O.; Jager, T.; Kooijman, S. A. L. M.; Kammenga, J. E. Responses to stress of *Caenorhabditis elegans* populations with different reproductive strategies. *Funct. Ecol.* **2005**, *19*, 656-664.
(18) Byerly, L.; Cassada, R. C.; Russell, R. L. The life cycle of the nematode *Caenorhabditis elegans*. I. Wild-type growth and reproduction. *Developmental Biology* **1976**, *51*, 23-33.
(19) Hirose, T.; Nakano, Y.; Nagamatsu, Y.; Misumi, T.; Ohta, H.; Ohshima, Y. Cyclic GMP-dependent protein kinase EGL-4 controls body size and lifespan in *C. elegans*. *Development* **2003**, *130*, 1089-1099.
(20) Wieser, W. Benthic studies in Buzzards Bay II. The meiofauna. *Limnol. Oceanogr.* **1960**, *5*, 121-137.
(21) Knight, C. G.; Patel, M. N.; Azevedo, R. B. R.; Leroi, A. M. A novel mode of ecdysozoan growth in *Caenorhabditis elegans*. *Evolution & Development* **2002**, *4*, 16-27.
(22) Höss, S.; Schlottmann, K.; Traunspurger, W. Toxicity of ingested cadmium to the nematode *Caenorhabditis elegans*. *Environ. Sci. Technol.* **2011**, *45*, 10219-10225.
(23) Ritz, C.; Streibig, J. C. Bioassay analysis using R. *Journal of Statistical Software* **2005**, *12*, 1-22.
(24) Cedergreen, N.; Svendsen, C.; Backhaus, T. In *Encyclopedia of Environmental Management*; Jorgensen, S. E., Ed.; Taylor & Francis: New York, 2013; pp 2601-2610.
(25) Williams, P. L.; Dusenbery, D. B. Aquatic toxicity testing using the nematode, *Caenorhabditis elegans*. *Environ. Toxicol. Chem.* **1990**, *9*, 1285-1290.

(26) ASTM *Standard guide for conducting laboratory soil toxicity tests with the nematode Caenorhabditis elegans*; American Society for Testing and Materials, **2001**; Vol. E2172-01.
(27) Zimmer, E. I.; Jager, T.; Ducrot, V.; Lagadic, L.; Kooijman, S. A. L. M. Juvenile food limitation in standardized tests: a warning to ecotoxicologists. *Ecotoxicology* **2012**, *21*, 2195-2204.
(28) Ashauer, R.; Agatz, A.; Albert, C.; Ducrot, V.; Galic, N.; Hendriks, J.; Jager, T.; Kretschmann, A.; O'Connor, I.; Rubach, M. N.; Nyman, A. M.; Schmitt, W.; Stadnicka, J.; Van den Brink, P. J.; Preuss, T. G. Toxicokinetic-toxicodynamic modeling of quantal and graded sublethal endpoints: a brief discussion of concepts. *Environ. Toxicol. Chem.* **2011**, *30*, 2519-2524.