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Toxic Mixtures in Time—The Sequence Makes the Poison

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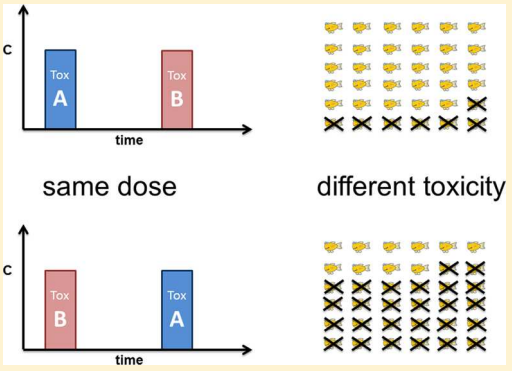
Supporting Information

ABSTRACT: "The dose makes the poison". This principle assumes that once a chemical is cleared out of the organism (toxicokinetic recovery), it no longer has any effect. However, it overlooks the other process of re-establishing homeostasis, toxicodynamic recovery, which can be fast or slow depending on the chemical. Therefore, when organisms are exposed to two toxicants in sequence, the toxicity can differ if their order is reversed. We test this hypothesis with the freshwater crustacean *Gammarus pulex* and four toxicants that act on different targets (diazinon, propiconazole, 4,6-dinitro-*o*-cresol, 4-nitrobenzyl chloride). We found clearly different toxicity when the exposure order of two toxicants was reversed, while maintaining the same dose. Slow toxicodynamic recovery caused carry-over toxicity in subsequent exposures, thereby resulting in a sequence effect—but only when toxicodynamic recovery was slow relative to the interval between exposures. This suggests that carry-over toxicity is a useful proxy for organism fitness and that risk assessment methods should be revised as they currently could underestimate risk. We provide the first evidence that carry-over toxicity occurs among chemicals acting on different targets and when exposure is several days apart. It is therefore not only the dose that makes the poison but also the exposure sequence.



INTRODUCTION

The paradigm "the dose makes the poison", that is, that chemicals can be toxic or nontoxic depending on their dose, stems back to Paracelsus. Given that exposure to toxicants in the environment is often episodic and repeated[1,2] and organisms are exposed to several toxicants at the same time or after each other, it is important to also take into consideration the temporal aspects of toxicity.[3−5] The time required by an organism to recover from damage due to previous exposure events is defined as the organism recovery time.[6,7] Organism recovery results from toxicokinetic recovery, that is, biotransformation, distribution, and excretion of the toxicant, and toxicodynamic recovery, that is, re-establishment of homeostasis. In previous studies,[7−9] we developed toxicokinetic-toxicodynamic models using toxicants from different chemical classes and found that certain toxicants exhibit slow toxicodynamic recovery in *Gammarus pulex*. The modeling suggested that even after the substance was eliminated from the organism, more time was necessary to re-establish homeostasis. The conventional wisdom, as reflected by most current chemical risk assessment practices,[10] is that when a chemical is eliminated from an organism (toxicokinetic recovery), it no longer exerts an effect. This overlooks a subtler process of toxicodynamic recovery, which is determined by the mode of toxic action. Both aspects of recovery are related to the characteristics of the substance,[3,9] where toxicokinetic parameters are related to hydrophobicity[11] and toxicodynamic parameters to mechanism of toxicity[9] and hence molecular structure.[12]

What if subsequent exposure events occur in intervals that are sufficient for toxicokinetic recovery but not for toxicodynamic recovery? We already know that toxicodynamic buildup of damage can lead to carry-over toxicity from subsequent pulses of the same toxicant[7] and from subsequent pulses of different toxicants that act on the same target.[6] Therefore, toxicokinetic-toxicodynamic models were developed recently, that extend the classical dose paradigm by complementing internal concentrations with scaled damage as the dose metric.[9,13] These early insights into toxicokinetics and toxicodynamics led to the sequence effect hypothesis: when organisms are exposed to two toxicants in sequence, the toxic effects may differ if that sequence is reversed.[6]

To date this hypothesis has only been tested once, with carbaryl and chlorpyrifos in *G.pulex*, two toxicants that both inhibit the same enzyme. The empirical evidence supported it

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Table 1. Key Characteristics of the Test Compounds[a]

compound (references for models)	CAS no.	abbreviation	mode of toxic action in *G. pulex*	toxic molecule(s)	time required for 95% elimination of toxic molecule (toxicokinetic recovery)	time required for 95% recovery of toxicodynamic damage (toxicodynamic recovery)
diazinon[7]	333−41−5	DIAZ	acetylcholinesterase inhibition[15]	diazoxon	1.1 days	20−168 days
propiconazole[8,16]	60207−90−1	PCZ	baseline toxicity[16]	propiconazole	0.4 days	1.3−3.0 days
4,6-dinitro-*o*-cresol[9,21]	534−52−1	DNOC	uncoupling[17]	4,6-dinitro-*o*-cresol	2.7 days	0.1−2.7 days
4-nitrobenzyl chloride[9,14]	100−14−1	NBCl	reactive toxicity[18]	NBCl as reactive, sum of metabolites as baseline toxicants[b]	0.03 days	>29 000 days

[a]We calculated the times required for 95% elimination and 95% recovery using previously published toxicokinetic and toxicodynamic models. [b]Toxicity of the NBCl was modeled as reactive toxicant and its metabolites as baseline toxicants as in a previous study[9] (see also section on mixture toxicity modeling below).

but it could be explained by a buildup of inhibited enzyme[6] and therefore did not challenge the current paradigm. More recently we gained a much deeper understanding of toxicokinetics[14] and toxicodynamics[9] of a diverse range of toxicants in *G. pulex*, in particular how toxicokinetic and toxicodynamic recovery times differ among chemicals. Based on this new knowledge we now propose a significant broadening of the sequence effect hypothesis: it also applies to toxicants that act on different targets and via different mechanisms. Would carry-over toxicity also occur after subsequent pulses of different toxicants that act on different targets with different toxic modes of action? This is an important question because sequential exposure to toxicants is the norm, but the current risk assessment paradigm pays little attention to possible interactions of different toxicants over time. We think that substances acting on different targets can cause carry-over toxicity, which we view as a proxy for reduced organism fitness. If the sequence effect occurs for a wide range of toxicants with different mechanisms of toxicity, then we are potentially severely underestimating risk from chemicals.

The sequence effect hypothesis can be tested experimentally because it predicts that the toxicity of two subsequent pulsed exposure events is more pronounced if we first expose organisms to the substance with a slower recovery time, followed by exposure to the substance with a faster recovery time. Conversely, exposure to the substance with a shorter recovery time followed by the substance with a longer recovery time will result in less carry-over toxicity and have a weaker impact on the organism.

We tested the sequence effect hypothesis in the amphipod crustacean *Gammarus pulex* using four model toxicants that differ in their toxic mode of action. *G.pulex* are particularly suitable for this type of study because of their relatively long life-span and sufficiently large size to measure toxicant body residues. Diazinon [DIAZ] is an insecticide that inhibits acetylcholinesterase,[15] propiconazole [PCZ] is a fungicide that likely acts as a baseline toxicant in *G. pulex*,[16] 4,6-dinitro-o-cresol [DNOC] is an uncoupler of oxidative phosphorylation,[17] and 4-nitrobenzyl chloride [NBCl] is a reactive toxicant that forms covalent bonds with proteins and other biomolecules.[18] Therefore, each toxicant acts according to a different mode of action, and their mixture toxicity should be explained by the model of independent action.[19,20] Previously, we determined the times required for 95% elimination of the toxicologically relevant metabolites and for 95% recovery of toxicodynamic damage in *G. pulex*[7,8,14,21] for each compound individually (Table 1). All four substances have toxicokinetic recovery times of less than 3 days, whereas the toxicodynamic recovery times are short for PCZ (<3 days) and DNOC (<3 days), but long for DIAZ (20−168 days) and NBCl (>29 000 days). Based on this knowledge, we designed four experiments, each involving two toxic experimental groups and a control group. The two toxic experimental groups employed two subsequent pulsed exposures to two different toxicants (Figure 1). The two toxic experimental groups in each experiment differed only in the sequence of the toxicants applied.

We chose a pulse interval that was long enough to allow toxicokinetic recovery (such that no residual chemical remained in the body at the onset of the second pulse (Supporting Information (SI) Figure S1−S4)), but too short to allow complete toxicodynamic recovery after DIAZ and NBCl exposure. Hence, we expected carry-over toxicity for those experimental groups in which the first applied pulse was a toxicant with slow toxicodynamic recovery (DIAZ or NBCl) but not in the groups in which the first applied pulse was a toxicant with fast toxicodynamic recovery (PCZ and DNOC). Consequently, we expected to observe a sequence effect, that is, differences in survival at the end of the experiment, in the first, second, and third experiments (Figure 1). In the fourth experiment, we expected both experimental groups to exhibit carry-over toxicity due to slow toxicodynamic recovery of both toxicants, and therefore did not expect the sequence effect.

We also carried out toxicokinetic-toxicodynamic mixture toxicity modeling. This dynamic modeling expands the applicability domain of mixture toxicity models to pulsed or time-variable exposures. The model structure was derived by assuming that the toxicants act on different targets and therefore independently. The model parameters were taken from previous studies. This enables us to compare model forecasts with new experimental data from this study to assess how good the model predictions are.

MATERIALS AND METHODS

Experiments. Experimental procedures closely followed established protocols.[6,7,9] Adult *G. pulex* were collected in a headwater stream in the Itziker Ried, Switzerland near Zurich (E 702150, N 2360850) and acclimatized to 13 °C (SI Table S2). *G. pulex* were fed horse-chestnut leaf discs conditioned with *Cladiosporum herbarum*[22] fungi. Experiments were performed in pyrex beakers filled with 500 mL preaerated artificial pond water[22] and at 13 °C under a 12:12 h light:dark cycle. Each beaker initially contained 10 *G. pulex* and the total number of test organisms in each toxic experimental group is

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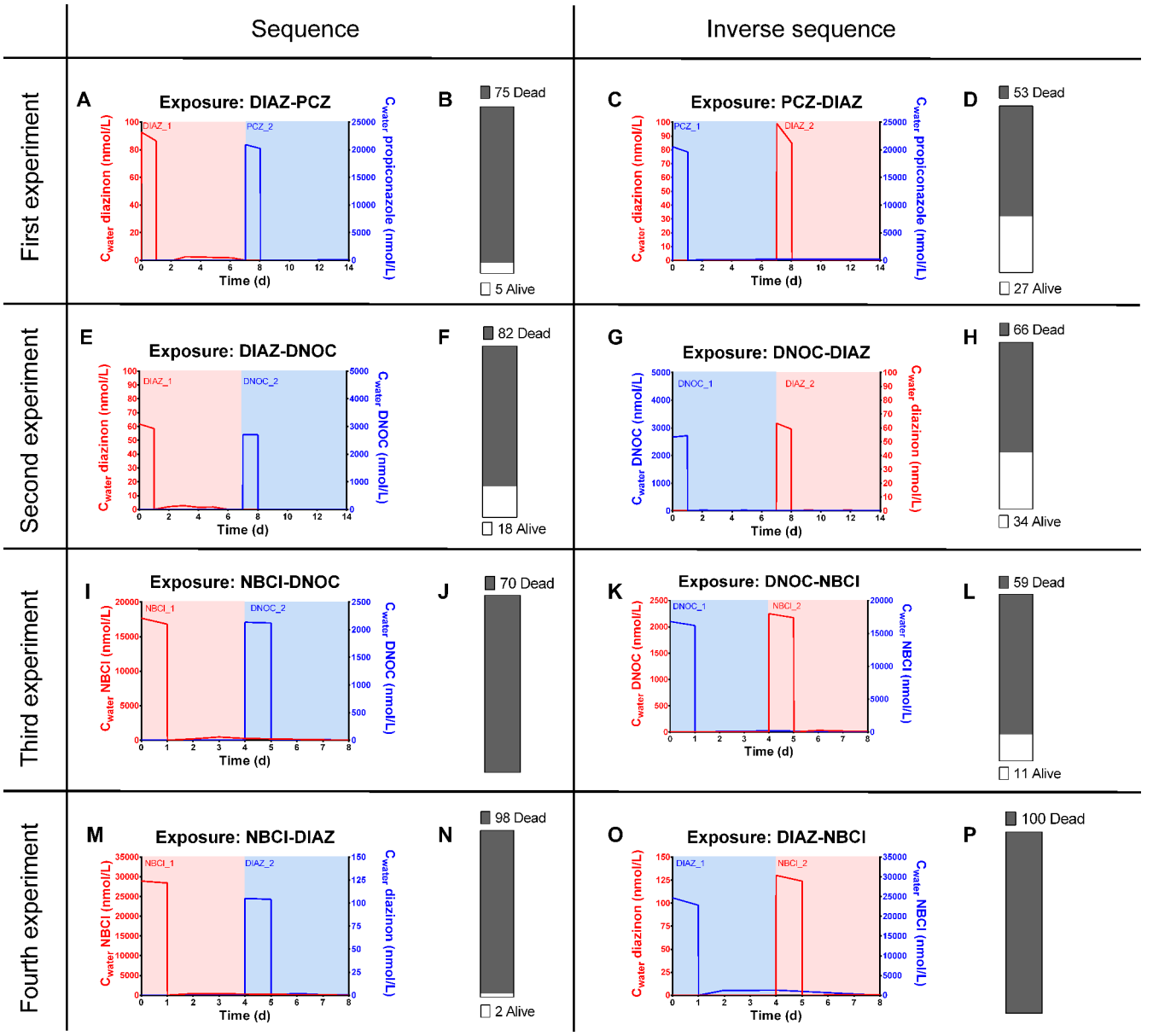

Figure 1. The sequence effect. A, C, E, G, I, K, M, O Sequence of exposure pulses and measured exposure concentrations. Each exposure consists of two 1 day pulses with recovery in clean water in between pulses and recovery in clean water after the second pulse. The dose is the same in both experimental groups, but the order of exposure to the two substances is reversed. B, D, F, H, J, L, N, P Ratios of dead to living organisms at the end of the experiment (numbers refer to *G.pulex* individuals). Differences between sequence and inverse sequence are significant in the first, second and third experiment ($p < 0.0001$, $p = 0.0151$, $p = 0.0006$, respectively), but not the fourth ($p = 0.4975$). The odds ratios and their 95% confidence intervals are 7.6 (2.8−21), 2.4 (1.2−4.5), and 27.3 (1.6−473) in the first, second, and third experiment, that is, *G. pulex* were about 8, 2, and 27 times as likely to die in the "sequence" compared to the "reverse sequence" treatment. Sequence: exposure to slow recovery substance first. Reverse sequence: exposure to fast recovery substance first. Exception: Fourth experiment—both exposures are slow recovery substances, similar likelihood of death in both treatments.

given in SI Tables S6, S 8, S10, and S12. Test organisms were a natural mixture ratio of adult males and females and the numbers used in each experiment varied depending on the availability of organisms at the Itziker Ried. Leaf discs were added for ad libitum feeding, ranging from three to five leaf discs per beaker depending on the number of alive *G. pulex*. Live organisms were counted by gently prodding with a spatula and observing their resulting movement (SI Tables S6, S8, S10, and S12). Each experiment included also a control group which was not treated with toxicants. No blinding was employed. Any neonates that were released by females during the experiments were not counted. Dead individuals were removed.

The test concentrations were chosen based on previous toxicity studies[7−9] to achieve partial mortality after the first pulse. The test chemicals were dosed as a mixture of ^{14}C-labeled and unlabeled substances to allow quantification of exposure concentrations. Radiolabeled DIAZ and PCZ were supplied by the Institute of Isotopes (Budapest, Hungary). Radiolabeled DNOC and NBCl were sourced from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled chemicals were of analytical grade and supplied by Sigma-Aldrich (Buchs, Switzerland). Dosing stocks of labeled and unlabeled test compounds were prepared in acetone as solvent. Each beaker was dosed by pipetting the required volume of stock into the

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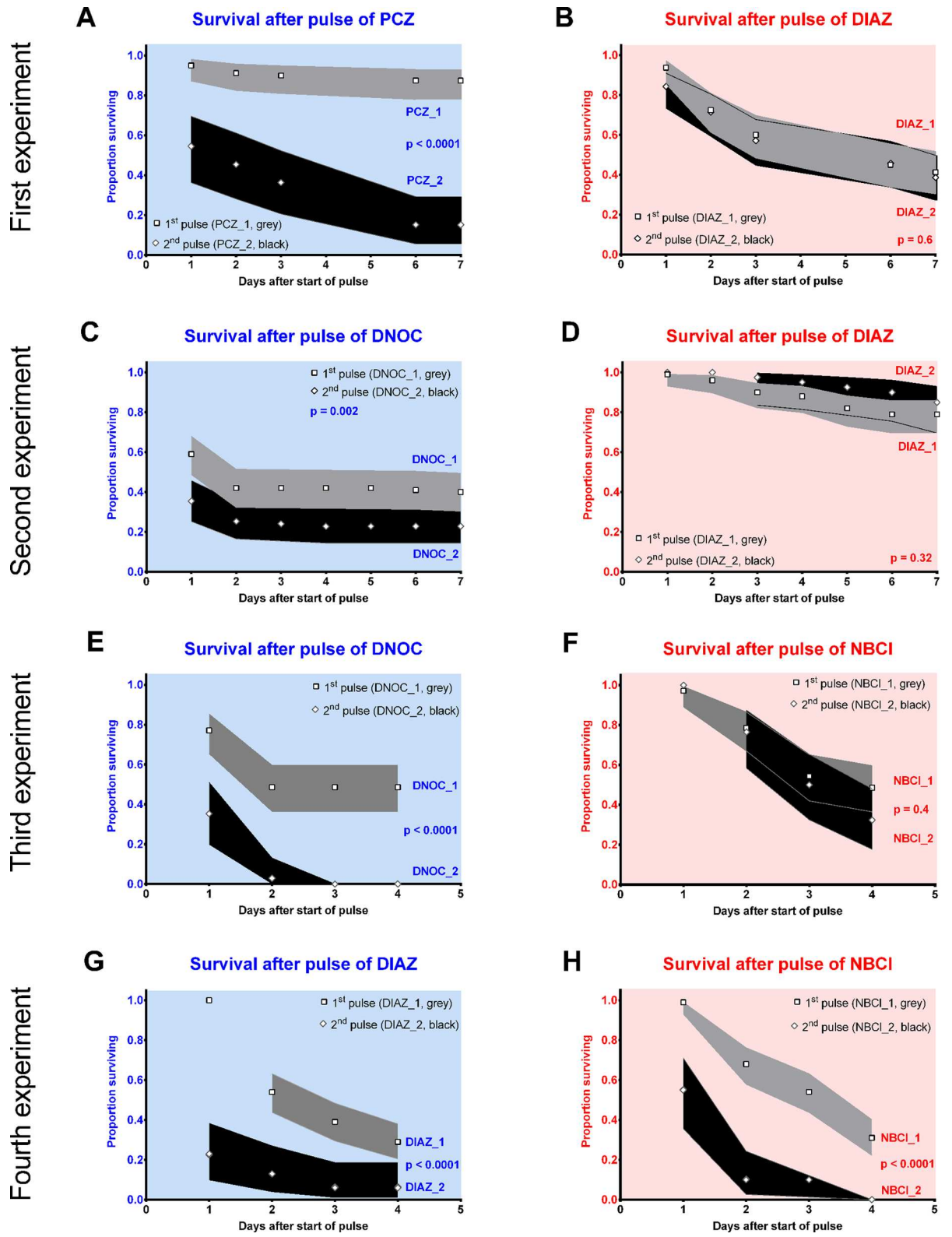

Figure 2. Carry-over toxicity. Partial survival curves following exposure to the same toxicants after 1-day pulse. Comparing the curves tests for carry-over toxicity (first pulse vs second pulse, Gehan-Breslow-Wilcoxon test, *p*-values on graphs, 95% confidence intervals plotted). See Figure 1 for exposure pulse sequences and exposure concentrations and SI for full survival curves.

medium, followed by gentle stirring with a glass rod. Concentrations of the solvent acetone in the test medium were 0.1% or lower (SI Table S4), that is, three orders of magnitude below concentrations toxic to crustaceans.[23] Actual exposure concentrations were measured by liquid scintillation counting (Tri-Carb 2200CA, Packard) using 1 mL aliquots of the medium (SI Tables S5, S7, S9, and S11), correcting for efficiency using internal standards, subtracting background activity using control medium samples and converting radioactivity to molar concentrations by using the known mixture

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ratio of labeled and unlabeled substance. Immediately before and at the end of an exposure pulse, organisms were separated from the medium by using a tea strainer, the test medium was discarded, and the organisms were rinsed with clean water and then placed in beakers with fresh artificial pond water and fresh leaf discs. The pH, conductivity and dissolved oxygen concentration of the medium were monitored (SI Table S4).

Our experimental design aimed to maximize the number of test organisms that we could handle so that we would maximize experimental power. However, no formal power analysis was carried out and the experimental design was constrained by the practicalities of the experiment.

Statistical Analysis. Statistical testing was performed using GraphPad Prism (v6.03, GraphPad Software Inc., La Jolla, CA). To detect the sequence effect, we compared the numbers of dead and live individuals at the end of the experimental groups using contingency table analysis, more specifically, Fisher's exact test (Figure 1B, D, F, H, J, L, N, P). For evaluation of carry-over toxicity, we compared the survival curves after the first and second pulses for each experimental group using the Gehan-Breslow-Wilcoxon test (Figure 2). These survival curves were constructed by recording the absolute number of alive *G. pulex* at the start of a pulse (this could be the first or the second pulse in an experimental group) as the initial number of organisms and then following each group until either the end of the experiment or the onset of the second pulse. In other words: we compared survival curves during and following the pulsed exposure to the same toxicant in different groups of the same experiment, once as the first pulse and again as the second pulse. If there was a difference, then we concluded that carry-over toxicity had occurred because we used the same dose for a single toxicant in both of its exposure pulses within each experiment. The only difference was that the order was reversed in the second experimental group. All four experiments were analyzed following the same method.

Toxicokinetic-Toxicodynamic Modeling. *Toxicokinetics: Modeling the Internal Dose.* The toxicokinetic model was calibrated previously using time series data of measured internal concentrations in pulsed exposure experiments.[7−9,14] Here, the time-course of internal concentrations in *G. pulex* was simulated using the previously established toxicokinetic models and parameters from those studies:

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

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

,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 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 the formation of metabolite j [1/d], and $k_{out,j}$ is the elimination rate constant of the biotransformation product j. Internal concentrations of propiconazole (PCZ) and 4,6-dinitro-*o*-cresol (DNOC) were directly used as inputs in the toxicodynamic model; by contrast, for diazinon (Diaz) and 4-nitrobenzyl chloride (NBCl), biotransformation products were the driving variables for the toxicodynamic model (see Table 1 and SI Table S1, also for model parameter values). In the case of 4-nitrobenzyl chloride, the metabolites are unidentified and considered to act as baseline toxicants.[9] For diazinon, metabolite 1 is diazoxon, which is the toxicologically relevant molecule (Table 1). For propiconazole and 4,6-dinitro-*o*-cresol, we included only the parent compounds in the model, because biotransformation in *G. pulex* is negligible.[8,14]

Toxicodynamics: Modeling Survival. We used the two limit cases stochastic death (SD) and individual tolerance (IT) of the General Unified Threshold model of Survival (GUTS)[13] with explicitly modeled internal concentrations and scaled damage[9,13] as the dose metric. These models have been described previously,[9,13] but we repeat their description here for clarity. The toxicodynamic model parameters are given in SI Table S2. Scaled damage is a proxy for the toxicodynamic state of the organism, is generally inferred by model fitting[9,13] and it is calculated as follows:

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

,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 of Scaled Damage on Survival in GUTS-SD. The hazard rate is the probability of an organism dying at a given point in time and is calculated as follows:[8,13,24]

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

,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 during the experiment, see Table S3) [1/d]. The survival probability, i.e. the probability of an individual surviving until time t, is given as follows:

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

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

Effects of Scaled Damage on Survival in GUTS-IT. We assume a log−logistic distribution of the threshold in the study population (individual tolerance).[8,13] Then, the cumulative log−logistic distribution of the tolerance threshold in the study population, which changes over time as individuals die, is calculated as follows:

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

,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

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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 as follows:[8,13]

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

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

Mixture Toxicity Model. *Mixture Toxicity Model Assumptions.* Mixture toxicity models generally assume either that the toxicants act on the same target or on different targets.[25,26] Independent action is the model for the second assumption, which applies to our combinations of toxicants. Traditional mixture toxicity models are designed for exposure to multiple toxicants at the same time and for constant concentrations. We expand the applicability domain of the mixture toxicity modeling to fluctuating, pulsed, or sequential exposures using a dynamic model, GUTS.[9,13] As we assume independent action for compounds with different modes of action, we sum the hazards from the different toxicants,[9,27] which is equivalent to multiplying the survival probabilities.

Mixture Toxicity Model Equation. The same mixture toxicity model is used for GUTS-SD and GUTS-IT:

$$S_{mix}(t) = e^{-\sum H_i} \quad (8)$$

which is equivalent to

$$S_{mix}(t) = \prod S_i(t) \quad (9)$$

$S_{mix}(t)$ is the survival probability under toxic stress from i toxicants. In eq 8, we sum the hazards, which is equivalent to multiplying the survival probabilities (eq 9) and corresponds to the assumption that toxicants act on different target sites or via different toxicity pathways.

Treatment of NBCl Metabolites in the Toxicity Model. In the case of NBCl, the internal concentration of the parent compound was modeled as a reactive toxicant, whereas the sum of the internal concentrations of the metabolites was modeled as a baseline toxicant, as in a previous study.[9] We used the toxicodynamic parameters of 1,2,3-trichlorobenzene, a well-known prototype baseline toxicant, to simulate the effects of the metabolites assumed to act via baseline toxicity.[9] As we assumed the two modes of toxic action are independent, we modeled separate scaled damages for each using eqs 3−7 and then multiplied the resulting survival probabilities:

$$S_{combined}(t) = S_{baseline\ toxicity}(t) \times S_{specific\ toxicity}(t) \times e^{h\_controls \times t} \quad (10)$$

RESULTS AND DISCUSSION

Mixture Toxicity Model Prediction. We compared the survival prediction determined by the mixture toxicity models with the observed survival (SI Figure S1−S4). The agreement or disagreement between the predicted and observed survival curves after the first pulses is a measure of the interexperimental variability. In half of the cases, the predicted survival after the first pulse did not match the observed survival very well (SI Figure S1G, S3G, S4G, H). Only in experiment two was survival after the first pulses predicted accurately in both groups (SI Figure S2G, H); in this experiment, survival after the second pulses was also predicted accurately (SI Figure S2G, H). The disagreement between the predicted and observed survival after the second pulse in experiment one (Diaz, SI Figure S1H) or three (NBCl, SI Figure S3H) is consistent with the disagreement seen after the first pulse and is not necessarily an indicator that the mixture toxicity models fail to predict full recovery after PCZ (experiment one) or DNOC (experiment three). In experiment four the model predicted that all organisms would die almost immediately following exposure to NBCl, thus assessment of the predicted mortality after DIAZ in this experiment is difficult.

We also compared the mixture toxicity model prediction with the observed survival curve in the five experimental halves where we found evidence of carry-over toxicity (survival after PCZ, DNOC, DNOC, DIAZ, and NBCl in experiments one, two, three, four, and four, respectively; Figure 2 A, C, E, G, H) and observed in four out of five cases that the observed mortality following the respective second pulses exceeded the predicted mortality (SI Figure S6 to S10). From that observation we conclude that the toxicants interact, even if exposure is days apart, and that the observed mortality following the second pulses is more than just continued mortality from the other toxicant of the respective first pulses in all the "sequence" treatments and the "inverse sequence" treatment of experiment four (i.e., those with carry-over toxicity).

We need more studies of this type to better understand how the model performs for different substances and classes of chemicals and where the limits of its predictive capabilities are.[28] Uncertainty arising from calibration experiments themselves can be included in the model predictions[28] to better understand interexperimental variability. Interesting questions arise also from the temporal mismatch in the onset of predicted and observed mortality (particularly NBCl, see SI Figure S3 and S4) as well as our choice of toxic molecules (Table 1).

In summary, we found no evidence that the mixture models fail to predict carry-over toxicity or the sequence effect. However, we did find large interexperimental variability evidenced by a disagreement between the predicted and observed mortalities. Since the models were calibrated based on previously published experiments, this disagreement only indicates that the variability in survival from one pulsed exposure experiment to the next is too large to assess the performance of the model prediction. However, the experimental evidence depicted in SI Figures S1−S4 fully supports the sequence effect hypothesis. Conclusions drawn from comparisons within an experiment, such as in this study, are not subject to the limitations of interexperimental variability. Variability between experiments could possibly be reduced by using laboratory-cultured organisms.

Furthermore, we identified a knowledge gap in the suite of toxicokinetic-toxicodynamic mixture toxicity models available. We provide equations for summing up hazard rates for toxicants which are assumed to act independently. However, it is also conceivable to model the combined effects of toxicants that act on different targets by summing up the state variable scaled damage. However, there are two challenges. First, summing up damage has been previously used to model combined effects of toxicants that act on the same target.[6,29] This raises a question: How can we reconcile that previous work with any future use of the same approach for toxicants acting on different targets? The task of reconciling this previous work with newer studies is further complicated by subtle issues

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and inconsistencies in the models used in the older studies.[13] Second, the model equations for summing up scaled damage in GUTS-SD are straightforward to derive, however the model equations for summing up scaled damage in GUTS-IT are unknown and not trivial to derive.

The Sequence Effect. We observed the sequence effect in experiments one, two and three (Figure 1). Contingency table analysis of the number of dead and alive individuals at the end of each experimental group indicated a sequence effect for the combinations of DIAZ and PCZ ($p < 0.0001$, first experiment), DNOC and DIAZ ($p = 0.0151$, second experiment), and NBCl and DNOC ($p = 0.0006$, third experiment), but not for the NBCl and DIAZ combination ($p = 0.4975$, fourth experiment). There was no difference in the toxicant concentrations, test durations or intervals between the experimental groups, but survival was significantly different at the end (Figure 1). The odds ratios and their 95% confidence intervals are 7.6 (2.8−21), 2.4 (1.2−4.5), and 27.3 (1.6−473) in the first, second, and third experiment. This means that organisms in these experiments were about 8, 2, and 27 times as likely to die in the "sequence" compared to the "reverse sequence" treatment (Figure 1). The confidence interval of the odds ratio in the fourth experiment extends from 0.01 to 4.2, indicating similar likelihood of death in both treatments. These results clearly support our hypothesis that besides the dose, the sequence of exposure also affects toxicity of combinations of toxicants with different mechanisms of action.

Carry-over Toxicity and Slow Recovery. This led us to the question of whether toxicodynamic interactions due to slow recovery of toxicodynamic damage were the underlying cause of the sequence effect. To test this we compared survival curves following exposure to the same toxicants (Figure 2). In each experiment and for each toxicant we monitored survival over time. Hence we have survival curves for the duration of half of the total experiment's duration that can be compared, with the only difference being whether the toxicant under consideration was applied as first or second pulse. If the survival curves differ, it must be due to the effect of the pre-exposure to the respective other toxicant on the survival following the second exposure. For example, there is a significant difference in the survival curves following PCZ exposure (Figure 2B, PCZ_1 vs PCZ_2, $p < 0.0001$). The most plausible explanation is, that this difference must have been caused by the previous exposure to DIAZ_1 in DIAZ_PCZ (Figure 1A). In fact, we observed carry-over toxicity after DIAZ exposure for all tested chemicals, as demonstrated by the significantly different survival curves (Figure 2C, H, DNOC_1 vs DNOC_2, $p = 0.002$; NBCl_1 vs NBCl_2, $p < 0.0001$). Exposure to NBCl also caused carry-over toxicity, as demonstrated by the significantly different survival curves following pulses of DNOC (Figure 2E, DNOC_1 vs DNOC_2, $p < 0.0001$) and DIAZ (Figure 2G, DIAZ_1 vs DIAZ_2, $p < 0.0001$).

The observation that both DIAZ and NBCl caused carry-over toxicity explains why we did not observe a sequence effect in experiment four. The carry-over toxicity observed following exposure to DIAZ and NBCl is most likely due to incomplete recovery of toxicodynamic damage because buildup of toxicodynamic damage is the most plausible explanation. Since we cannot measure toxicodynamic damage directly we constructed toxicodynamic mixture toxicity models (SI) for independently acting toxicants[25−27,30] with scaled damage as dose metric.[9,13] Here we modeled carry-over toxicity as a buildup of scaled damage, which was done for single substances before[9,13] but is now used to model a mixture of toxicants in sequence.

Which chemicals do not cause carry-over toxicity? Exposure to PCZ or DNOC did not cause carry-over toxicity, presumably because the interval between pulses was long enough for complete toxicodynamic recovery. For PCZ, this was evidenced by the lack of difference in the survival curves following DIAZ exposure (Figure 2B, DIAZ_1 vs DIAZ_2, $p = 0.6$). For DNOC, the survival curves following DIAZ or NBCl exposure did not differ (Figure 2D, DIAZ_1 vs DIAZ_2, $p = 0.32$; Figure 2F, NBCl_1 vs NBCl_2, $p = 0.4$). Classifying chemicals by their mode of toxicity is a good starting point for identifying those that are unlikely to cause carry-over toxicity because of fast toxicodynamic recovery. Since the rate of toxicodynamic recovery is related to the mode of toxic action,[9] it is possible to predict the chemical structures that would cause carry-over toxicity and for which sequence effects are likely.

This study clearly shows that the exposure sequence is important for *G. pulex* survival, specifically in cases when organism recovery is slow relative to the interval between exposure pulses. Slow organism recovery can be caused by slow toxicodynamic recovery, which likely occurs only for certain toxicological mechanisms of action, such as irreversible inhibition of enzymes (e.g., acetylcholinesterase inhibition) or reactive toxicity (e.g., covalent binding to proteins). Our interpretation rests on the assumption that total body residues are a good proxy for toxicant concentrations at the target site. This assumption, that is, the idea that the concentration at the target site is proportional to whole body residues, seems plausible, but we are not aware of any studies that provide empirical support for it. If the time-course of toxicant concentrations at the cellular target sites differs markedly from our modeled whole body toxicokinetics, for example by much slower elimination, then carry-over toxicity could also be explained as a simple buildup of concentrations at the target site over time. More research is needed to clarify this point.

Implications for Chemical Safety Assessment. Our experiments have shown, for the first time, that the sequence effect and carry-over toxicity occur among chemicals acting on different targets and via exposure several days apart. This effect is currently not considered in environmental risk assessment[10] but has wide reaching implications. This is because exposure to multiple toxicants and in highly variable sequences is a real-life scenario for humans as well as all other organisms.[1,2,31−33] It also means that we need novel concepts for environmental mixture toxicity assessment,[26] because current environmental mixture toxicity assessments are all based on the assumption that toxicants that occur after each other do not interact.[25,30] Our observation that not only the dose but also the sequence makes the poison shows the need to improve human and environmental safety assessment of chemicals. We provide a tool to combine predictions of survival not only for time-variable exposure to one toxicant but several, which could be applied in chemical risk assessment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b06163.

The data reported here is available in the Supporting Information. Further information on test compounds, model parameters, experimental details, figures showing

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modeled toxicokinetics and toxicodynamics and model code are also provided. The modeling figures are also provided separately as high resolution images (ZIP)

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