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Mixture Toxicity in DEB-IBM

Development and Validation of a Mixture Toxicity Implementation in DED-IBM: Effects of Copper and Zinc on *Daphnia Magna* Populations

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ABSTRACT: Mechanistic population models are gaining considerable interest in ecological risk assessment. The dynamic energy budget approach for toxicity (debtox) and the general unified threshold model for survival (guts) is a well-established theoretical framework that describes sub-lethal and lethal effects of a chemical stressor, respectively. However, there have been limited applications of these models for mixtures of chemicals, especially to predict long-term effects on populations.

Here, we used DEBtox and GUTS in an Individual-Based Model (IBM) framework to predict both single and combined effects of copper and zinc on *Daphnia magna* populations. The model was calibrated based on standard chronic toxicity test results with

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the single substances. A mixture toxicity implementation based on the general independent action model for mixtures was developed and validated with data from a population experiment with copper and zinc mixtures.

Population-level effects of exposure to individual metals were accurately predicted by DEB-IBM. The DEB-IBM framework also allowed to identify the potential mechanisms underlying these observations. Under independent action the DEB-IBM was able to predict the population dynamics observed in populations exposed to the single metals and their mixtures ($R^2 > 65\%$ in all treatments).

Our modelling shows that it is possible to extrapolate from single-substance effects at the individual level to mixture toxicity effects at the population level, without the need for mixture toxicity data at the individual level from standard mixture toxicity tests. The application of such modelling techniques can increase the ecological realism in risk assessment.

Keywords: Copper (Metals), Zinc (Metals), Mixture toxicity, *Daphnia magna*, Dynamic Energy Budget (DEB), Individual Based Model (IBM), Metal risk assessment, Ecological modelling, Population model

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INTRODUCTION

The use of population models for risk assessment has gained increasing interest from the scientific community as the relevance of these models is increasingly understood (EFSA 2018; Forbes et al. 2016; Galic et al. 2010; Hommen et al. 2015; Raimondo et al. 2017). Currently, advanced computational techniques enable intensive and complex calculations, e.g. to track the behaviour of organisms within complex dynamic systems. Additionally, the relevance of population models for risk assessment is mentioned in the protection goals of several EU guidelines and legislations, i.e. the Water Framework Directive (WFD), REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), Plant Protection Products Regulation (PPP), Biocidal Products Regulation (BPR), and others (Galic et al. 2010; Hommen et al. 2010). Nonetheless, most studies and tests in routine risk assessment of chemicals occur at the individual level under laboratory conditions (Hommen et al. 2010) and only consider one chemical at a time. In higher-tier risk assessment, the construction of a Species Sensitivity Distribution (SSD) is often required or even the performance of a mesocosm experiment to assess community-level effects (EC 2018). The reality is, however, that individuals within populations and communities are exposed to mixtures of chemicals. Given the complexity of the situation, mechanistic population models have often been proposed as potential alternatives for time- and resource-consuming experimental population studies (Forbes et al. 2011).

Current risk assessment of chemicals is still very substance-based (Carvalho et al. 2014). For example, under the WFD, if the concentration of all single (priority) substances is below its Environmental Quality Standard (EQS) value, it is assumed the environment has a “good chemical status” and is protected from any adverse effects of chemicals (EC 2000; Carvalho et al. 2014). However, in the environment, organisms (living in populations and communities within complex ecosystems) are exposed to mixtures of chemicals that can vary over time, both in concentration and composition (Schreiner et al. 2016; Brack et al. 2017). The current approach based on individual EQS values completely disregards the potential combined action of co-occurring chemicals in mixtures and their effects on higher levels of organisation (i.e. populations or communities) (Carvalho et al. 2014). Many man-made, harmful chemicals enter aquatic ecosystems as mixtures (e.g. an active substance with co-formulants) or occur as unintended mixtures (e.g. two substances existing in the same water body) in the environment (Brack et al. 2017). It is challenging to assess the toxic effects of these complex mixtures and predict their joint mixture toxicity effects and their impact on populations or aquatic ecosystems based on individual-level, single-substance toxicity tests.

The use of mechanistic effect models in regulations up to now is very limited. The OECD in 2006 has already described mechanistic models as a biology-based approach to analyse toxicity data (OECD 2006). However, modelling is herein merely considered as an additional tool to conventional statistical analyses (e.g. the dose-response curve). More recently, the European Food and Safety Authority (EFSA) has published a scientific opinion on the use of TKTD models for regulatory risk assessment of pesticides in aquatic ecosystems (EFSA 2018). Within this opinion, models such as DEBtox (Jager and Zimmer 2012) and GUTS (Jager et al. 2011; Jager and Ashauer 2018) are evaluated as possible tools for the risk assessment of pesticides. GUTS models simulate toxicokinetic (TK) and toxicodynamic (TD) processes leading to toxicity at the organism level and can describe the time-course of effects that lead to mortality (Jager et al. 2011). TKTD models can also be used for sub-lethal effects with Dynamic Energy Budget (DEB) models (Kooijman 2010). DEB models describe the energy flows in an organism, from the assimilation of energy from food to growth, maturation, and reproduction (Kooijman 2010). DEBtox (an extension of DEB; Jager and Zimmer 2011) computes how stressors can ultimately affect these DEB energy flows, predicting sub-lethal effects at the organism level (i.e. a reduction in growth or reproduction). Thus, mechanistic frameworks, such as DEBtox and GUTS, are powerful tools that can integrate effects of stressors to eventually predict effects for untested scenarios. Moreover, integrating DEBtox and GUTS in a population model, e.g. an Individual-Based Model (IBM), can help us predict how a chemical stressor will affect populations in the field (Martin et al. 2013a; Martin et al. 2013b; Pereira et al. 2019; Vlaeminck et al. 2019).

So far, applications of GUTS and DEBtox models for mixture toxicity in a population context are, to our knowledge, non-existing. Jager et al. (2010) have conceptualized the mixture toxicity approach for DEBtox models, while Jager and Ashauer (2018) have explored the possibility of using GUTS models for mixtures. However, GUTS and DEBtox applications in literature are mostly based on a single-substance approach for a

specific species of interest (EFSA 2018; Vighi et al. 2019). There is an opportunity to extend these models to account for mixture toxicity effects, in addition to extrapolating these effects to the population level (Murphy et al. 2018; Vighi et al. 2019).

Considering the environmental protection goals defined in several EU legislations, there is a need to move from the current single-substance individual-level approach to the assessment of multiple chemical stressors at the population level. To show the relevance of GUTS and DEBtox models for risk assessment, we aim to demonstrate the use of modelling to predict population-level effects of chemicals and their mixtures and highlight the applicability of these models for mixture toxicity risk assessment.

The research question we put forward in this study is: Can mixture toxicity at the population level be predicted, with a DEB-IBM calibrated on the basis of data from standard toxicity tests at the individual level alone? In this study, we focus on two compounds, the trace metals copper (Cu) and zinc (Zn), and their effects on *Daphnia magna*. We calibrated GUTS and DEBtox models for Cu and Zn based on their effects on survival, growth and reproduction in a standard, 21-day toxicity test (OECD 2012). The calibrated GUTS and DEBtox models were integrated in an IBM to predict population dynamics of *D. magna* and we further extended our implementation to account for mixture toxicity effects. Based on the independent action (IA) reference model for mixture toxicity, an analogous implementation was constructed in the IBM. This implementation accounts for chemicals that could act through dissimilar modes of action (i.e. independent action). In contrast to IA, concentration addition (CA) is applicable when compounds act on the same biological target. However, in the current study the focus will be on the IA approach. For Cu, the observed mechanisms for toxicity to *D. magna* is related to the Na-metabolism (De Schamphelaere et al. 2007a), in contrast to Zn which interferes with the Ca-regulation (Muyssen et al. 2006). Based on the observed mechanisms of toxicity to *D. magna* and experimental evidence, it is reasonable to assume that Cu and Zn are independently acting chemicals. Eventually, the model was validated against experimental data with *D. magna* populations exposed to mixtures of Cu and Zn. In an additional analysis (see additional TRACE document – paragraph 7.2) we explain the observed trends in the population experiment by using the model. Finally, we highlight the applicability of this model for the population-level risk assessment of chemical mixtures.

MATERIAL AND METHODS

Individual-level experiment

A 21-day chronic life cycle test with *D. magna* (clone K6) was performed to assess apical effects of Cu and Zn. The OECD guideline no. 211 for *Daphnia magna* chronic reproduction test (OECD 2012) was followed. For the *Daphnia* culture and the experiment, a modified M4 medium as described in Pereira et al. (2017) was used. Prior to exposure, the daphnids were acclimated for at least two generations to the modified M4 medium.

Neonate daphnids (<24 h) were randomly selected from the culture and divided over plastic cups containing 50 mL of medium. The daphnids were exposed to 5

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concentrations of Cu (nominal concentrations: 32, 64, 128, 256 and 512 µg/L – chemical form: CuCl₂·2H₂O, CAS: 10125-13-0) and 5 concentrations of Zn (nominal concentrations: 64, 128, 256, 512 and 1024 µg/L – chemical form: ZnCl₂, CAS: 7646-85-7) for 21 days. The daphnids were fed daily with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) with daily addition of 125 µg C per daphnid in the first week, and 250 µg C in the following weeks. The experiment was conducted under a 16:8h light-dark photoperiod. Three times per week the medium was replaced. Mortality and reproduction (number of neonates produced) were recorded daily. Length of the daphnids was recorded 4 times during the 21-day period (on days 0, 7, 14 and 21) using a stereomicroscope (Olympus SZX10) and a camera (Olympus UC30).

The pH was measured once a week, both in the fresh and old medium. Once a week, samples from the fresh and old medium were collected for analysis of total (only fresh medium) and dissolved (after filtration over a 0.45 µm membrane) metal concentrations. Metal concentrations and concentrations of the major cations (Na, K, Ca and Mg) were analysed using iCAP 7000 Series ICP-OES (Thermo Scientific – limits of quantification: 5.0 µg Cu/L, 2.0 µg Zn/L, 50 µg Ca/L, 50 µg Mg/L, 100 µg Na/L, and 100 µg K/L – limits of detection: 2.0 µg Cu/L, 0.5 µg Zn/L, 15 µg Ca/L, 15 µg Mg/L, 30 µg Na/L, and 30 µg K/L). The DOC samples were analysed with TOC-L CPH (Shimadzu, Duisburg, Germany). The pH remained stable during the experiment (mean pH of 7.9, standard deviation of 0.3, n=28). The daily average temperature of the incubation room of the experiment was 20.2 °C (sd=0.6 °C, n=38). The average DOC concentration was 2.6 mg/L (sd=0.3, n=3). Further analysis was performed using dissolved metal concentrations (average of old and new medium). For Cu, the measured dissolved (diss.) concentrations were 6.8 (control), 16.6, 25.0, 42.1, 86.9 and 149.2 µg diss. Cu/L. For Zn, the measured dissolved concentrations were 8.2 (control), 23.2, 36.2, 62.7, 175.9, 357.4 and 790.5 µg diss. Zn/L.

Statistical analysis was performed in RStudio (RStudio Team 2016). Dose-response curves were fitted using the *drc* package (Ritz et al. 2016). A two-parameter log-logistic curve was chosen for the dose-response curves. Effect concentrations causing x% effect (ECx values) were derived from the fitted dose-response curves. The raw data of the individual-level experiment can be downloaded from Figshare (see link in supplemental information).

Population-level experiment

A mixture-toxicity population experiment was conducted, exposing *D. magna* populations to two concentrations of Cu (a low and a high concentration; nominal concentrations of 50 and 200 µg/L) and Zn (a low and high concentration; nominal concentrations: 100 and 250 µg/L) and their mixtures (low-low, low-high, high-low, high-high). The concentrations of Cu and Zn were based on effects observed at the individual level (i.e. the experiment described in paragraph 2.1). The Cu concentrations correspond approximately to the 21-day EC10 and EC50 values observed at the individual level on chronic reproduction. For Zn, the concentrations correspond to the 21-day LC10 and LC50 values on chronic survival observed at the individual level. In total there were 9 treatments: 4 single treatments, 4 mixtures and a control treatment (no Cu or Zn added), each in replicates of 4 (36 aquaria in total).

The initial population density in each aquarium was 6 egg-carrying females (3 weeks of age) and 10 neonates (<24h of age). The organisms were transferred to plastic aquaria

(water volume of 2L) with M4 medium (same medium as the individual-level experiments) that were aerated constantly. The daphnids were fed *Raphidocelis subcapitata* with a daily addition of 5 mg C food per aquarium. The populations were kept in a 16:8-h light-dark photoperiod regime. Half of the medium was renewed twice a week (every Tuesday and Friday). Population density (total, adult and juvenile) was recorded twice a week using an image analysis protocol (see protocol in SI). The total exposure duration of the population experiment was 75 days.

Metal samples and pH measurements were carried out once per week, both for the fresh and old medium. The pH remained stable during the experiment (average pH of 7.9, $sd=0.1$, $n=198$). The aquaria were placed in warm water baths, keeping the temperature stable at an average of 19.8 °C ($sd=1.0$, $n=415$). The average DOC concentration was 2.4 ($sd=0.2$, $n=2$) mg/L. Average (of old and new medium) dissolved metal concentrations were considered: 4.9 (control), 20.8 and 46.4 µg diss. Cu/L, and 7.2 (control), 88.7 and 182.5 µg diss. Zn/L.

Statistical analysis was performed using R-Studio (RStudio Team 2016). Dunn's test was performed at each timepoint to analyse differences between control and the exposure treatments (i.e. the singles and the mixtures) using the *dunn.test* package (Dinno 2017). The raw data of the population experiment is available on Figshare as well (see link in supplemental information).

Model considerations

To extrapolate the effects of Cu and Zn to the population level, an individual-based model (IBM) based on the dynamic energy budget theory (DEB) was used. An existing DEB-IBM implementation for *D. magna* by Pereira et al. (2019) was modified and used to calibrate the toxic effects of Cu and Zn based on the results of the individual-level experiment (for the endpoints: growth, reproduction and survival). Similar methods to extrapolate effects from the individual to the population level with DEB-IBM have been applied in the past (Martin et al. 2013b; Vlaeminck et al. 2019; Pereira et al. 2019). A TRACE document was developed (see supplemental information) containing detailed information on the model equations, development, and rationale, as well as in-depth analysis and evaluation of the model. The model itself can be downloaded from Figshare (see link in supplemental information).

Adaptations to the DEB-IBM

A generic DEB-IBM for *D. magna* was developed by Martin et al. (2012) in NetLogo (Wilensky 1999). We used the DEB parameters for *D. magna* from the Add-my-pet database (Table 1; Add-my-pet 2016). Specific adaptations made to the model were (1) addressing the difference in clonal and feeding conditions, (2) implementation of a starvation sub-model, and (3) implementing mixture toxicity effects of Cu and Zn.

Firstly, a recalibration of the DEB model was introduced to take into account the differences in algal food and *Daphnia magna* clone (i.e. species-specific physiological characteristics) compared to the studies used to derive the DEB parameters (see Pereira et al. 2019 for methodological details). The maximum surface-specific assimilation rate (p_{Am}) was recalibrated by fitting the model to the observed growth and reproduction of

the control data. The recalibrated value for p_{Am} was $254.37 \text{ J d}^{-1} \text{ cm}^{-2}$ (log-likelihood of -51.181) instead of the original value of $315.611 \text{ J d}^{-1} \text{ cm}^{-2}$ (for details, see TRACE document – paragraph 6.2). In addition, the *shape-factor* parameter was recalibrated (a value of 0.221 instead of 0.264). The *shape-factor* relates structural length (one of the DEB state variables) to actual measured length. In the current publication, *Daphnia* length was measured from the base of the spinal antenna to the eye. In the studies used to derive the DEB parameters for *D. magna* it is not specified how the length measurements were performed (Add-my-pet 2016).

Secondly, a starvation sub-model was integrated in the DEB-IBM. In DEB, mild starvation occurs when there is no food input (Kooijman 2010). The energy assimilation flux decreases to 0 and the energy reserves will not increase. However, energy will still be mobilized and is still used for maturation, growth and reproduction. When the reserves become too low, there might not be enough energy available to pay maintenance costs. Prolonged starvation will thus potentially lead to mortality. A starvation sub-model in the DEB-IBM was implemented to predict the effects of starvation-induced mortality. Prolonged starvation was simulated by increasing the mortality as the energy reserves deplete (Martin et al. 2013a). The probability of starving to death within a timestep of an individual p_{starv} [-] is considered proportional to the scaled energy reserve density (e_{scaled} [-]) and a reserve-dependent mortality coefficient ($mort_{const}$ [-]):

$$p_{starv} = (1 - e_{scaled}) * mort_{const} \quad (1)$$

Finally, toxic effects were implemented in the *D. magna* DEB-IBM. We used toxicokinetic-toxicodynamic (TKTD) modelling to predict the effects of Cu and Zn over time. Sub-lethal effects (effects on growth and reproduction) were modelled using the DEBtox approach (Jager and Zimmer 2012), and lethal effects using the GUTS approach (Jager et al. 2011).

TKTD models often describe the link between external water concentrations and internal concentrations in the body of the organism. However, internal concentrations of Cu and Zn are typically not measured in standard toxicity tests. In addition, for some metals it has been shown that the internal concentration reveals little on the toxicity of the metal (Borgmann et al. 1993; De Schampelaere et al. 2004; De Schampelaere and Janssen 2004). Therefore, scaled damage (D_w) was implemented as a new state variable (Jager et al. 2011). In the original GUTS paper, scaled damage is considered analogous to an internal concentration (Jager et al. 2011). However, more recently, Jager and Ashauer (2018) argue their preference for “scaled damage” over “internal concentration”, as these authors consider it to be more consistent in terminology. Internal concentration implies that it is a concentration that can be measured. Scaled damage implies a more abstract concept that is not directly measurable (e.g. it integrates all the toxicological, relevant processes at the physiological and biochemical level). We consider scaled damage, as defined here, as analogous to the internally available concentration of the chemical at the target site of toxicological action. For consistency with Jager and Ashauer (2018), we also use the terminology “scaled damage”.

In this study, the toxicokinetics and the toxicodynamics are modelled together in a single step (reducing complexity of the model). The change in scaled damage D_w [$\mu\text{g L}^{-1}$] over time is determined as:

$$\frac{dD_w}{dt} = k_d(C_w - D_w) \quad (2)$$

where C_w [$\mu\text{g L}^{-1}$] is the external (i.e. water/exposure) concentration of the metal, and k_d [h^{-1}] is the dominant rate constant. Note that Equation 2 assumes no dilution of damage through growth, nor are there any surface-volume ratio effects taken into consideration. The dominant rate constant determines the damage dynamics, as it relates to the time needed for the damage to reach steady state with the external concentration (Jager and Ashauer 2018). We assume that the damage state is explicitly shared by both the DEBtox and GUTS processes. This means that a single damage state is used to calculate both the lethal and sub-lethal effects of a specific compound.

As the damage increases, effects will become visible. The lethal and sub-lethal effects are modelled differently. The DEBtox approach for sub-lethal effects states that a chemical stressor will impact a Physiological Mode of Action (PMoA), affecting the energy flows in the DEB model (Jager et al. 2010). The PMoA has been described by Ashauer and Jager (2018) as “the distinct way in which a chemical interferes with the energy fluxes in an organism, and thereby affects life history traits”. As the stress increases, one or two DEB parameters will increase or decrease in value, depending on the PMoA (see Table 2). Typically, five DEB PMoAs are distinguished (Martin et al. 2013b), i.e. the decrease in energy assimilation (1), the increase in maintenance costs (2), the increase in costs of growth (3), the increase in the cost per egg (4), and the decrease in survival during the embryonic period (5).

The damage-dependent change in a DEB parameter results in a change in the life cycle of the organism (e.g. reduced growth and/or reproduction). Mathematically, this is implemented using the stress s (Equation 2; Table 2) as a multiplier on a DEB parameter. We assumed that the unitless s increases linearly with the level of damage, but only above a certain level:

$$s = \frac{1}{c_T} * \max(0, D_w - NEC) \quad (3)$$

where D_w [$\mu\text{g L}^{-1}$] is the scaled damage, NEC [$\mu\text{g L}^{-1}$] the no-effect concentration, and c_T [$\mu\text{g L}^{-1}$] the tolerance parameter. The assumption here is that the organism has an internal threshold for damage before effects will be visible, represented by the NEC value (Jager et al. 2010). Above the NEC value, damage will lead to effects. The simplest relationship, a linear relationship, is used to model the damage-dependent effects (Jager 2017), and therefore the stress on the PMoA will thus linearly increase with the damage. Table 2 shows the effect of the stress variable on DEB parameters for the different PMoAs.

For lethal effects, the GUTS approach is used, which is based on hazard modelling (Jager and Ashauer 2018). The hazard rate will increase linearly with the damage level. Again, the hazard will only increase above a certain damage level. Each organism has its own

damage threshold (drawn from a log-logistic distribution). The hazard rate of an individual h_z [h^{-1}] is calculated as:

$$h_z = b_w * \max(0, D_w - z_w) \quad (4)$$

where z_w [$\mu\text{g L}^{-1}$] is the individual's damage threshold, and b_w [$\text{L } \mu\text{g}^{-1} \text{h}^{-1}$] the killing rate. The one-hour survival probability (p_s , the probability to survive from one hourly timestep to the next) is then calculated from the hazard rate (see derivation of Equation 5 in the TRACE document – paragraph 2.8):

$$p_s = e^{-h_z} \quad (5)$$

The implementation presented here corresponds to the reduced GUTS model with stochastic death (GUTS-RED-SD) (Jager and Ashauer 2018). A discretized version of GUTS-RED-SD is implemented in NetLogo. A detailed derivation of the discretized GUTS sub-model as well as an example and comparison to the analytical solution of GUTS is presented in the TRACE document on the adapted DEB-IBM (TRACE – paragraph 2.8 and 5.4).

Mixture toxicity implementation

Mixture toxicity of a binary mixture has been implemented following the general theories for DEBtox (Jager et al. 2010) and GUTS (Jager et al. 2011). In DEBtox, a distinction is made between independent action (analogous to the Independent Action (IA) reference model for mixture toxicity) and damage addition (analogous to the reference model for Concentration Addition (CA) in mixture toxicity) (Jager et al. 2010). For GUTS the approach is similar, distinguishing independent action (also referred to as survival probability multiplication) and damage addition (Jager and Ashauer 2018).

IA considers that both compounds will act on different biological targets. Each compound will have its own damage state variable (Equation 2). For DEBtox the effect (i.e. stress) of each compound is thus calculated separately (Equation 3). The easiest example is if two compounds act on a different PMoAs, e.g. one increases the cost per egg and the other increases the cost of growth (Figure 1; IA (1)). Note that it is possible for two chemicals to act on the same PMoA but still through IA. If the biological pathway of toxicity of both compounds is different (e.g. they inhibit different digestive enzymes), but in the end both disrupt energy assimilation, then the compounds will act independently but on the same PMoA (Figure 1; IA (2)). For GUTS, in the case of independent action, all calculations are performed independently, i.e. the hazard rates (Equation 4) of each compound are calculated individually (with the separate damage states from each of the compounds) and the survival probabilities (Equation 5) can be multiplied (Jager and Ashauer 2018).

In contrast to IA, concentration addition (CA) would be applicable for chemicals that act on the same biological targets. Often the compounds are then treated as dilutions of each other. Since the compounds act on the same target, they exert a similar effect (i.e. the same form of damage). For DEBtox, this means they could affect the same PMoA

(Figure 1; damage addition - DA). However, for the case presented here, we only focus on the IA approach for the modelling of mixture toxicity effects of Cu and Zn. It makes sense to treat Cu and Zn as independently acting chemicals since the mechanism of toxicity to *D. magna* are known to be different. Cu interferes with the Na-metabolism (De Schampelaere et al. 2007a), whereas Zn interferes with the Ca-homeostasis (Muysen et al. 2006). The difference in mechanism and the observed link with toxicity data gives preference to the IA approach over the DA approach for mixtures of Cu and Zn.

Model calibration

All calculations are based on measured dissolved metal concentrations (in $\mu\text{g L}^{-1}$). Calibration was performed for the two metals (Cu and Zn) individually. Both data on growth over time, reproduction over time and survival over time were considered for the calibration. Both the starvation sub-model, the DEBtox sub-model (for sub-lethal effects), and the GUTS sub-model (for lethal effects) were fitted simultaneously. Model parameters were optimized by maximizing the log-likelihood between data and predictions. The log likelihood of a given endpoint is calculated as:

$$\ell(\theta|Y) = -\frac{n}{2} * \ln SSE = -\frac{n}{2} * \ln(\sum_{i=0}^n (y_i - \hat{y}_i)^2) \quad (6)$$

where *SSE* is the sum of squares between prediction (for parameter set θ) and dataset *Y*, *n* the total number of data points, y_i the data, and \hat{y}_i the associated predictions with parameter set θ .

Approximate Bayesian Computation (ABC) was used to calibrate the starvation, DEBtox and GUTS parameters (van der Vaart 2016). The NetLogo model was configured for the 21-day toxicity experiment, which was then run for 10 000 iterations, with randomized values of the parameters in each iteration (drawn from the *prior* distribution).

Optimization was performed in R-Studio (RStudio Team 2016). Based on the log-likelihood (Equation 6), the 100 best iteration fits to the data are selected for which the mean and confidence intervals of the parameters were calculated (the *posterior* distribution). This procedure was performed in several sequential iterations to further optimize the parameter values. First, wide *prior* distributions were selected, which were narrowed with each ABC iteration.

Model validation

The model was validated with the data from the population experiment. The experimental conditions of the experiment were imposed to the DEB-IBM, i.e. an initial population density of 6 adults (assuming on average 15 ± 3 eggs in the eggs pouch – average for a 4th brood of this clone under the culture conditions) and 10 juveniles, daily feeding, two-weekly media renewal, etc. The Cu and Zn exposure concentrations were imposed as measured during the population experiments.

Comparison between model predictions and data was first assessed visually by plotting the predictions against the raw data (i.e. population density over time). Validation plots were also made, plotting the predicted density against the observed density. Next to this,

a qualitative model fitness criterion was used. We define an R-squared (similar as the R-squared used in linear regression curves) as indicator of model fitness. The R-squared values were based on log-transformed densities. For the R-squared values, the sum of squares of the residuals (SSR) and the sum of squares explained (SSE) by the model were calculated:

$$R^2 = 1 - \frac{SSR}{SSE+SSR} \text{ with } SSR = \sum_{i=0}^n (y_i - \hat{y}_i)^2 \text{ and } SSE = \sum_{i=0}^n (\hat{y}_i - \bar{y})^2 \quad (7)$$

where y_i is the observed log-transformed density, \hat{y}_i the log-transformed predicted density on day i , and \bar{y} the average log-transformed density of the treatment.

RESULTS

Individual-level experiment

Cu reduced survival and reproduction of *D. magna* significantly. Growth was impacted as well, but to a lesser extent. The 21-day EC10 values for reproduction and growth respectively are 19.0 and 35.2 µg diss. Cu/L (Table 3). The 21-day LC10 is lower, with a value of 12.5 µg dissolved Cu/L. Zn showed slight positive effects on reproduction and growth at lower concentrations. No significant negative sub-lethal effects of Zn were observed at the tested concentrations. Only survival at day 21 was significantly impacted, with an LC10 value of 89.0 µg diss. Zn/L (Table 3).

Population-level experiment

The control data shows that the total population density over time first increases until it reaches a peak after 22 days (Figure 2). Then, the population density decreases, and a steady state density is reached from day 36 until day 75.

The population experiment showed significant effects on total population density only for the high Cu concentration treatments (46.4 µg diss. Cu/L) (Figure 2; see Table S2 in SI for detailed results on statistics). Only the treatments with high Cu show significant differences with the control treatment. More specifically, the population density increases more slowly (significantly lower total densities between days 9 and 22) and the equilibrium density is also lower (from day 40 onward in the high-high mixture treatment; Table S2). Zn did not lead to significant effects on total population density. Occasionally, higher peak densities were observed in the Zn treatments, but these were not significantly different from the control treatment.

The statistical analysis was also performed for the juvenile and adult densities (Figures S1 and S2; Tables S3 and S4). The effects on juvenile density were similar to that of total population density (Figure S1 and Table S3). In the Zn treatments (high Zn, and the mixture treatments low-high, high-low and high-high) a significant positive effect of Zn on juvenile density was observed in the final three weeks of the experiment (days 54 and onward). Significant negative effects of Cu were observed on total adult density in the high Cu treatment (high Cu, and the mixture high-low and high-high) (Figure S2 and

Table S4). The low Cu and Zn treatments, nor the mixture of the two lows showed significant deviations from the control treatment.

Modelling results of the single substances

Calibration was performed based on the data obtained at the individual level (paragraph 3.1) to derive effect parameters for Cu and Zn. The starvation sub-model, together with the GUTS and DEBtox sub-model were calibrated simultaneously. In Table 4 the optimized parameter values for both the GUTS and DEBtox sub-models are given. For Cu, only the PMoA for the decrease in energy assimilation was able to predict both the decrease in growth and reproduction with increasing Cu concentrations (Figure S5). For Zn, no significant individual-level effects were observed. Therefore, no PMoA for sub-lethal effects of Zn for *D. magna* could be derived. The fits of the GUTS model for Cu and Zn and the DEBtox for Cu can be found in the supplemental information (Figures S3 to S8).

The control predictions at the population level follow the trends observed in the control data of the population experiment (Figure 3). First, there is a population growth phase, after which an equilibrium density in the population is reached. The trends in the control treatment are governed by competition for food and starvation-induced mortality predicted by the model, since there is no exposure to Cu or Zn.

Examining the metal effects on the population (Figure 4), the model captures the observed trends well for the majority of the test. The predictions and experimental data were plotted relative to their respective control (i.e. control-normalized) to highlight the effects of Cu and Zn at the population level (Figure 4). Only for the high Cu treatment significant population effects were observed. In this treatment, initially, there is a large effect of Cu. There is a clear delay in the growth phase and the population equilibrium density is lower when the *D. magna* population is exposed to high Cu (Figure 4, top right). However, towards the end of the experiment, there seems to be a recovery of the population density. The DEB-IBM was able to capture this trend and predict the recovery. The predictions for the Zn treatments are more or less in line with the experimental data. The model slightly underpredicts the absolute population density in the high Zn treatment at certain timepoints (Figure S9 – day 20 to 40). The peak density is also underpredicted, but this was underpredicted in the control treatment as well (Figure 3). The absolute density at the end of the experiment matches the observations (Figure S9). Based on relative effects (Figure 4), no significant effects at the population level were observed in the population experiment with *D. magna* exposed to high Zn.

The total, juvenile and adult densities over time are plotted separately for visual evaluation (Figures S9, S10 and S11). Overall, the juvenile densities are well predicted by the model for the single metal exposures (Figure S10). The adult density is well predicted as well (Figure S11). For further validation, the model predictions are plotted against the observations (Figure S12). The closer the points are to the 1:1-relationship, the better the predictability of the model. For the single metal treatments, the R^2 on log-transformed total population density are higher than 75%, meaning at least 75% of the variation in the data could be explained by the model (Table 5). The high Cu treatment

shows the lowest R-squared value of the single substance treatments. This is caused by the overprediction of the adult density in the high Cu treatment (Figure S11; Table 5).

Mixture toxicity modelling at the population level

To predict mixture toxicity effects, the independent action (IA) approach for GUTS was used to model the combined lethal effects of Cu and Zn. Because no sub-lethal effects of Zn were observed in the individual-level tests, only sub-lethal effects of Cu were considered, i.e. via a decrease in energy assimilation with increasing Cu since this PMoA gave the best fit for the individual-level data. The mixture toxicity approach was thus only considered to predict lethal effects of Cu and Zn at the population. Overall, the mixture toxicity treatments are well predicted under IA (Figure 4). The effects at the population level in the mixture treatment are mainly dominated by Cu. The recovery of effects that was observed in the treatment with both high Cu and high Zn was correctly predicted as well (Figure 4). The model shows deviations in the high Cu and low Zn treatment, where there is a significant underprediction of the peak density in the early phase of the experiment. The adult density correlates well in this treatment (Figure S11), but the juvenile density is underpredicted (Figure S10). Overall, there was a high correlation between data and predictions in the mixture toxicity treatments, with R-squared values of 65% and higher (Table 5). The lowest R-squared value was found for the treatment with high Cu and high Zn.

DISCUSSION

Modelling of metal effects at the population level

The main goal of the present study was to extrapolate the effects from single substances at the individual level, to mixture toxicity effects at the population level. We calibrated a GUTS and DEBtox model to integrate respectively the lethal and sub-lethal effects of Cu and Zn at the individual level in a DEB model. The DEB-IBM framework allows for predictions of *D. magna* population dynamics under chemical stress. Overall, the predictions at the population level reflect the trends from the population experiment.

For Zn, there were no significant effects observed at the population level at the highest tested concentration of the experiment (Figure 2). The DEB-IBM accurately predicted the absence of effects at this Zn concentration (Figure 4). The peak density is underpredicted in the Zn treatment, but this is also the case for the control treatment (Figure 3). The density at the end of the experiment is well predicted in the high Zn treatment. Pereira et al. (2019) have reported similar results with a *D. magna* population exposed to Nickel (Ni). At the highest tested Ni concentrations, they observed no consistent, significant effects at the population level, even though they imposed concentrations that affected reproduction at the individual level significantly. Their detailed analysis of the mortality causes in a population simulation, suggested that the increase in mortality due to chemical stress decreases the mortality due to starvation. Because more individuals died due to chemical exposure, there was relatively more food remaining for the survivors, which resulted in an increase in resource efficiency in the population. Detailed analysis of our model reveals similar population-level mechanisms at hand for our case (see TRACE

– paragraph 7.2). In the control scenario, initially, all mortality is caused by starvation, which decreases over time as more ageing mortality occurs. When the population reaches equilibrium, the main cause of death is ageing (about 70% of the total mortality; TRACE – paragraph 7.2, Figure 16). As the Zn concentration increases the proportion of mortality due to starvation decreases as the mortality due to Zn increases. At the end of the simulation, the largest portion of the total predicted mortality is caused by chemical mortality due to Zn exposure (about 38%) and ageing (also about 38%) (TRACE – paragraph 7.2, Figure 16). Starvation mortality has a relatively smaller impact on the population (just under 25% of the total mortality). This is also reflected in the assimilation flux, where the predicted average assimilation flux per individual is higher at high Zn exposure than at lower Zn concentrations (TRACE – paragraph 7.2, Figure 17). In the model, there is thus a compensation process at the population level, where an increase in chemical mortality decreases starvation mortality. This could explain why in the high Zinc treatment (182.5 µg diss. Zn/L) in the population experiment no significant effects at the population level are observed. Dedicated experiments with similar Zn exposure but different levels of food competition (i.e. different levels of starvation) could help investigate the extent of this compensation mechanism for *D. magna* populations.

For Cu, there were significant effects observed on the *D. magna* population at the highest concentration tested (46.4 µg diss. Cu/L, the 21d-EC50 on reproduction). There was a large effect in the first weeks of the experiment, where the population did not reach a distinct peak in contrast to the control (Figure 2). The DEB-IBM was able to capture the effects of Cu at the population level. The model predicts the absence of a peak in the first weeks. When observing the relative effects (Figure 4), we can see the model is able to capture the recovery observed over time. The additional analysis (TRACE – paragraph 7.2) of the model reveals how this recovery emerges over time. At 40 µg diss. Cu/L (close to the measured concentration in the high Cu treatment), initially the main cause of mortality is starvation, which decreases as the chemical mortality due to Cu increases (TRACE – paragraph 7.2, Figure 13). Eventually, the proportion of mortality due to Cu in the population surpasses the proportion of starvation mortality and becomes the dominant driver for mortality in the population. However, once the population density reaches a specific size, all available food is consumed. This is predicted after about 15 days of exposure in the simulated population (TRACE – paragraph 7.2, Figure 14). At that moment, due to the lack of food, the proportion of starvation mortality increases again. The proportion of mortality due to Cu decreases and becomes less dominant. At the equilibrium state, the ageing mortality is more important than the mortality due to Cu-exposure or starvation at this concentration. The proportion of Cu-induced mortality and starvation is more or less equal (each about 25% of the total mortality in the population; TRACE – paragraph 7.2, Figure 14). The switch from starvation mortality to Cu-induced mortality, and back from Cu-induced mortality to starvation mortality when food becomes limiting, explains the predicted population dynamics in the Cu treatment. Initially, the effect on the population is large due to Cu. After a while, Cu becomes less important as the starvation and ageing mortality increase again, leading to the predicted recovery over time at this concentration. Dedicated experiments would be helpful to confirm whether these processes are actually occurring.

Validation of the model with the (independent) data from the population experiment showed a high predictive capacity of the model. Plotting the predictions against the population data (relative to their respective control), we find a high correlation between the model and our experimental observations (see Table 5 and Figure 4). In all the single-metal treatments, the R-squared value is higher than 75% (based on log-transformed, total population density).

Applications of DEBtox-based IBMs for *D. magna* populations exposed to chemical stressors have been discussed already in literature (Martin et al. 2013b; Pereira et al. 2019). The effects of different PMoAs on *D. magna* populations have been theoretically explored before (Martin et al. 2014). These authors observed that the PMoA for assimilation leads to a reduction of population equilibrium abundance in a simulated population. The most-likely PMoA for Cu that explains the observed decrease in reproduction and growth at the individual level (standard test) was the decrease in energy assimilation (paragraph 3.3). Indeed, the effect of Cu on *D. magna* has been suggested to be related to reduced energy acquisition (De Schampelaere et al. 2007b; Martins et al. 2017) or declining filtration rates (Ferrando et al. 1993). This decrease in energy assimilation due to Cu eventually resulted in a significant decrease of population growth in the early phases of the experiment. Based on an additional simulation without the DEBtox sub-model for Cu (Figure S13), the predicted population dynamics differ in the early growth phase from the simulation with sub-lethal effects included (Figure S9). The decrease in energy assimilation causes the individuals to invest less energy in growth and reproduction, decreasing the overall population growth rate during the early phase of the experiment. In addition, less energy is invested into the energy reserves, increasing the starvation mortality in the population. Indeed, the population is predicted to grow slower in the beginning when accounting for sub-lethal effects of Cu (Figure S9). However, the sub-lethal effects of Cu did not affect the final equilibrium density. This potentially indicates that the *D. magna* population dynamics at equilibrium under Cu stress are mostly governed by the lethal effects, whereas sub-lethal effects of Cu strongly influence population growth rates.

Pereira et al. (2019) developed a similar *D. magna* population model to account for Ni toxicity. However, the authors used an implementation that directly extrapolates observed lethal concentrations (LCx values) to calculate survival probabilities under constant exposure, therefore disregarding the kinetics and damage dynamics of the modelled compound. GUTS models have proven successful for predicting dynamic survival patterns under time-variable exposure (Jager et al. 2011). In addition, some individuals can show tolerance for stressors (Jager and Ashauer 2018), which can be predicted with GUTS as well assuming IT. However, for our case, we used the SD approach. The main advantage of our approach is that the complete dataset (i.e. survival, reproduction and growth over time) is used to calibrate the combined GUTS and DEBtox model, in contrast to Pereira et al. (2019) where only survival on the last day of the experiment is used. In addition, using both lethal and sub-lethal data during calibration of effects at the individual level yields greater confidence in parameter values (e.g. the dominant rate constant (k_d), which gives information on the damage dynamics of both lethal and sub-lethal effects). Combining GUTS with DEBtox in an IBM-context thus provides a

holistic, state-of-the-art approach (combining lethal and sub-lethal effects) to extrapolate effects of a chemical stressor from the individual to the population level. Additionally, we were able to put forward (testable) hypotheses about the mechanisms behind certain trends that were observed.

Evaluation of a mixture toxicity implementation in DEB-IBM

The DEB-IBM framework presented here contains several building blocks. We use a DEB model as the basis for our organism, describing the metabolic processes, ranging from energy assimilation, to maturation, maintenance, growth and reproduction (Kooijman 2010). The DEBtox extension includes sub-lethal effects of stressor (Jager and Zimmer 2011). By changing some of the energy flows in the DEB model with the damage of a stressor, sub-lethal effects (i.e. reduction in growth or reproduction) can be predicted. GUTS is then used to calculate the survival probability over time. Combining DEB, DEBtox and GUTS in an IBM framework allows for predictions of *D. magna* populations under chemical stress, including both lethal and sub-lethal effects.

The current implementation was extended to account for mixture toxicity effects of Cu and Zn. For the mixture toxicity effects, we tested a mechanistic implementation of the independent action (IA) model. The (mechanistic) implementation is based on the general (statistical) mixture toxicity theory of IA (based on Jager and Ashauer 2018) based on the same logic. The theory states that two compounds acting on two different chemical targets, exert an effect that is independent of the other compound (SCHER 2011). For the IA approach it suffices to calibrate the effect parameters for the two substances individually and calculate their individual effect independently in the DEB-IBM. The mixture toxicity effects at the population level seem to emerge as expected based on the effects calibrated at the individual level. The IA approach was able to predict the observed mixture toxicity effects well (Figure 4). The R-squared values of the mixture treatments were higher than 70%, except for the high Cu and high Zn mixture treatment (Table 5). In both these treatments, the lower R-squared can be largely explained by the underprediction of the peak density in the early phase of the experiment. This increases the absolute values of the error in the SSE, thus decreasing the R-squared in these treatments, whereas model deviations from observations at lower population densities contribute less to the SSE and R-squared. The adult densities in these treatments are quite well predicted (Figure S11), in contrast to the juvenile density being underpredicted (Figure S10). This lower juvenile density is caused by the reduced assimilation due to Cu (less energy available to produce eggs) and the increased competition of food as the density increases starvation mortality. A potential explanation for the discrepancy between observed and predicted juvenile density could be crowding-related processes, which are not included in the current extended DEB-IBM. At higher densities, the daphnids could be triggered by the presence of other individuals around them, potentially affecting their reproduction rates (Burns 1995).

Across a wide set of metal mixtures and aquatic organisms, concentration addition (CA) is generally more conservative than IA for predicting chronic metal mixture effects at the individual (apical) level, i.e. mostly additive or antagonistic effects relative to CA are predicted compared to what is observed, whereas IA is 'on average' a better chronic

metal mixture toxicity predictor (Nys et al. 2018). The mechanistic IA approach here is based on the same assumption as traditional IA: the relative effect of the mixture can be predicted based on the relative effects of the substances individually (Nys et al. 2017). However, the approach presented here is mechanistic: the two compounds will exert their effect at the level of the PMoA, i.e. internal damage leading to an effect. In traditional IA, the approach is statistical: the effect concentrations of the individual mixture components are used to calculate the mixture toxicity effect at the apical level (SCHER 2011).

It is important to not project conclusion on the use of a certain type of mixture toxicity model at one level of biological organisation to the predictive abilities of an analogous model at another level of organisation. Here, the IA model applied at the PMoA level makes relatively accurate predictions at the population level. However, this does not mean the CA model needs to be rejected at the individual (apical) level. Additionally, it also means that applications of IA on individual (apical) level data do not provide conclusive information on the appropriateness of the use of IA at the PMoA-level for predicting population-level effects with DEB-IBM. Assessment of capability of selecting IA or CA at the PMoA-level for accurately predicting population-level effects should be considered within the modelling cycle, i.e. a calibration with individual-level data (reproduction, survival and growth over time) and a validation at the population level (see current study and TRACE document). Conversely, judgement of predictive ability of (statistical) IA or CA for metal mixtures at the individual level (including Cu-Zn mixtures to *D. magna*) is best done with individual-level data (e.g. Nys et al. 2018).

An analogous, mechanistic implementation of CA could prove insightful for investigating (metal) mixture toxicity in general and their effect on population dynamics. CA assumes chemicals affect the same toxicological target. For instance, for DEBtox-based mechanistic models, this means they affect the same PMoA. CA methods for mechanistic models have been proposed, e.g. damage addition as suggested by Jager et al. (2018) or body residue addition (Jager et al. 2010). Applicability of CA (and IA) at the PMoA level can provide us information on the conditions under which CA or IA are applicable for mixtures in mechanistic (population) models. Eventually, an integrated comparison could be made between mixture toxicity modes across different levels of organisation, i.e. PMoA-level vs. the apical level.

A limitation of the current implementation is that it does not account for potential chemical interactions. The DEB model, however, inherently considers potential physiological interactions (Jager et al. 2011). Indeed, if a specific DEB process is impacted (e.g. Cu affects energy assimilation) it will automatically influence other processes further down the chain, e.g. a reduction in growth, which in turn affects food energy assimilation as well (smaller individuals assimilate less food). As Jager et al. (2011) stated, the strength of a biology-based approach, such as DEB, is that it can separate the physiological interactions from the chemical interactions. Chemical interactions that happen outside the organism, could be taken into account by using speciation or bioavailability models (e.g. Windermere Humic Aqueous Model – WHAM; Tipping et al. 2011). Chemical interactions inside the organism that potentially cause augmented effects (synergism) or diminished effects (antagonism) are disregarded. With our modelling approach here, we assumed no chemical interference in the toxicokinetics

or the toxicodynamics of Cu and Zn (i.e. explicit separation of damage state of Cu and Zn – Equation 1). In addition, we applied the independent action approach in DEB-IBM to calculate the lethal effects of Cu and Zn on the *D. magna* population.

Mechanistic effect models for mixture toxicity

Mechanistic models, contrary to descriptive or statistical models (e.g. dose-response curves), describe effects through mechanisms (Jager and Ashauer 2018). The advantage of mechanistic DEB-based IBMs is that we can precisely track for each individual the metabolic energetics and the influence of stressors on these. With increasing concentrations of Cu and Zn, the mortality due to Cu and Zn will play a larger role in governing the population dynamics in the population. In addition, there is an effect on energy assimilation due to Cu. Knowing how these factors mechanistically influence individuals, gives us more insight into the mechanisms that affect populations (see additional analysis in TRACE – paragraph 7.2). For instance, for Zn the effect on the population is minor – at least for the conditions tested here – even at toxicologically relevant concentrations at the individual level. For Cu, the combination of a decrease in energy assimilation and an increase in mortality causes the population to grow slower. The mixture toxicity effects of Cu and Zn at the population level emerge from the effects of the two individual compounds. Based on the effects of the single substances at the individual level, the approach here can predict mixture toxicity effects at the population level and explain the outcomes mechanistically. This has a clear advantage over the classical, descriptive mixture toxicity models (i.e. IA and CA) and dose-response curves.

Based on the methods described above, a general scheme for mechanistic models for population-level effects of mixtures can be proposed (see Figure 5). The main approach is based on a calibration of the model at the individual level, followed by a validation at the population level. Effects of the individual compounds at the individual level need to be calibrated first. This can be done based on standard toxicity test data (i.e. survival, growth and reproduction over time). For most chemicals, toxicity data is already available, albeit that size is not a default endpoint that is measured, nor is it standardized in testing guidelines. The EFSA opinion on TKTD models prescribes an explicit, additional validation of GUTS models at the individual level, using time-variable exposure (i.e. pulsed exposure) (EFSA 2018). The next step would then be using the model to predict population-level effects. The methods described in the current study only consider IA as mixture toxicity approach but, based on model validation, other mixture toxicity models could be evaluated. Validation at the population level can be done by using population-level data. Finally, the model can be applied to predict population-level effects of chemical mixtures under different scenarios.

CONCLUSION

Using the DEB-IBM, we could mechanistically explain the trends observed in the population experiment. The absence of adverse effects due to Zn was explained through compensation mechanisms happening at the population level. The increase in mortality due to Zn decreased the starvation mortality in the *Daphnia* population. For Cu, the switch from Cu-induced mortality to starvation mortality when food becomes limiting at

high densities, explained the recovery that is observed over time. The mechanistic framework presented here can thus help understand the specific mechanisms of toxicity that are going on in a population context. Based on the effects observed in standard tests (single substances tested on individual organisms), we are able predict mixture toxicity effects at the population level (multiple substances and many individuals of the same species together). Application of these models for relevant scenarios will allow for more realism and ecological relevance in the risk assessment of chemicals and their mixtures.

Supplemental Data--The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability Statement-- Data, associated metadata, and calculation tools are available from the corresponding author (Karel.Vlaeminck@ugent.be).

This article has earned an Open Data/Materials badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [provided URL]. Learn more about the Open Practices badges from the Center for Open Science: <https://osf.io/tvyxz/wiki>.

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Figure 1. Conceptual scheme of a mixture toxicity implementation in DEBtox. A distinction is made between independent action (IA) and damage addition (DA). In IA, chemical A and B will each have its own damage state variable, D_A and D_B . In the DA approach, the two chemicals will cause the same damage D_{mix} . In IA, a further distinction is made between two chemicals that affect a different PMoA (IA 1) or the same PMoA (IA 2).

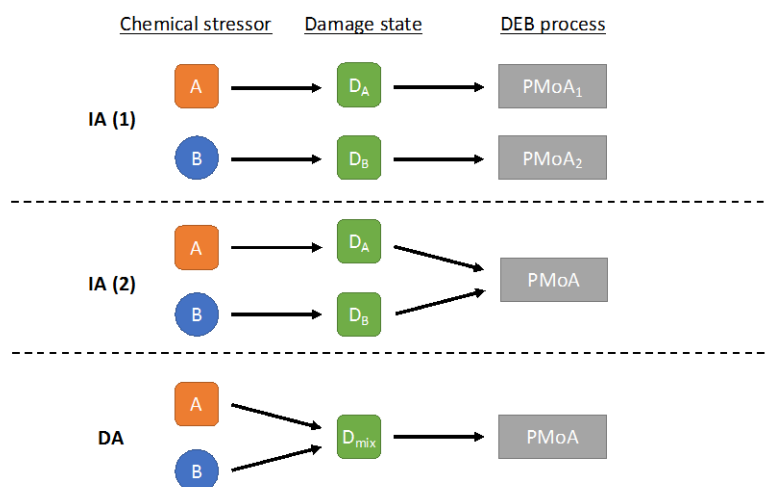


Figure 2. Results of the population experiment with a *D. magna* population exposed to mixtures of Cu and Zn. Total population density (number of individuals) is shown over time for the different treatments. The error flags indicate the standard deviation of 4 replicates. 9 treatments in total are shown: the blanks, low Cu (CuL), high Cu (CuH), low Zn (ZnL), high Zn (ZnH), the mixture of low Cu and low Zn (CuL/CuL), low Cu and high Zn (CuL/CuH), high Cu and low Zn (CuH/CuL), high Cu and high Zn (CuH/CuH).

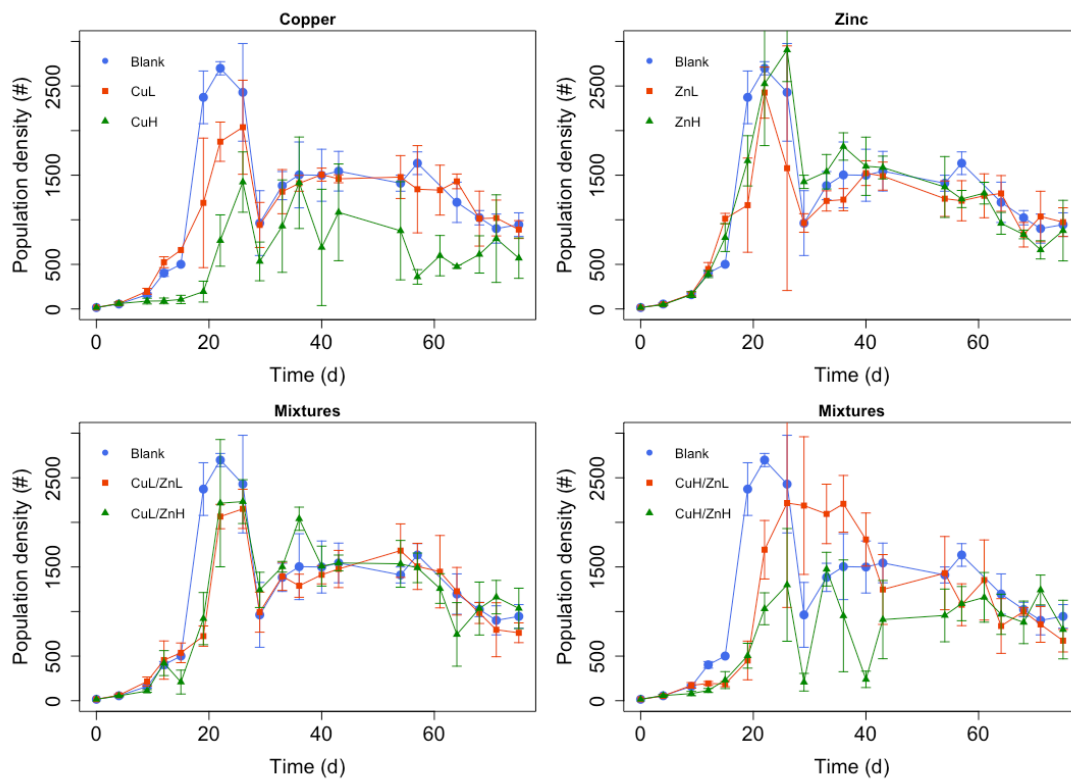


Figure 3. DEB-IBM model fit against the population data of the control treatment. The blue dots represent the total population density over time measured in the population experiment. The grey areas represent the DEB-IBM predictions of 100 simulation iterations (indicated by the minimum and maximum density predicted).

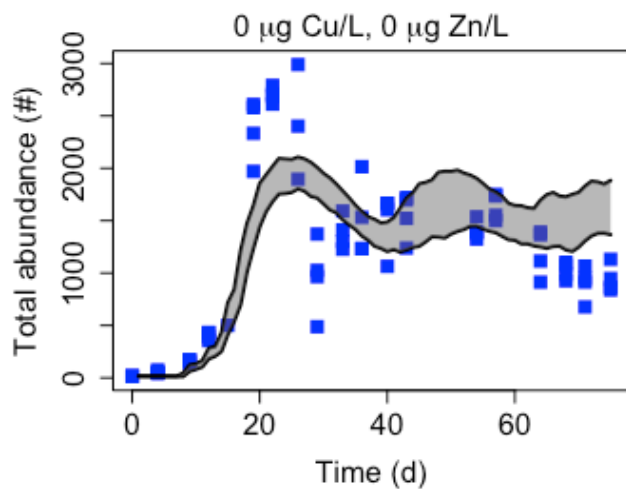


Figure 4. Results of the Independent Action approach. The DEB-IBM model predictions (relative to the control predictions) are plotted against the population data (relative to the control data). The blue dots represent the total population density over time (average of 4 replicates) measured in the population experiment relative to the control. The blue area around the dots indicates the standard deviation on the data. The grey areas represent the DEB-IBM predictions of 100 simulation iterations (indicated by the minimum and maximum density predicted) relative to the control. Going from left to right, the Cu concentration increases from 0, to 20.8 and 46.4 $\mu\text{g diss. Cu/L}$. Moving from up to down, the Zn concentration increases from 0, to 88.7 to 182.5 $\mu\text{g diss. Zn/L}$.

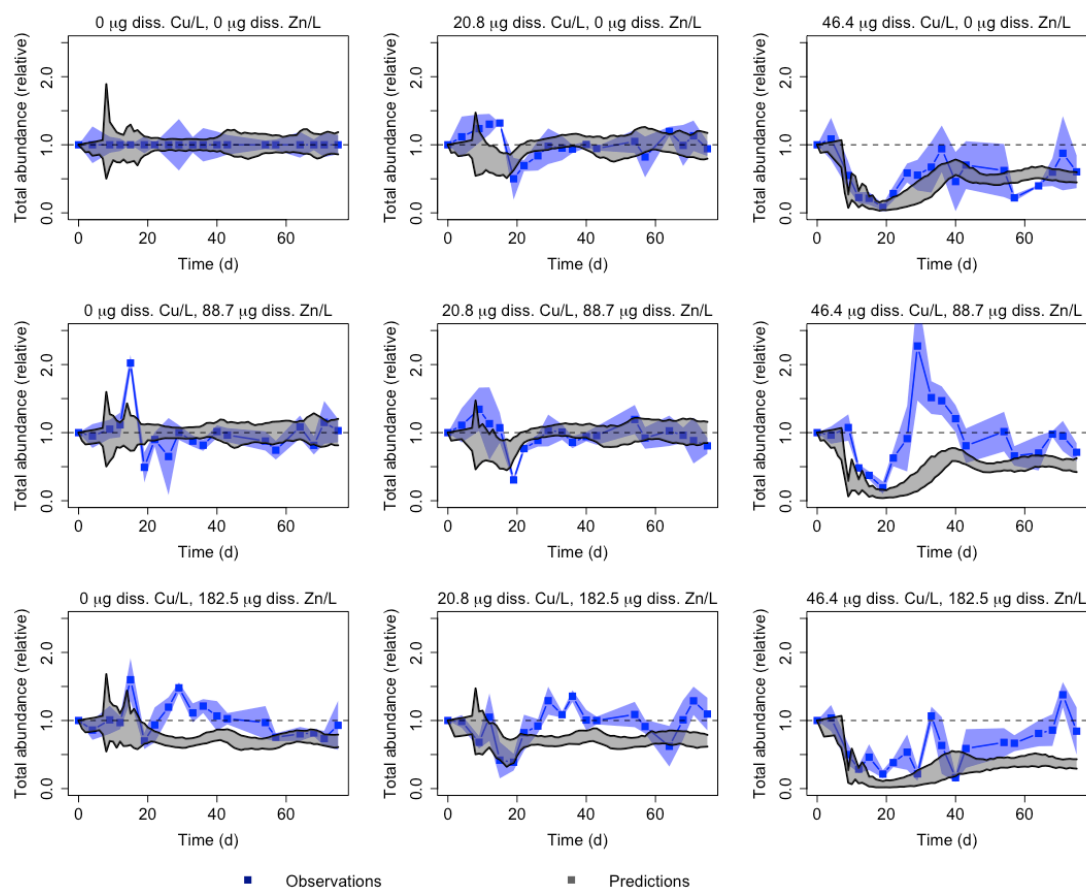


Figure 5. General scheme for effect assessment with mechanistic models for population-level effects of chemical mixtures (independent action approach for mixture toxicity).

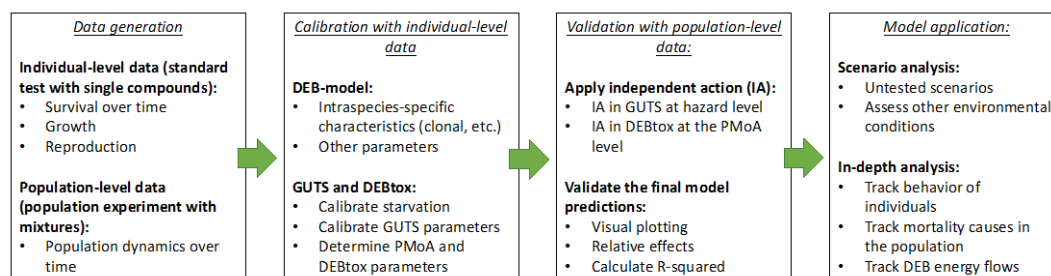


Table 1. DEB parameters for *D. magna* from the Add-my-pet database (Add-my-pet 2016). Note that these are not the most recent DEB parameters from the *D. magna* entry in the Add-my-pet database. An older set of DEB parameters was used (2016 version).

Parameter	Symbol	Value	Unit
Maximum surface-specific assimilation rate*	p_{Am}	254.37	J/(d*cm ²)
Maximum surface-specific searching rate	F_m	6.5	L/(d*cm ²)
Energy conductance	v	0.1584	cm/d
Allocation fraction to the soma (structural growth and structural maintenance)	κ	0.61	-
Reproduction efficiency	κ_R	0.95	-
Volume-specific somatic maintenance rate	p_M	1453	J/(d*cm ²)
Specific costs for structure	E_G	4400	J/cm ³
Maturity maintenance rate	k_J	0.002	d ⁻¹
Maturity at birth	E_H^b	0.0139	J

Maturity at puberty	E_H^p	0.3211	J
Weibull ageing acceleration	h_a	0.0003105	d ⁻²
Gompertz stress coefficient	s_G	-0.3	-

*The value for the surface-specific assimilation rate was recalibrated (see SI – paragraph 1). The original value of p_{Am} from the Add-my-pet database was 315.611 J/(d*cm²).

Table 2. DEB parameters under stress, taken from Martin et al. (2013b).

PMoA	Description	Affected parameter	Stressed value
Feeding/assimilation	Decrease in energy assimilation efficiency	f	$f_s = \frac{f}{(1 + s)}$
Maintenance	Increase in maintenance costs	k_M	$k_{M,s} = k_M * (1 + s)$
		k_J	$k_{J,s} = k_J * (1 + s)$
Growth costs*	Increase in overhead costs of growth	k_M	$k_{M,s} = \frac{k_M}{(1 + s)}$
		g	$g_s = g * (1 + s)$
Reproduction costs	Increase in cost per egg	κ_R	$\kappa_{R,s} = \frac{\kappa_R}{(1 + s)}$
Embryonic hazard	Decrease in survival during embryonic period	κ_R	$\kappa_{R,s} = \kappa_R * e^{-s}$

Table 3. Effect concentrations (ECx and LCx values) for Cu and Zn assessed at the individual, based on a standard 21-day toxicity test with *D. magna*. ECx and LCx values are derived from a 2-parameter log-logistic dose-response curve (drc package in R-Studio – RStudio Team 2016; Ritz et al. 2016). Effect concentrations (\pm the standard error) are given in μg dissolved Cu/L and μg dissolved Zn/L.

Compound	Endpoint	Effect concentration	Value
Cu	Reproduction	EC10	13.7 (\pm 3.6)
		EC20	17.6 (\pm 3.3)
		EC50	26.9 (\pm 3.8)
	Reproduction* (only survivors)	EC10	19.0 (\pm 8.3)
		EC20	27.6 (\pm 7.5)
		EC50	52.3 (\pm 20.2)
	Growth	EC10	35.2 (\pm 7.7)
		EC20	53.7 (\pm 20.7)
		EC50	110 (\pm 97.0)
	Survival	LC10	12.5 (\pm 3.8)
		LC20	17.8 (\pm 4.0)
		LC50	32.8 (\pm 4.9)
Zn	Survival	LC10	116 (\pm 15)

		LC20	150 (± 14)
		LC50	230 (± 24)

*Effect on reproduction was calculated in two ways. For reproduction here, the individuals that died during the experiment were excluded from the analysis – hence, the derived ECx value for 21-day reproduction only accounts for a true, mechanistic effect on reproduction (i.e. not a reduction caused by an increased mortality).

Table 4. Calibrated effect parameter values for Cu and Zn. A distinction is made between the starvation parameters, TKTD parameters, GUTS parameters and DEBtox parameters. The 95% confidence intervals are given between parentheses.

Parameter	Symbol	Value for Cu	Value for Zn	Unit
<i>Starvation related parameters</i>				
Mortality constant	$mort_{const}$	$1.0 * 10^{-3}$ ($0.5 * 10^{-3} - 1.6 * 10^{-3}$)	$1.1 * 10^{-3}$ ($0.5 * 10^{-3} - 1.5 * 10^{-3}$)	-
<i>TKTD parameters</i>				
Dominant rate constant	k_D	$1.7 * 10^{-2}$ ($1.1 * 10^{-2} - 2.9 * 10^{-2}$)	$8.8 * 10^{-3}$ ($8.3 * 10^{-3} - 9.0 * 10^{-3}$)	h^{-1}
<i>GUTS parameters</i>				
Threshold for damage	z_w	38.2 (32.9 – 40.6)	143.0 (140.2 – 151.6)	$\mu g / L$
Killing rate	b_w	$4.3 * 10^{-4}$ ($1.7 * 10^{-4} - 10.0 * 10^{-4}$)	$6.8 * 10^{-5}$ ($6.5 * 10^{-5} - 7.0 * 10^{-5}$)	$L / (\mu g * h)$
<i>DEBtox parameters</i>				
No effect concentration	NEC	12.3 (5.5 – 18.1)	NA*	$\mu g / L$
Tolerance rate	c_T	0.011 (0.007 – 0.019)	NA*	$\mu g / L$

*NA: not applicable. DEBtox was not considered for Zn, since the observed sub-lethal effects were not significant.

Table 5. Results of the validation of the DEB-IBM against the population density of the population experiment with *D. magna* exposed to mixtures of Cu and Zn. R-squared values (%) were calculated based on log-transformed densities. A distinction is made between the total, juvenile and adult population density.

Treatment	R-squared (%)		
	Total	Juvenile	Adults
Control	90.14	79.57	79.74
Low Cu	87.84	83.23	78.94
High Cu	76.45	72.79	68.35
Low Zn	86.98	83.53	81.36
High Zn	89.28	83.03	81.44
Mixture: Low Cu / Low Zn	86.93	82.09	79.40
Mixture: High Cu / Low Zn	70.05	68.25	79.40
Mixture: Low Cu / High Zn	88.30	84.98	80.06
Mixture: High Cu / High Zn	66.39	66.14	62.35