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Death Dilemma and Organism Recovery in Ecotoxicology

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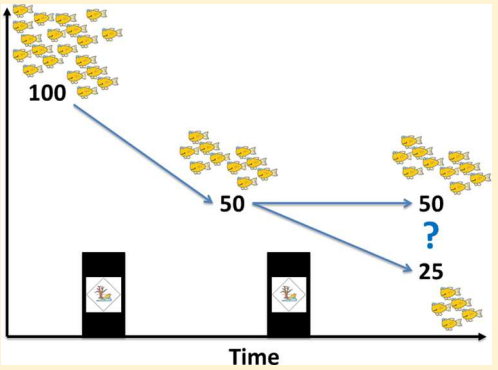
Supporting Information

ABSTRACT: Why do some individuals survive after exposure to chemicals while others die? Either, the tolerance threshold is distributed among the individuals in a population, and its exceedance leads to certain death, or all individuals share the same threshold above which death occurs stochastically. The previously published General Unified Threshold model of Survival (GUTS) established a mathematical relationship between the two assumptions. According to this model stochastic death would result in systematically faster compensation and damage repair mechanisms than individual tolerance. Thus, we face a circular conclusion dilemma because inference about the death mechanism is inherently linked to the speed of damage recovery. We provide empirical evidence that the stochastic death model consistently infers much faster toxicodynamic recovery than the individual tolerance model. Survival data can be explained by either, slower damage recovery and a wider individual tolerance distribution, or faster damage recovery paired with a narrow tolerance distribution. The toxicodynamic model parameters exhibited meaningful patterns in chemical space, which is why we suggest toxicodynamic model parameters as novel phenotypic anchors for in vitro to in vivo toxicity extrapolation. GUTS appears to be a promising refinement of traditional survival curve analysis and dose response models.



INTRODUCTION

Why Do Not All Individuals Die at the Same Dose?

The sigmoidal shape of concentration−response curves raises the question: why do not all individuals in the tested population die at the same concentration or time? Either, the tolerance threshold is distributed among the individuals in a population, and its exceedance leads to certain death, or all individuals share the same threshold above which death occurs stochastically.[1,2] Recent theoretical advances have established a mathematical relationship between the two assumptions and resulted in the General Unified Threshold model of Survival (GUTS).[3] Both assumptions lead to the typically observed sigmoidal concentration−response curves, but the time course of mortality differs markedly. More importantly, stochastic death would result in systematically faster compensation and damage repair mechanisms than individual tolerance. Thus, we face a circular conclusion dilemma because inference about the death mechanism is inherently linked to the speed of damage recovery. Two complementary assumptions describe the limit cases of what is, probably, a mix of both in reality: (i) the tested individuals share a common tolerance threshold and when that is exceeded they die at random (stochastic death), or (ii) each individual has its own tolerance threshold above which it dies immediately, and the tolerances of many individuals follow a statistical distribution in the tested population (individual tolerance)[3] (Figure 1A).

Mathematical models of both assumptions have been unified in GUTS; however, inference about the prevalent nature of death in a given set of mortality or survival data is impossible without considering the rate of toxicodynamic recovery. The classic example from Newman & McCloskey[2] illustrates the challenge: Consider the hypothetical example of 100 fish exposed to a pulse of toxicant that kills half of them. The surviving fish are transferred to clean water for a duration that is long enough to allow full recovery and then they are exposed to the exact same pulse of toxicant again. How many fish will survive the second pulse? The answer depends: Stochastic death (SD) would predict that 25 fish survive, whereas assuming an individual tolerance (IT) distribution predicts that all 50 fish survive the second pulse (Figure 1A). Crucially, this thought experiment requires full organism recovery between the pulses otherwise carry-over toxicity could cause 25 fish to die also under the assumption of IT. The experiment with the two pulses might seem like a good test for carry-over

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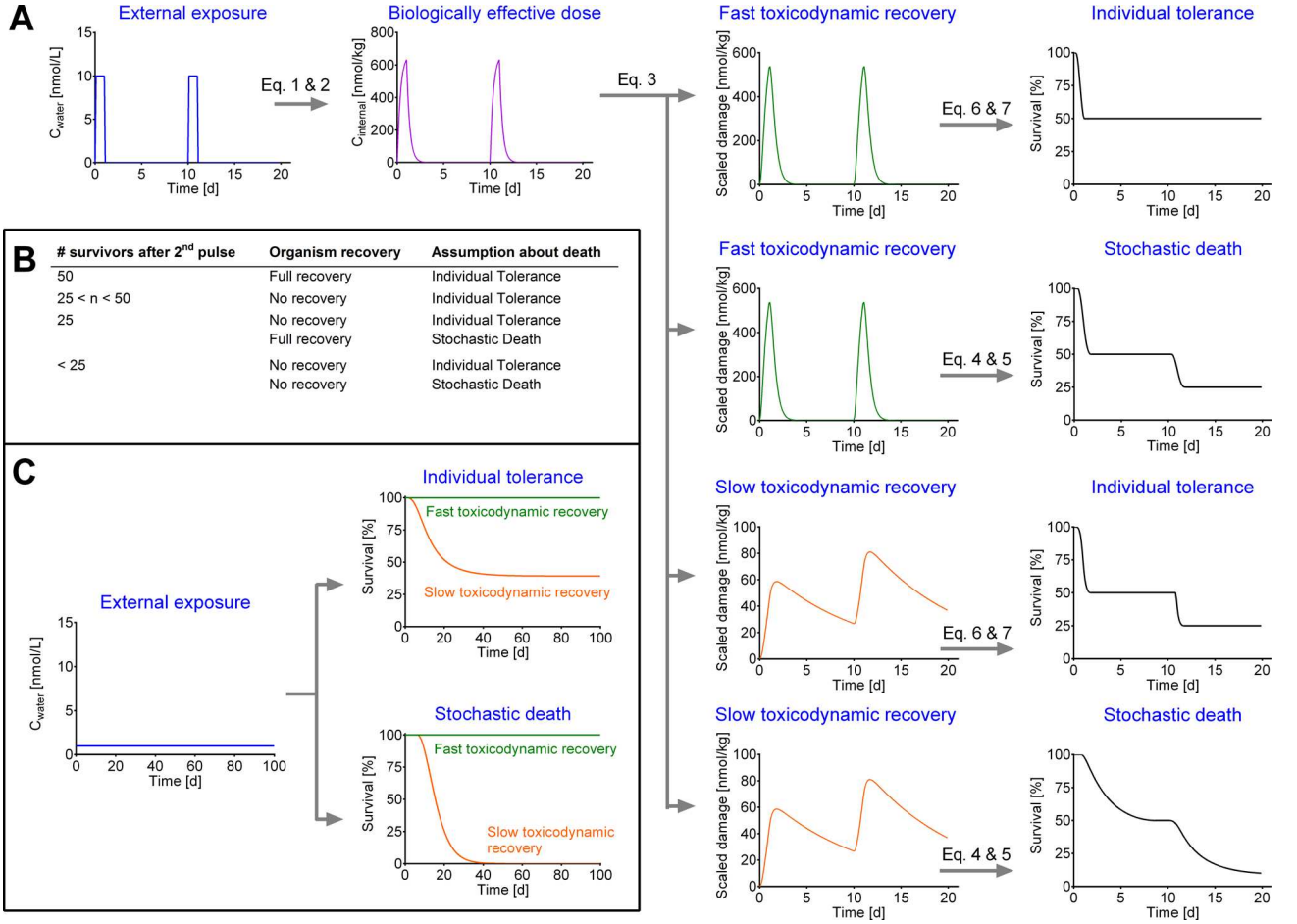

Figure 1. Toxicodynamic recovery is intertwined with assumptions about death (stochastic vs individual tolerance). (A) Toxicokinetics determine the biologically effective dose whereas toxicodynamic recovery shapes the survival curve. (B) Different combinations of assumptions about death and toxicodynamic recovery can explain the same observation. (C) Assumptions about death also affect survival after constant exposure. The parameter values used in the simulations shown here are provided in the Supporting Information (SI).

toxicity and organism recovery, but the only unambiguous outcome would be the observation of all 50 fish surviving the second pulse, which can be rationalized by IT. All other outcomes can be explained by different combinations of the nature of death and organism recovery (Figure 1B). This is the "death dilemma". A constant exposure to a toxicant or other stressor will also lead to different outcomes: any exposure that kills at least one individual will eventually kill the whole population when assuming SD, whereas the assumption of IT will result in mortality leveling off at toxicokinetic and toxicodynamic steady-state (Figure 1C, aka: incipient lethal level[4]).

How to Study Toxicodynamic Recovery? Toxicodynamic recovery is a result of cellular and physiological compensating mechanisms and repair in an organism's stress response to the chemical insult. However, when we quantify toxicodynamic recovery in a model, the above considerations imply slower organism recovery in the case of IT, and faster recovery in the case of SD (Figure 1). Thus, when analyzing survival data we face the dilemma of having to infer the stochastic or deterministic nature of death and at the same time quantifying the speed of toxicodynamic recovery. This entanglement requires explicit representation of recovery and assumptions about death in the toxicodynamic model.

The Toxicodynamic Clustering Hypothesis. In 2007 we hypothesized that "compounds with the same mode of action cluster together in the toxicodynamic parameter space",[5] but testing this hypothesis required solving the "death dilemma" first. In 2009 Jager & Kooijman demonstrated patterns in the parameters of GUTS-SD for fish, but without clear separation of toxicokinetics and toxicodynamics.[6] The GUTS framework provides the theory to solve the "death dilemma" and enables systematic experimentation to test two related hypotheses in this study: (i) assuming individual tolerance results in slower toxicodynamic recovery, and (ii) toxicodynamic parameters cluster according to the chemical mode of action. In other words: to better understand toxicodynamic parameters we mapped them across chemicals and toxic modes of action, analyzed patterns in their values and tested if the differences in recovery rates between IT and SD as predicted by theory manifested themselves in survival data.

Study Overview and Rationale. The first step is to quantitatively differentiate toxicokinetic processes (uptake, biotransformation and elimination) from toxicodynamic processes (interaction with biological target and toxic effect). In previous work we experimentally quantified the uptake, biotransformation and elimination rates of 14 synthetic organic chemicals in the aquatic invertebrate *Gammarus pulex* and built a toxicokinetic model.[7] With the additional toxicity experiments

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presented here we are able to construct a toxicokinetic-toxicodynamic model for each compound where the toxicokinetic part simulates the time course of the internal concentrations. The sums of internal concentrations of the toxicologically active chemicals over time are the biologically effective doses and serve as inputs for the new toxicodynamic model part (SI Figure S3). The toxicodynamic model links the biologically effective dose to the survival data. In a second step we fitted two toxicodynamic models for each of the 14 compounds: GUTS-IT assumes individual tolerance, and GUTS-SD assumes stochastic death.[3,8] This analysis enables us to learn about the nature of death and its relation to toxicodynamic recovery; and extract mechanistic signals from survival data.

MATERIALS AND METHODS

Chemicals, Test Organisms, and Experiments. We measured the time-course of survival of the freshwater amphipod *Gammarus pulex* when exposed to 14 toxicants separately (listed below) in repeated pulsed toxicity tests.[8,9] *G. pulex* were collected from a headwater stream [Itziker Ried, 20km southeast of Zürich, Switzerland, E 702150, N 2360850], acclimatized to laboratory conditions (preaerated artificial pond water (APW), 13 °C, 12:12 light:dark) and fed horse-chestnut leaf discs (preconditioned with the fungi *Cladiosporium herbarum*).[10] Experimental treatments consisted of seven or eight replicate beakers (600 mL) filled with 500 mL APW, ad libitum leaf discs and initially ten individual *G. pulex* per beaker. Survival was usually measured daily (see Model Fits: SI Figures S6−S19 and raw data file). We dosed a mixture of ^{14}C-labeled and unlabeled material and frequently measured the actual exposure concentration during the experiments by Liquid Scintillation Counting (LSC) in 1 mL aliquots of the test solutions.[8,9] *G. pulex* were exposed to each test chemical in separate experiments.

Additional survival data was taken from four day toxicity tests with constant exposure[11] and previous studies[9,12] (SI Table S1). Overall we analyzed data for 14 compounds: 2,4-dichloroaniline, 1,2,3-trichlorobenzene, 2,4-dichlorophenol, pentachlorophenol, 2,4,5-trichlorophenol, 4,6-dinitro-o-cresol, aldicarb, carbofuran, carbaryl, diazinon, malathion, chlorpyrifos, sea-nine and 4-nitrobenzyl-chloride. The CAS numbers can be found in SI Table S2. We used the same batches of ^{14}C-labeled compounds as in the toxicokinetic studies.[7,9] The ^{14}C-labeled chlorpyrifos, pentachlorophenol, carbaryl, malathion, aldicarb, carbofuran, imidacloprid were supplied by the Institute of Isotopes, Budapest, Hungary. 2,4-Dichloroaniline, 2,4-dichlorophenol, 1,2,3-trichlorobenzene, 4,6-dinitro-o-cresol, 2,4,5-trichlorophenol, ethyl acrylate, 4-nitrobenzyl-chloride were supplied by American Radiolabeled Chemicals, St. Louis, MO. Sea-nine (4,5-dichloro-2-octyl-3-isothiazolone) was supplied by Amersham (GE Healthcare), UK. Unlabeled material of these compounds was of analytical grade and purchased from Sigma-Aldrich, Buchs, Switzerland, except for Sea-Nine (>97% purity, Rohm and Haas), which was a gift of Christ Chemie AG, Rheinach, Switzerland.

The pulsed toxicity tests generally followed the design established previously,[8,9] in which test organisms were exposed to pulses of toxicant of 1 day duration, then transferred to clean medium for intervals of varying duration (treatment), followed by a second pulse of the same concentration and duration. Survival was measured daily throughout the experiment, including a follow-up period after the last pulse. This design allows for depuration of toxicant and different degrees of recovery between pulses. The overall durations of the pulsed toxicity tests were between 6 and 28 days (SI Table S1). Raw data is provided in the Supporting Information, including measured time series of exposure concentrations and survival in the pulsed toxicity tests (SI Figures S6−S19).

Modeling Approach. The time-course of the sums of internal concentrations of toxicologically active chemicals in the tested organisms (SI Table S3) was simulated using previously established toxicokinetic models[7,9] and served as input for GUTS-IT and GUTS-SD.[3,8] Scaled damage was the dose metric in both toxicodynamic models (for an explanation of scaled damage see the original GUTS publication[3]). Toxicodynamic parameters were estimated by maximizing the likelihood (see eq 8 below) with a combination of Monte Carlo sampling of the parameter space and optimization with the downhill simplex algorithm.

The models were implemented in ModelMaker (version 4, Cherwell Scientific Ltd., Oxford, UK). First the background hazard rate was fitted to the control survival data from each experiment, assuming constant background hazard through time (this can be replaced with a different model for background hazards that change through time, for example, Weibull[13]). Then the toxicodynamic parameters were fitted to the survival data from all the treatments. Wherever possible we used data from the pulsed toxicity tests in conjunction with data from standard toxicity tests[11] because the combination of pulsed and constant exposures maximizes the information gain for this type of model.[14]

History and Assumptions of GUTS. GUTS is the current synthesis of a range of assumptions and toxicokinetic-toxicodynamic models traditionally used to describe survival data in (eco)toxicology.[3,15] Historically these models[16−22] evolved out of the need to understand and describe the time course of toxicity data and they have been reviewed previously.[3,15,23,24] Common to these models is that they relate a dose metric to mortality, where the dose metric is allowed to vary over time. Common dose metrics are external concentrations (i.e., concentration in experimental media), scaled internal concentrations, internal whole body concentrations, scaled damage and damage. GUTS does not prescribe the use of any of these dose metrics, it can be used with any dose-metric as long as there is consistency within one study. Here, we chose to simulate internal whole body concentrations and use the scaled damage as dose metric because we want to quantify toxicodynamic parameters without confounding them with toxicokinetics.

Traditionally effect models relate mortality to the maximum concentration, critical body residue or the area under the curve. Inherent in these choices are assumptions about the speed of toxicodynamic recovery, for example using the maximum concentration assumes very fast toxicodynamic recovery and using the area under the curve assumes very slow toxicodynamic recovery. It has been shown that these assumptions, when expressed mathematically, translate into a subset of the traditionally used toxicodynamic models.[3,15] As these assumptions hold only for certain groups of chemicals and are difficult to ascertain as discussed in the introduction, the suitability of the corresponding models is often not given or unclear. GUTS resolves this problem because it does not require a priori assumptions about the speed of toxicodynamic recovery.[3] This is why it has been suggested that GUTS is applicable to a wide range of chemicals with diverse modes of

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action and speeds of toxicodynamic recovery.[3] However, GUTS requires the assumption that toxicodynamic recovery can be approximated with first order kinetics, and that the link between the dose metric and mortality can be modeled with a proportionality constant (killing rate constant, see below) between the dose metric and the hazard rate and a distribution for the threshold.[3] Here we assumed a log−logistic distribution of the threshold (see eq 6 below), but other distributions are possible too. Further we assume time-invariable model parameters, which implies that organisms do not change substantially during the experiment (e.g., no physiological adaptation) and we ignore variability in toxicokinetics between organisms.

Toxicokinetic Model. The time-course of internal concentrations in *G. pulex* was simulated using a previously established toxicokinetic model:[7,9]

$$\frac{dC_{internal,p}(t)}{dt} = C_w(t) \times k_{in,p} - C_{internal,p}(t) \times k_{out,p} - \sum_j (C_{internal,p}(t) \times k_{met,j}) \quad (1)$$

$$\frac{dC_{internal,j}(t)}{dt} = C_{internal,p}(t) \times k_{met,j} - C_{internal,j}(t) \times k_{out,j} \quad (2)$$

Where $C_{internal,p}(t)$ is the concentration of the parent compound in the organism [nmol/$kg_{w.w.}$], $C_w(t)$ is the concentration of the parent compound in the water [nmol/L], $C_{internal,j}(t)$ is the concentration of biotransformation product j in the organism [nmol/$kg_{w.w.}$], $k_{in,p}$ is the uptake clearance coefficient [L/($kg_{w.w.}$ × d)], $k_{out,p}$ is the elimination rate constant of the parent compound [1/d], $k_{met,j}$ is the first-order biotransformation rate constant for formation of metabolite j [1/d] and $k_{out,j}$ is the elimination rate constant of the biotransformation product j.

As there was a maximum of three different biotransformation products, j took values between 1 and 3. For some parent compounds we did not detect any biotransformation[7] and for others we modeled the toxicity using the sum of the internal concentrations of parent compound and biotransformation products as driving variable for the toxicodynamic model (e.g., for baseline toxicity, see SI Table S3). In both those cases the toxicokinetic model reduces to eq 1 without the biotransformation term.

Dose Metric. The dose metric is the state variable which is compared with the threshold (SI Figure S2). We used the two limit cases of GUTS, GUTS-SD, and GUTS-IT, both with scaled damage as dose metric. The scaled damage is a proxy for the toxicodynamic state of the organism[3] and is calculated based on the sums of the internal concentrations of the toxicologically active chemicals (SI Table S3):

$$\frac{dD_{scaled}(t)}{dt} = k_r \times (C_{sum\ tox,organism}(t) - D_{scaled}(t)) \quad (3)$$

Where $D_{scaled}(t)$ is the time course of the scaled damage [nmol/$kg_{w.w.}$], t is time [d], $C_{sum\ tox,\ organism}(t)$ is the time course of the sums of the internal concentrations of the toxicologically active chemicals in the organism [nmol/$kg_{w.w.}$] and k_r is the damage recovery rate constant [1/d]. The damage recovery rate constant captures the time course of toxicodynamics and we use two different parameters k_r SD and k_r IT for the two limit cases of GUTS.

Effects on Survival in GUTS-SD. Here, the hazard rate is the probability of an organism dying at a given point in time and, assuming it increases linearly with the damage above the threshold, is calculated as[3,8,22]

$$\frac{dH(t)}{dt} = k_k \times \max(D_{scaled}(t) - z, 0) + h\_controls \quad (4)$$

Where $dH(t)/dt$ is the hazard rate [1/d], k_k is the killing rate constant [$kg_{w.w.}$/(nmol × d)], $D_{scaled}(t)$ is the time course of the scaled damage [nmol/$kg_{w.w.}$], t is time [d], z is the threshold [nmol/$kg_{w.w.}$] and h_controls is the background hazard rate (control mortality rate, assumed to be constant through time) [1/d]. The survival probability, that is, the probability of an individual to survive until time t, is given by

$$S(t) = e^{-H(t)} \quad (5)$$

Where $S(t)$ is the survival probability [unitless].

Effects on Survival in GUTS-IT. Here we assume a log−logistic distribution of the threshold in the tested population (individual tolerance).[3,8] Then the cumulative log−logistic distribution of the tolerance threshold in the test population, which changes over time as individuals die, is calculated as

$$F(t) = \frac{1}{1 + \left(\frac{\max_{0<\tau<t} D_{scaled}(\tau)}{\alpha}\right)^{-\beta}} \quad (6)$$

Where $F(t)$ is the cumulative log−logistic distribution of the tolerance threshold over time [unitless], $D_{scaled}(t)$ is the time course of the scaled damage [nmol/$kg_{w.w.}$], t is time [d], τ is time [d], α is the median of the distribution [nmol/$kg_{w.w.}$] and β is the shape parameter of the distribution [unitless]. Under the assumption of individual tolerance the survival probability is then given by[3,8]

$$S(t) = (1 - F(t)) \times e^{-h\_controls \times t} \quad (7)$$

Where $S(t)$ is the survival probability [unitless] and h_controls is the background hazard rate (control mortality rate, assumed to be constant through time) [1/d].

Likelihood Function and Parameter Estimation. The parameters for GUTS-SD and GUTS-IT were found by maximizing the ln(likelihood) function:[3]

$$\ln l(\boldsymbol{\theta}|\mathbf{y}) = \sum_{i=1}^{n+1} (y_{i-1} - y_i) \ln(S_{i-1}(\boldsymbol{\theta}) - S_i(\boldsymbol{\theta})) \quad (8)$$

Where l is the likelihood, y is the time series of the number of survivors, i is sampling date, n is the number of sampling dates, θ is the vector of model parameters and $S(\theta)$ is the survival probability given θ. Note that n is the last sampling date and n +1 is infinity. This means y_n refers to the number of survivors at the end of the test, who will die between then (n) and infinity (n+1). The likelihood was calculated for each treatment and the likelihoods for all treatments were summed up.

Then the −ln(likelihood) was minimized using a combination of Monte Carlo sampling of the parameter space and optimization with the downhill simplex algorithm. For the GUTS-SD model the parameters $k_{r,\ SD}$, kk and z were calibrated and for the GUTS-IT model the parameters $k_{r,\ IT}$, α, and β were calibrated. The parameter k_r was constrained to $0.001 < k_r < 1000\ d^{-1}$ because this translates to recovery times between 4 min and 30 000 days. All other toxicodynamic parameters were constrained to positive values.

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The 95% confidence intervals are calculated from transects of the ln(likelihood) surface. Each parameter is varied separately, the resulting ln(likelihood) values are compared to the best-fit ln likelihood value and then the parameter value on each side of the best fit that corresponds to a 1.92 difference (half of 3.84, the corresponding value of the χ^2 distribution for 1 degree of freedom) to the best-fit ln(likelihood) is taken as the 95% confidence interval.

Model for Effects of Internal Mixtures with Different MOAs. There were two parent compounds for which we modeled two separate, independent modes of toxic action because the biotransformation of the parent compound with a specific MOA led to metabolites that we assumed to act via baseline toxicity. Details on the biotransformation pathways, rates, and products can be found in the biotransformation study.[7] In the cases of 4NBCl and Sea-nine the internal concentrations of the parents were modeled as reactive toxicants, whereas the sum of the internal concentrations of the metabolites was modeled as baseline toxicant. The other specifically acting compounds were either modeled based on the sum of the internal concentrations of parent and metabolites combined or based on the internal concentrations of the parent compound alone (SI Table S3).

In cases of toxicodynamic models with two different MOAs (parent compounds: 4NBCl and Sea-nine) we used toxicodynamic parameters of 123TCB (this is a well-known prototype baseline toxicant) to simulate the effects of the metabolites that are assumed to act via baseline toxicity. We kept the toxicodynamic parameters for the metabolites fixed at baseline toxicity values while fitting the toxicodynamic parameters for reactive toxicity (4NBCl and Sea-nine). As we assumed independence of the two MOAs we modeled separate scaled damages for each using eqs 3 to 7 and then multiplied the resulting survival probabilities:

$$S_{\text{combined}}(t) = S_{\text{baselinetoxicity}}(t) \times S_{\text{specificMOA}}(t) \times e^{-h\_\text{controls}\times t} \quad (9)$$

Multiplying $S(t)$ is equivalent to adding hazard rates in GUTS-SD. Internal mixtures of compounds acting via the same MOA can be modeled by summing the scaled damages.[25]

Disappearance Times and Damage Recovery Times. The disappearance time is an approximation of how fast the organisms eliminate the toxicants. The damage recovery time is an approximation of how fast the scaled damage declines after toxic insult and can be viewed as an indication of how fast toxicodynamic repair and recovery processes are. Times to 95% disappearance of the toxicologically active chemicals (disappearance time) were calculated with the toxicokinetic model and the time to 95% toxicodynamic recovery (damage recovery time) were calculated assuming a first-order model represents toxicodynamic recovery as

$$t_{\text{damage recovery}} = -\ln 0.05/k_r \quad (10)$$

RESULTS AND DISCUSSION

Chemicals and Mode of Action Classification. In aquatic toxicology toxicity is typically represented by the concentration of chemicals in the aqueous phase that kills 50% of the test organisms after a fixed time (LC_{50}). Internal exposure concentrations represent the biologically effective dose and account for toxicokinetics.[26] Toxicity is then reported as internal lethal concentration that kills 50% of the test organism, ILC_{50} (SI Figure S1). The ILC_{50} much better reflects the intrinsic toxicity, i.e., toxicodynamics, and thus allows classification of chemicals according to groups of modes of action, which is important in itself, but also for understanding mixture toxicity,[27] species sensitivity differences[28] and for environmental risk assessment.[29]

The baseline toxicity is the minimum toxicity any chemical can exhibit. It corresponds to a constant internal concentration independent of chemical and biological species with ILC_{50} in the order of a few mmol of chemical per kg of organism, e.g, 2 to 8 $\text{mmol/kg}_{\text{fish}}$[29,30] or 0.9 to 3.1 $\text{mmol/kg}_{\text{water flea}}$.[31] The mode of action underlying baseline toxicity is narcosis, i.e., the mechanism is the nonspecific partitioning of chemicals in biological membranes and membrane-protein interfaces.[31,32]

Because the bioaccumulation is dependent on the hydrophobicity of a chemical, the LC_{50} of baseline toxicants is quantitatively related to hydrophobicity descriptors such as the octanol–water partition coefficient K_{ow},[32] the lipid membrane-water partition coefficient K_{lipw}[33] or, for ionizable chemicals, the speciation-corrected lipid membrane-water distribution ratio $D_{lipw}(pH)$.[29,31] In SI Figure S1A, the $D_{lipw}(pH7)$ is used as hydrophobicity descriptor because the test set of chemicals includes some weak organic acids. The LC_{50} values of all baseline toxicants lie on the green line in SI Figure S1A, which is the baseline Quantitative Structure Activity Relationship for the model organism *G. pulex*.

Chemicals that act according to a specific (i.e., receptor-mediated) mechanism or exhibit reactive toxicity have a lower LC_{50} than the corresponding baseline LC_{50}. A measure of the degree of enhancement of effect is the toxic ratio TR, which is the quotient of the baseline LC_{50} to the experimental LC_{50}.[34] The TR values typically range around 1 ($0.1 < TR \leq 10$) for baseline toxicity and $TR > 10$ for specifically acting and reactive chemicals.[34,35]

The TRs derived from the experimentally determined LC_{50} values after 48h of exposure ranged from 0.2 to 30 000 (SI Table S2). The known baseline toxicants had TR matching the criterion for baseline toxicity but the expected uncouplers 245TCP and PCP[31] and the expected reactive toxicant 4NBCl classified in *G. pulex* merely as baseline toxicants. All other expected specifically acting compounds were confirmed by the TR analysis (SI Table S2). Most chemicals were extensively metabolized and for some MOAs not the parent is the toxicologically active species but rather an internal metabolite. This is the case for 4NBCl as well as for the organothiophosphates where only the oxidized species inhibits the acetylcholinesterase. The subsequent toxicokinetic-toxicodynamic modeling resulted in similar toxicodynamic parameters for PCP, 245TCP and DNOC when using GUTS-IT (Figures 2 and 3C,D), but 245-TCP did not cluster with PCP and DNOC when using GUTS-SD (Figures 2B and 3A,B). The clustering for GUTS-IT (Figure 3C,D) would support the interpretation that all three are uncouplers, but the TR analysis indicates otherwise and the clustering for GUTS-SD would suggest that PCP acts via baseline toxicity (Figure 3A,B). In case of these three "controversial uncouplers" the rate limiting process for organism recovery is damage recovery for GUTS-IT (Figure 4B) and toxicant elimination for GUTS-SD (Figure 4C). In case of 4NBCl the toxicokinetic-toxicodynamic modeling revealed that most of the observed toxicity can already be simulated as result of the baseline toxicity of the metabolites, which explains why the LC50 based TR analysis (SI Table S2)

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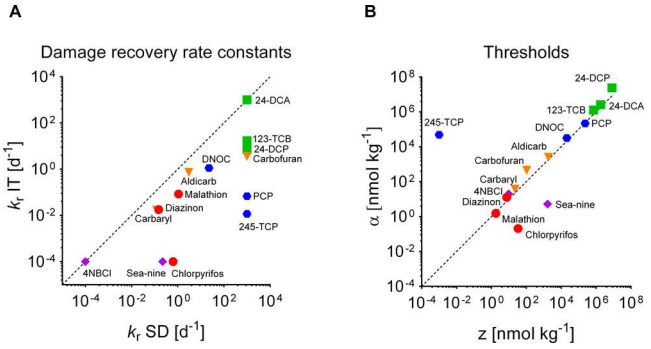

Figure 2. Patterns in toxicodynamic parameters: (A) Faster damage recovery when assuming stochastic death, slower damage recovery when assuming individual tolerance. (B) Decreasing thresholds (α for GUTS-IT and z for GUTS-SD) from baseline toxicants to reversible and irreversible MOAs. Green squares: baseline toxicity, blue hexagons: uncoupling of oxidative phosphorylation, orange triangles: AChE inhibition (carbamate), red circles: AChE inhibition (organophosphates), purple diamonds: reactive toxicity.

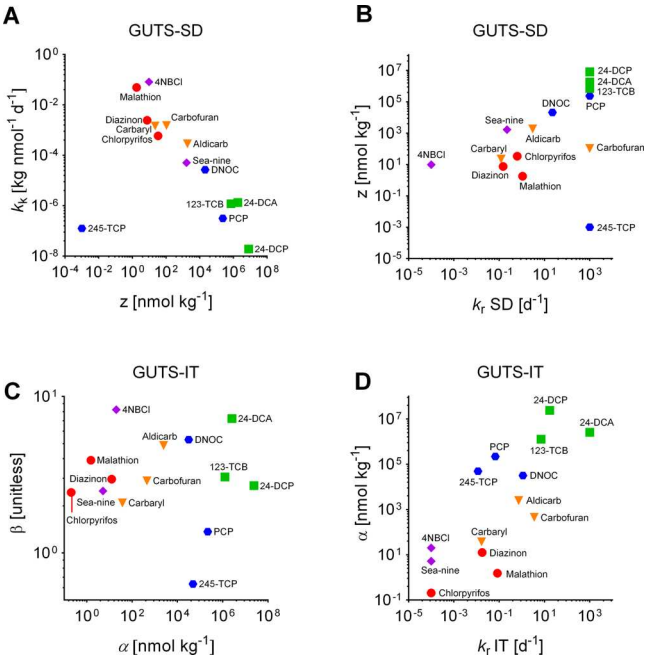

Figure 3. Patterns in toxicodynamic parameters. Clustering of toxicodynamic parameters according to chemical MOA is more pronounced for GUTS-IT (panels C, D) than for GUTS-SD (panels A, B). (A) Parameters z and k_r in GUTS-SD, (B) Parameters k_r and z in GUTS-SD, (C) Parameters α and β in GUTS-IT, (D) Parameters k_r and α in GUTS-IT. Green squares: baseline toxicity, blue hexagons: uncoupling of oxidative phosphorylation, orange triangles: AChE inhibition (carbamate), red circles: AChE inhibition (organophosphates), purple diamonds: reactive toxicity.

indicates baseline toxicity and the ILC50 based analysis (SI Figure S1B) indicates specific toxicity.

The ILC_{50} were calculated from the experimental LC_{50} using a comprehensive toxicokinetic model[7] and refer only to the concentrations of the toxicologically active species (parent or metabolite or combination thereof) (SI Table S2 and S3). The intrinsic potency (i.e., TR) shifted when the biologically effective internal dose was taken into account rather than the parent concentration in the aqueous phase (SI Table S2). The TR derived from ILC_{50} can be considered more toxicologically relevant as it is already corrected for toxicokinetic differences and is a pure measure of toxicodynamic excess toxicity.[29,35]

Learning about the Toxic Mechanism from Survival Data. A cornerstone of toxicology is the idea that the mode of action (MOA) is related to the chemical structure[29] and that MOAs are often conserved across biota because they are triggered by common molecular initiating events.[36] We hypothesized that toxicodynamic parameters reflect the MOA and cluster according to chemical class.[5,6] Thus, we look for patterns in toxicodynamic parameter space. Both toxicodynamic models contain a generic state variable "damage". An increase in the internal concentration of the toxicologically active chemicals is translated into an increase of damage, and when a damage threshold (parameter α in GUTS-IT, z in GUTS-SD) is exceeded then the organism dies (GUTS-IT) or the hazard rate increases (GUTS-SD) (SI Figure S2). The recovery of "damage" represents biochemical and physiological recovery, approximated as a first-order system, and is quantified by the recovery rate constant k_r. The killing rate k_k is the slope between the damage and the hazard rate in GUTS-SD, whereas β is the slope of the log−logistic distribution of α in GUTS-IT. The interplay of the three parameters in each model determines the shape of the survival curve. The toxicodynamic parameters were found by fitting both GUTS models to survival data from standard and pulsed exposure toxicity tests for our 14 study compounds. The parameter values at the smallest -ln likelihood are the best-fit parameter values given in SI Tables S4 and S5.

Since both models can describe the experimental data adequately (SI Figures S4, S6−S19), we compared the recovery rates. For all chemicals IT was associated with smaller recovery rates than SD (Figure 2A), which confirms the expectation that recovery is slower in case of IT as compared to SD (first hypothesis). The same pattern was observed in fathead minnow and carp, although with only one toxicant.[37] This is evidence that it matters whether one uses a model based on the assumption of SD or IT. It means that any given survival data can be explained by either slower toxicodynamic recovery and a wider individual tolerance distribution, or faster recovery paired with a narrow tolerance distribution in the test population. This finding not only has implications for toxicology, pharmacology and epidemiology but because selection has implications for IT but not for SD it might also contribute to our understanding of evolutionary processes.[38]

The effect thresholds α and z were very similar in both models (Figure 2B) and the toxicodynamic parameters tend to cluster according to the MOA (Figures 2 and 3). For both SD and IT, the thresholds decreased from baseline toxicity over reversible (uncoupling, carbamate AChE inhibition) to irreversible MOAs (reactive toxicity, organophosphate AChE inhibition). Within each model, the MOAs also tend to cluster when the recovery rates were plotted against the thresholds (Figure 3B,D), although IT more strongly differentiated between the different MOAs. Baseline toxicity is characterized by the combination of large thresholds with fast recovery in both GUTS-SD and −IT (Figure 3B, D) as well as small killing rates in GUTS-SD (Figure 3A).

When using GUTS-SD with scaled internal concentration as dose metric it is the same as the classic DEBtox acute model.[3,22] When using GUTS-SD with modeled internal concentrations as input and scaled damage as dose metric, killing rates are expected to be smaller and thresholds larger as compared to the classic DEBtox acute model for bioaccumulative chemicals (i.e., where internal concentrations are larger than external

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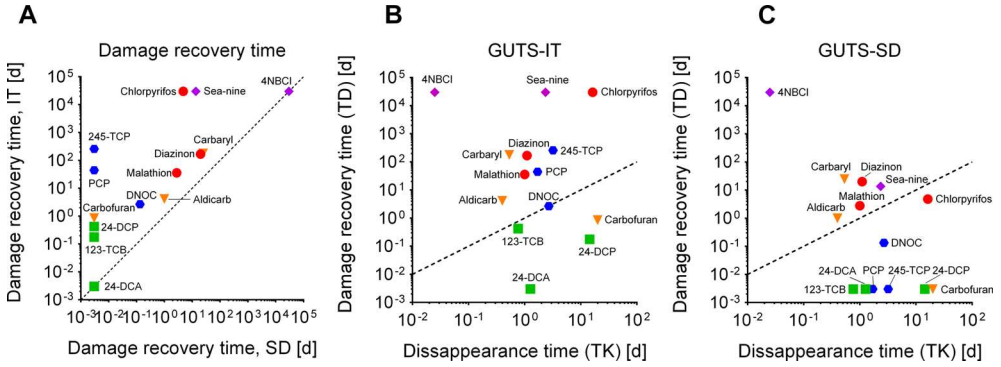

Figure 4. (A) Damage recovery time for IT and SD model. Importance of toxicokinetics and toxicodynamics for organism recovery: individual tolerance (B) and stochastic death (C). Dotted: 1:1 line. Green squares: baseline toxicity, blue hexagons: uncoupling of oxidative phosphorylation, orange triangles: AChE inhibition (carbamate), red circles: AChE inhibition (organophosphates), purple diamonds: reactive toxicity.

concentrations). Mindful of this caveat we compared our results with GUTS-SD and *G. pulex* to previous DEBtox acute modeling with baseline toxicants and reactive chemicals in fathead minnows (*Pimephales promelas*)[6] and baseline toxicants in *D.magna*.[39] Killing rates for *G. pulex* in this study cover a narrower range than with *P. promelas* and sit in the lower range of baseline toxicity killing rates for *P. promelas*, but in the upper range of reactive toxicity killing rates for *P. promelas* (compare Figure 3A in this study with Figure 4 in Jager & Kooijman[6]). For baseline toxicity this shift is as expected (due to the different dose metrics) and is also consistent with the higher killing rates for baseline toxicants in *D. magna*.[39] The *G. pulex* threshold values (z in our study, NEC in Jager & Kooijman[6]) for baseline toxicity are higher than for *P. promelas* and *D. magna*, whereas the *G. pulex* z values do extend to lower values for specifically acting compounds. Interestingly, in our study most compounds appear to fall on a straight line when plotting z against k_k, but we did not observe the parallel lines for baselines toxicants and reactive chemicals (shifted to lower left) observed for *P. promelas*.[6] To learn more about species sensitivity differences from interstudy comparisons one would have to account more rigorously for the differences in dose metrics and the covariation of model parameters (e.g., correlated killing rates and thresholds). Due to this covariation it is unclear how meaningful a comparison of each parameter by itself across studies with different dose metrics is, but threshold values would be expected to have larger values for bioaccumulative chemicals when scaled damage is used as it is done here as opposed to scaled internal concentration.[6,39]

From baseline toxicity to reversible to irreversible MOAs the thresholds and recovery rates decreased in GUTS-SD and GUTS-IT (Figure 3), whereas killing rates increased for GUTS-SD (Figure 3A) but no clear pattern for β emerged for GUTS-IT (Figure 3C). A comparison of thresholds (z and α) with critical organism concentrations for a wide range of aquatic species and toxicants[40] revealed that values from this study are similar but confined to narrower ranges. This could mean that the three toxicodynamic parameters of GUTS are better able to discriminate between MOAs than a single critical organism concentration, but this notion requires further study with a wider range of species and toxicants.

What Is Rate Limiting: Toxicokinetics or Toxicodynamics? The interplay of toxicokinetics and toxicodynamics determines the time-course of toxicity. The question of toxicokinetics versus toxicodynamics is fundamental because toxicokinetics are driven by physicochemical properties whereas toxicodynamics are related to the chemical reactivity and three-dimensional structure of the active chemical species. Complete organism recovery can be defined as when damage falls sufficiently below the threshold that a subsequent exposure would not cause excess damage accumulation and carry-over toxicity.[9] Thus, organism recovery depends not only on toxicokinetics but also on the threshold and damage recovery rates and it is dominated by whichever is slower, toxicokinetics or toxicodynamics.

Shorter damage recovery times were calculated when assuming SD, longer times for IT (Figure 2A). In general for IT, toxicodynamics appear more rate limiting, while for SD toxicokinetics and toxicodynamics are equally rate limiting (Figure 4B,C). The classes, which generally discriminate mode of action of the toxins, show the same clusters but they are shifted in TK-TD space. For both patterns of mortality organism recovery from baseline toxicants was driven by toxicokinetic rates, that is, biotransformation and elimination. Toxicodynamic recovery dominated overall organism recovery for MOAs that are known to be slowly reversible (e.g., carbamates) or even irreversible (e.g., OPs, reactive chemicals) on the biochemical level. Exceptions were Carbofuran and Chlorpyrifos (GUTS-SD), for which organism recovery was also dominated by toxicokinetics instead of toxicodynamics due to unexpectedly slow elimination of a toxicologically active metabolite. For specific reversible (e.g., uncoupling) and reactive mechanisms IT and SD differed in outcomes.

Toxicity, Organism Recovery and Incipient LC50. GUTS does not require a priori assumptions about whether a maximum damage at a given time, an average or an area under the curve is critical, rather any of these cases are possible depending on the parameter values.[15] Here we used GUTS to study organism recovery, which is quantified by a first-order rate constant (k_r). This simple, generic model of toxicodynamics eqs 3−7 allowed a top-down comparison of damage recovery across different chemical classes. These features make the model applicable across chemical classes, but less accurate in each single, specific case. Previous studies have established the importance of recovery time, because incomplete organism recovery leads to delayed (latent) and carry-over toxicity.[5,9,12,25,41−45] Delayed effects occur after exposure ceases, whereas carry-over toxicity manifests itself in the increased toxicity of subsequent exposures.[5,9,25] Here we simply defined toxicodynamic recovery as the time needed to repair 95% of damage in our first-order model eq 10, however toxicokinetic-toxicodynamic modeling also enables calculation of the time

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that an organism needs to recover such that no delayed effects (e.g., damage falling below threshold) or carry-over toxicity occur anymore (e.g., damage falling below 5% of the threshold[5,9]). The former is a straightforward definition, but the latter is operationally defined and requires further investigation. Our results imply that assuming SD or IT will also result in different organism recovery times. A special case of time to steady state considerations is the "incipient" LC_{50}. Much effort in (eco)toxicology has been spent searching for "incipient" or "asymptotic" LC_{50} values when evaluating the time dependence of toxicity.[4,20,46,47] This search could be misdirected because an incipient LC_{50} is only possible in an experimental system that conforms entirely to the individual tolerance assumption. If stochastic death plays a role then incipient LC_{50} values cannot exist (Figure 1C, see also[6]), although we speculate that apparent incipient LC_{50} values may be observed due to adaptation or small sample size.

Temporal Aspects of Toxicity and Model Parsimony. Inclusion of the temporal dimension quickly reveals that GUTS requires far fewer fit parameters to describe toxicity than traditional concentration response modeling. This becomes obvious when predicting toxicity at different points in time. GUTS-IT and GUTS-SD can also be used with exposure quantified by external concentrations[8] and always only require three parameters each—independently of the number of time points modeled. The additional parameters of the toxicokinetic model do not count in this comparison, because that was a choice we made here so that we can learn about toxicodynamics. More to the point, using concentrations in the medium as driving variable for GUTS, for example fluctuating pesticide concentrations,[37] resulted in similar predictive power as when using internal concentrations in one previous study.[8] The most simple concentration−response model for four time points already requires eight parameters (four times slope and LC_{50}) or five parameters if a constant slope is assumed. For more time points and predictions over time GUTS quickly outperforms concentration response models. There are also other methods that account for the time course of mortality data with relatively few fit parameters, for example time-to-event modeling[48] or a modified Mancini-type model,[49] which differ in their assumptions and applicability scenarios. Because toxicokinetic-toxicodynamic models like GUTS are based on differential equations they are a natural choice for scenarios with fluctuating or time-variable exposure.[23,47,50−52] This sets them apart from conventional survival analysis,[53] time-to-event analyses,[48] modifications of Haber's law[54−58] or other methods that require constant exposure concentrations.[49,59,60] Our observation that GUTS-IT results in systematically slower damage recovery (Figure 4A) does not mean that it will necessarily result in worst case mortality predictions. Due to parameter covariance the calibration of GUTS-IT can result also in parameter combinations that predict less mortality than GUTS-SD and that outcome also depends on the exposure scenario.[37] As the theory underlying the effect model influences predictions for intermittent exposures[43] and it is difficult to know a priori which limit case (SD or IT) results in the worst case for which exposure scenario,[37] it seems prudent to always use both, GUTS-SD and GUTS-IT, or GUTS proper when doing environment risk assessment.[37] Here we modeled toxicodynamics of different MOAs with one model structure using the same toxicodynamic equations for all MOAs. This constitutes a parameter-sparse model and contributes to the development of overarching principles in ecotoxicology.[61]

Applications to Current Challenges in Ecotoxicology. Generally applications of toxicokinetic-toxicodynamic models range widely,[24,51,52] including assessment of time-variable exposures,[37,47,62,63] mixture toxicity[25,27,64,65] and potentially quantitative adverse outcome pathways.[44] The General Unified Threshold model of Survival can be viewed as a refinement of traditional survival curve analysis and dose response models. GUTS has also been used to study life-stage specific sensitivity,[66] species sensitivity differences,[67] the relation between biomarkers and survival[68] as well as the combined effects of toxicity and starvation[69,70] (i.e., multiple stressors). Mixtures pose another great challenge for chemical pollution and to date cannot be sufficiently addressed.[27,71,72] Here we include mixture effects by explicitly modeling the biotransformation products and their contribution to toxicity.

Our results support the hypothesis that toxicodynamic parameters cluster according to mode of action (Figures 2, 3, and 4), although the pattern for GUTS-IT is more pronounced than for GUTS-SD and there is substantial variability within modes of action. This variability could originate from interexperimental variability, from the use of field sourced test organisms, the ignorance of toxicokinetic variability among organisms or a host of other reasons. Also the model calibration is very sensitive to variability in the data and the choice of toxicologically active chemicals. Further studies are clearly needed to understand how robust the toxicodynamic patterns are and whether they also exist for other species, chemicals and end points (e.g., sublethal).

Assuming that the toxicodynamic clustering hypothesis holds true more widely it would suggest several applications. First, analyzing survival data in combination with toxicokinetic information (modeled or measured) for other chemicals using GUTS could indicate the mode of action. Second, it might be possible to predict the toxicity of untested chemicals of a known mode of action (e.g., from chemical structure) by reading GUTS parameters from Figure 3 and combine them with predicted toxicokinetics[73,74] to calculate a toxicity estimate. Third, we suggest toxicodynamic parameters as novel phenotypic anchors for in vitro to in vivo toxicity extrapolation. Toxicity extrapolation from in vitro to in vivo systems should aim at predicting TK-TD model parameters on the organism level as they have a biological interpretation and appear to reflect the biochemical mechanisms of toxicity. For the same reasons TK-TD parameters may be potentially powerful end points for novel Quantitative Structure Activity Relationships[6] and their study contributes to building theory in ecotoxicology.[29,52,75] Finally, we note that our approach to survival analysis can also be applied to stressors other than chemicals and entities other than organisms.

■ ASSOCIATED CONTENT

Supporting Information

The data reported here is available in the Supporting Information. Further experimental details, model parameter values, explanatory figures, parameter values used to create Figure 1 and model fits are also provided. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03079.

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Notes

The authors declare no competing financial interest.

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