Modeling Combined Effects of Pulsed Exposure to Carbaryl and Chlorpyrifos on *Gammarus Pulex*

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Aquatic risk assessment can be improved if we are able to quantitatively predict the effects resulting from sequential pulsed exposure to multiple compounds. We evaluate two modeling approaches, both extended to suit multiple compounds, the semi-mechanistic threshold damage model (TDM), and a model based on time-weighted averages (TWA). The TDM predicts that recovery of damage to *Gammarus pulex* from exposure to chlorpyrifos takes longer than that from exposure to carbaryl and consequently that the sequence of exposure matters. We measured survival of the freshwater invertebrate *Gammarus pulex* after sequential pulsed exposure to carbaryl and chlorpyrifos. Two groups of organisms were exposed to a first pulse of either carbaryl or chlorpyrifos for 1 day and then, after a recovery period of two weeks, to a second pulse with the other compound. The comparison of mortalities caused by each pulse, as well as combined mortalities in both treatments, show that the sequence of exposure to pulses of contaminants does indeed matter. Previous exposure to chlorpyrifos leads to significantly increased mortality from subsequent pulses of carbaryl, but not the other way round. The TDM facilitates a process-based ecotoxicological explanation by simulating the recovery dynamics and outperforms the TWA model.

Introduction

It has been recognized that aquatic nontarget organisms exposed to pesticides are typically exposed to sequential pulses with fluctuating concentrations (*1*, *2*), but current risk assessment relies on standard toxicity tests performed at constant concentrations and over fixed durations. We reviewed available models to relate fluctuating field exposures to laboratory effects data and found that there was no generally applicable and validated method available (*3*). Subsequently, we developed, evaluated, and compared two methods (*4*, *5*), the threshold damage model (TDM) and a method based on time-weighted average concentrations (TWA pulses). Both models performed well when simulating survival after fluctuating or sequential pulsed exposure to either chlorpyrifos or pentachlorophenol (*4*), whereas simulations with carbaryl demonstrated a better performance of the TDM (*5*). These models allow simulation of effects from realistic exposure patterns, but so far have only considered exposure to a single toxicant.

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The next step toward more realism in environmental effects assessment of toxicants is to consider fluctuating concentrations or sequential pulses of multiple compounds. Temporally staggered environmental fate processes and superimposition of multiple sources of contaminants lead to sequential pulses of multiple contaminants in natural water bodies (*6*−*9*). In this study we extend the two modeling approaches (TDM and TWA pulses) to describe effects from sequential exposure to multiple compounds. The TDM predicts that the recovery of damage to *Gammarus pulex* from exposure to chlorpyrifos takes longer than that from exposure to carbaryl (*5*). Lasting damage can cause increased mortality from subsequent exposures to the same compound, as shown for chlorpyrifos (*4*), but the TDM also predicts that lasting damage caused by one compound may increase mortality from subsequent exposure to another. Hence, if the recovery time between two exposures is long enough for sufficient recovery from damage caused by carbaryl, but not chlorpyrifos, then the sequence of exposure should matter. This study was designed to test this hypothesis.

The detailed objectives of the study were (i) to measure survival of the freshwater invertebrate *Gammarus pulex* after sequential pulsed exposure to carbaryl and chlorpyrifos, (ii) to test for increased mortality caused by previous exposure to the other compound, and (iii) to test whether mortality is different for chlorpyrifos followed by carbaryl compared to the reverse ordering. Furthermore, (iv) we evaluate the models with respect to the performance of their simulations of survival and their prediction or lack of a sequence effect.

Materials and Methods

Organisms and Exposure Water. The freshwater invertebrate *Gammarus pulex* is of ecological importance because it is involved in detritus processing in streams (*10*). It has been used in biomonitoring (*10*, *11*), laboratory toxicity studies (*12*, *13*) and microcosm experiments (*14*, *15*). For this study, *Gammarus pulex* (mixture of males and females, length ca. 5−10 mm) were collected from a small stream, Bishop Wilton Beck, ca. 20 km north-east of York, UK. Prior to experiments, organisms were kept for 4 days in aerated streamwater under the same conditions as in the experiments and were fed in excess with rehydrated horse chestnut leaves.

Streamwater was also collected from Bishop Wilton Beck and stored at 5 °C. Bishop Wilton streamwater (pH 9) was used in between pulses, but during the exposure pulses we used buffered streamwater from Keys Beck (North Yorkshire Moors, upstream catchment completely in drinking water protection area). Keys Beck water was buffered at pH 6.68 with 750 mg/L MOPS (3-N morpholino propane sulfonic acid, (*16*)) and NaOH. Buffering is necessary because carbaryl is not stable under alkaline conditions. The resulting changes in pH are likely to cause an additional stress on the organisms, but we assume that the effect is small compared to the toxic stress caused by the pesticides.

All experiments were carried out under static conditions in 600 mL pyrex beakers filled with 500 mL exposure solution. Beakers were kept in a cooling tank with water as coolant to maintain constant temperatures (12 ± 2 °C). The light regime was a cycle of 12 h light and 12 h dark. All beakers were sealed with Parafilm and aerated with pressurized air through Pasteur pipettes. Dissolved O_2 ranged between 9.7 and 9.9 mg/L (measured with a HI 9142 dissolved oxygen meter, Hanna Instruments) and pH ranged from 6.68 to 6.87 during the exposure pulses and from 8.91 to 9.36 mg/L in the elimination and recovery periods (measured with a Hanna pH 213 and HI 1131 electrode, Hanna Instruments).

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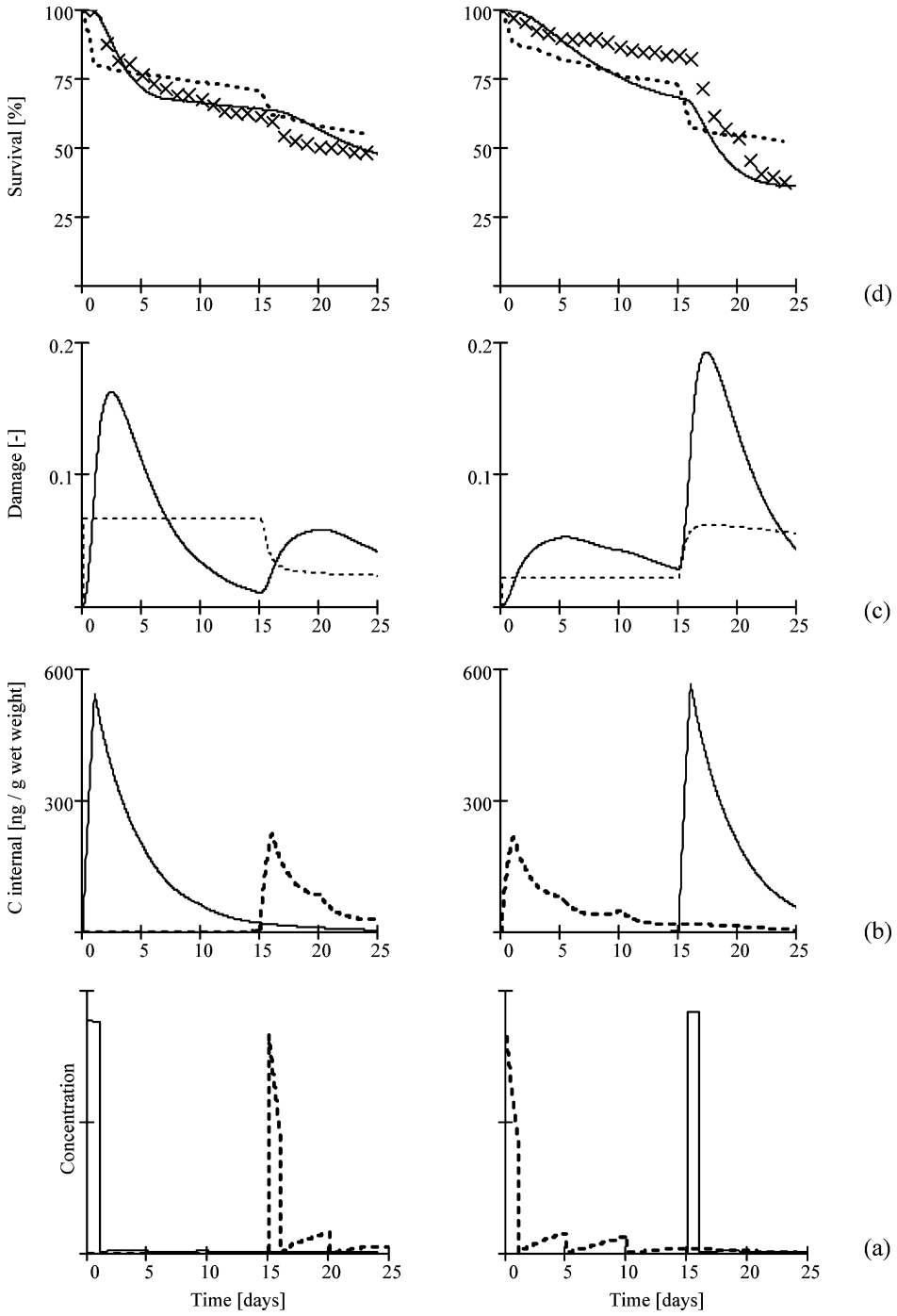

FIGURE 1. The graphs show treatment A (left) and treatment B (right). The sequence of the pulses with carbaryl (solid line) and chlorpyrifos (dashed line) is plotted to different scales (bottom graph, (a)). The initial aqueous concentrations of the pulses are 26.5 and 27.5 μg/L (carbaryl, A and B) and 0.5 μg/L (chlorpyrifos). The next graphs (second from bottom, (b)) show simulated internal concentrations of carbaryl (solid line) and chlorpyrifos (dashed line). The third graphs (from bottom, (c)) show the time course of the mixture damage (solid line, eq 3) together with the mixture threshold (dotted line, eq 4). The top graphs (d) show the observed survival ($\times$), the prediction by the TDM (solid line) and the prediction by the TWA model (dashed line).

Organisms were rinsed and transferred to clean water at the end of any exposure pulse (1 day) and more frequently such that the maximum duration between water changes was 5 days. The largest amount of methanol used in the treatments was 0.08% v/v (chlorpyrifos dosing). We assume that the methanol evaporated very quickly and had no effect on the organisms because all beakers were aerated with pressurized air.

Chemicals. Chlorpyrifos and carbaryl are both insecticides that cause toxicity through inhibition of the enzyme acetylcholinesterase (AChE). Chlorpyrifos is an organothiophosphate and carbaryl is a carbamate. AChE inhibited by carbamates shows faster reactivation than that after inhibition by organophosphates (*17*). ^{14}C-labeled carbaryl [1-naphthyl methylcarbamate] (ring-labeled, 100% purity, 503 MBq/mmol, batch no. XI/39) was purchased from Institute of Isotopes, Budapest, Hungary. Unlabeled carbaryl was purchased from Sigma-Aldrich Ltd. (Gillingham, UK, 99.8% purity). Dosing solutions were made in methanol by mixing labeled with unlabeled carbaryl. ^{14}C-labeled chlorpyrifos

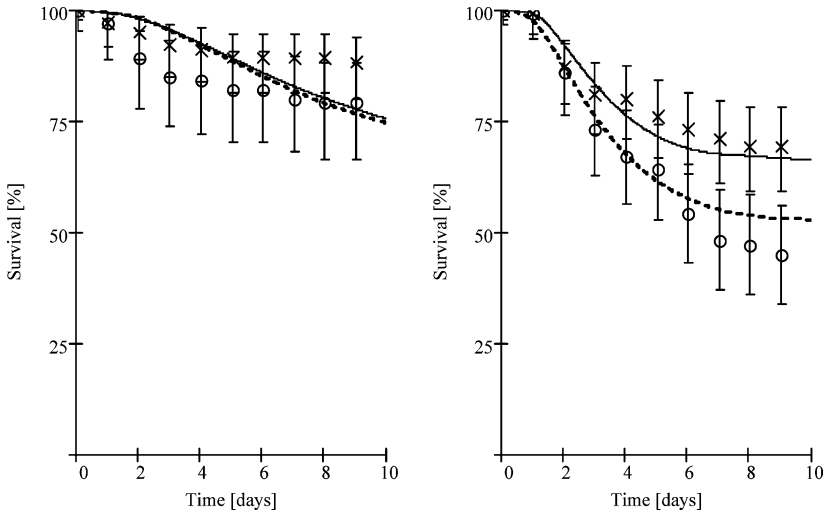

FIGURE 2. The graph to the left shows survival (and 95% confidence intervals of proportions) following the first (no preexposure, treatment B, ×) and second (14 days after preexposure, treatment A, ○) pulse to chlorpyrifos. There is no significant difference between the mortalities from the two chlorpyrifos pulses, because organisms were able to recover from the previous exposure to carbaryl in treatment A. The graph to the right shows survival (and 95% confidence intervals of proportions) following the first (no preexposure, treatment A, ×) and second (14 days after pre-exposure, treatment B, ○) pulse to carbaryl. The second pulse of carbaryl (treatment B) causes significantly more mortality than the first pulse (treatment A), because the organisms in treatment B have not yet recovered from the previous exposure to chlorpyrifos. Survival predicted by the TDM is shown for no preexposure (solid lines; left: treatment B, CPF; right: treatment A, CBL) and 14 days after pre-exposure (dotted lines; left: treatment A, CPF after preexposure to CBL; right: treatment B, CBL after preexposure to CPF).

[pyridine-2,6-^{14}C] (99% purity, 32 Ci/mol, lot no. 050107) was purchased from American Radiolabeled Chemical, Inc. (St. Louis, U.S.).

Experiments. Two groups of *Gammarus pulex* were exposed to a first pulse of either carbaryl or chlorpyrifos for 1 day and then, after a recovery period of two weeks, to a second pulse with the other compound respectively. The two treatments in our experiment are denoted A and B (left and right parts of Figure 1). Both treatments consisted of ten beakers with ten *Gammarus* in each beaker at the start of the experiment. Treatment A was dosed with a 1 day pulse of carbaryl first (initial concentration 26.5 μg/L), followed by 14 days of clean water and then a 1 day pulse of chlorpyrifos (initial concentration 0.497 μg/L), followed by 8 days in clean water. Treatment B was complementary to treatment A, with the same pulses of carbaryl and chlorpyrifos, but the order of the pulses reversed (Figure 1a). Hence. treatment B was dosed with a 1 day pulse of chlorpyrifos first (initial concentration 0.494 μg/L), followed by 14 days of clean water and then a 1 day pulse of carbaryl (initial concentration 27.5 μg/L), followed by 8 days in clean water.

Daily counts of surviving organisms were made in all beakers. Test solutions were sampled (1 mL) immediately after spiking and frequently thereafter to quantify actual pesticide concentrations by measuring the radioactivity present in the water. Radioactivity was quantified with liquid scintillation counting (Beckman LS6500 TA liquid scintillation counter, Beckman Instruments Inc., Fullerton, U.S.) after adding 10 mL of Ecoscint A scintillation cocktail (National Diagnostics, Hessle, UK). Samples were counted three times for 5 min. Sample counts were corrected for background activity by using blank controls. Counting efficiency and color quenching were corrected using the external standard ratio method.

Statistical Analysis of Mortality. Our experiment generated data for mortality following two pulses of carbaryl and two pulses of chlorpyrifos. The doses of both carbaryl pulses and the doses of both chlorpyrifos pulses were comparable, but for each compound there was one pulse where the organisms had not been previously exposed and one pulse

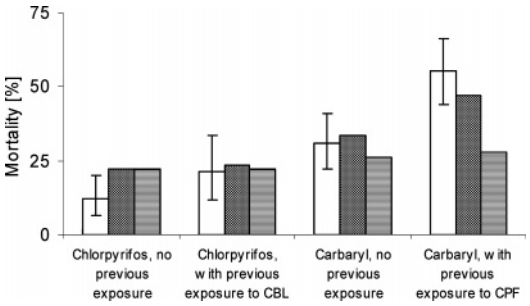

FIGURE 3. Comparison of the mortalities caused by chlorpyrifos and carbaryl with model predictions. The bars show observed (white, ± 95% CI) and predicted mortality (TDM: chequered, TWA: striped). From left to right: first pulse in treatment B, second pulse in A, first pulse in A and second pulse in B.

where they had been exposed to the other toxicant 14 days before (Figure 1). We compared the mortalities following exposure to the same toxicant, thus testing for differences caused by previous exposure to the other compound. Comparisons are made for the time course of survival during the pulse and over the following 8 days (Figure 2) and for the total mortality over the full 9 days (Figure 3).

Confidence intervals for proportions of dead organisms were calculated following (*18*). Differences between mortalities were tested for significance using the binomial test for two proportions (Software MINITAB, release 14.20).

Modeling

Approach. We used the semimechanistic threshold damage model (TDM) to simulate survival and compared it to a simpler model based on time-weighted averages (TWA pulses). Both models were first presented and discussed in ref *4*, where we also estimated the toxicodynamic parameters for chlorpyrifos. Uptake and elimination rate parameters for chlorpyrifos in *Gammarus pulex* were measured previously (*19*). The parameters for carbaryl and additional discussion of the two modeling concepts can be found in ref *5*. Here we

TABLE 1. Model Parameters

parameter	symbol	chlorpyrifos	carbaryl[c]	units[d]
uptake rate constant	k_{in}	747[a]	23.4	$L \times kg^{-1} \times day^{-1}$
elimination rate constant	k_{out}	0.45[a]	0.27	day^{-1}
killing rate constant	k_k	0.134[b]	0.42	$g_{wet.w.} \times \mu g_{a.i.}^{-1} \times day^{-1}$
recovery rate constant	k_r	0.169[b]	0.97	day^{-1}
threshold	threshold	0.022[b]	0.067	
scaling factor	f_TWA	321[b]	7.6	$L \times mg^{-1} \times day^{-1}$
background hazard rate	h_b	0.0071		day^{-1}

[a] From ref *19*. [b] Parameter set from fit to all data in ref *4*. [c] Parameter set from ref *5*. [d] $g_{wet.w.}$ is g wet weight and $\mu g_{a.i.}$ is μg of active ingredient.

extend both models to facilitate simulations with multiple toxicants (TDM_{mix} and TWA_{mix}) and apply the models using the previously estimated parameters (Table 1). Hence we are running independent, predictive simulations.

The Threshold Damage Model for Multiple Toxicants (TDM_{mix}). The TDM combines toxicokinetics (uptake and elimination) with toxicodynamics (damage accrual and recovery and exceedence of a damage threshold) in one consistent ecotoxicological model. We can simulate the processes leading from exposure to effect in a semimechanistic manner enabling us to assess fluctuating or sequential exposure.

Here, the basic TDM (*4*) is extended to simulate the survival following exposure to multiple toxicants. In the extension, TDM_{mix}, we calculate the internal concentrations and the damage for each toxicant individually (eqs 1 and 2). Then the total damage in the organisms is calculated by summing up the individual damages (eq 3). As the thresholds for different toxicants differ we calculate the mixture threshold in eq 4 as an average of the individual thresholds, weighted by the individual damages at any point in time. The hazard rate (probability of dying) increases when the total damage level rises above the mixture threshold (eq 5).

Equation 1 is the one-compartment first-order kinetics model,

$$\frac{dC_{int,i}(t)}{dt} = k_{in,i} \times C_i(t) - k_{out,i} \times C_{int,i}(t) \quad (1)$$

where $C_{int,i}$ (t) is the internal concentration at any point in time [amount $\times$ mass^{-1}], $C_i(t)$ the concentration in the water at any point in time [amount $\times$ volume^{-1}] and $k_{in,i}$ and $k_{out,i}$ the uptake rate constant [volume $\times$ mass^{-1} $\times$ time^{-1}] and the elimination rate constant [time^{-1}] of compound i, respectively. Equation 2 simulates the first part of the toxicodynamics as an accrual and, in the second term of eq 2, the recovery or repair of damage:

$$\frac{dD_i(t)}{dt} = k_{k,i} \times C_{int,i}(t) - k_{r,i} \times D_i(t) \quad (2)$$

where $k_{k,i}$ is a killing rate constant [mass $\times$ amount^{-1} $\times$ time^{-1}], $k_{r,i}$ is the rate constant for damage recovery or repair [time^{-1}] and $D_i(t)$ is damage [−] of compound i at any point in time, respectively. The following two equations constitute the extension of the TDM for multiple compounds. In eq 3 we sum up internal damage values for all toxicants:

$$D_{mix}(t) = \sum_i D_i(t) \quad (3)$$

where $D_{mix}(t)$ is the total amount of damage present in the organism at any point in time [−]. The damage $D_i(t)$ of each compound is linked to its threshold value. Therefore, we calculate the threshold for the total damage of the mixture as an average of the individual thresholds, weighted by the individual damages at any point in time:

$$\text{threshold}_{mix}(t) = \sum_i \left(\text{threshold}_i \times \frac{D_i(t)}{D_{mix}(t)} \right) \quad (4)$$

where $\text{threshold}_{mix}(t)$ is the mixture threshold [−]. The differential of $H(t)$, as used in eq 5 is the hazard rate, which is the probability of the organisms dying at a given time. The hazard rate rises above zero when the mixture threshold is exceeded by the mixture damage:

$$\frac{dH(t)}{dt} = \max[D_{mix}(t) - \text{threshold}_{mix}(t), 0] \quad (5)$$

where $H(t)$ is the hazard [−]. The threshold is due to compensating mechanisms as well as the change in scales from processes on the scale of cells or sites of action (eq 2) to the survival probability at the scale of the whole organism (eq 6). In eq 6 we use the standard approach of linking hazard to survival:

$$S(t) = e^{-H(t)} \times S_{background}(t) \quad (6)$$

where $S(t)$ is the survival probability [−] (probability of an organism surviving until time t) and $S_{background}(t)$ is the survival probability resulting purely from the background mortality [−].

The Time-Weighted Averages Model for Multiple Toxicants (TWA_{mix}). We compare the performance of the TDM with a modified time-weighted averages approach (TWA pulses model, (*4*)). The survival probability for each toxicant at any point in time is calculated as follows:

$$S_i(t) = 1 - f\_TWA_i \times \int_0^t C_i(t)dt \quad (7)$$

where f_TWA_i is the scaling factor [volume $\times$ mass^{-1} $\times$ time^{-1}] and C_i the concentration in the exposure solution [mass $\times$ volume^{-1}] for compound *i*. The scaling factors for chlorpyrifos and carbaryl are taken from previous studies (*4*, *5*). The overall survival probability for the mixture TWA model is calculated as follows:

$$S(t) = S_{background}(t) \times \prod_i S_i(t) \quad (8)$$

where $S_{background}(t)$ is the survival probability at any point in time resulting purely from the background mortality [−].

Correction for Background Mortality. The survival probabilities of both models were corrected for the background mortality in our test system. We did not have a control group in this experiment because we aimed to maximize the statistical power from our two treatments. Hence we estimated the background mortality by fitting eq 9 to background mortalities from experiments A, B, and D in ref *4* consisting of 15 replicate beakers (150 *Gammarus* initially, 10 beakers until day 20 and five beakers until day 24).

$$S_{\text{background}}(t) = e^{-h_b \times t} \quad (9)$$

where h_b is the background hazard rate [time^{-1}] and t is time [time].

Results and Discussion

Comparison of the TDM with the MDAM and Mixture Toxicity Theory. Up to eq 3 some aspects of the TDM are similar to the MDAM developed by Lee at al. (*20*). They developed a model that includes metabolism in the toxicokinetics and also has a damage term for the toxicodynamics but is used to predict lethal body residues. They did not use a threshold and they did not measure survival in experiments with repeated exposure pulses. Nevertheless, Lee at al. (*20*) showed that the assumption of damage addition, as used in the TDM and the MDAM, is equivalent to the independent action model for mixtures. However, as long as there are no toxicokinetic and toxicodynamic interactions between the compounds, the concept of damage addition is also equivalent to the concentration addition model (*20*). The assumptions of no interaction in the toxicokinetics and toxicodynamics between the compounds also hold for the TDM. The TDM can also be applied to simultaneous or overlapping peaks of pesticide concentrations, although this remains to be tested.

The MDAM was not tested in this study because it is not designed for simulating survival after repeated pulsed exposure. Furthermore, it needs to be reconsidered before general application because there is an inconsistency in the link between damage and survival (*3*).

It could be possible to fix the threshold parameter in the TDM_{mix} at a common value for compounds with the same mechanisms of action, but more research is required to confirm and yield a sufficiently supported common threshold value. Parametrizing the TDM for a range of compounds will show if there is an equal threshold level for related groups of toxicants.

Observed Mortality: The Sequence Matters. The time between the pulses was long enough for both compounds to be depurated (Figure 1b). The times for depuration of 95% of chlorpyrifos and carbaryl are 7 days (*19*) and 11 days (*5*), respectively. Even though 14 days are sufficient for depuration of chlorpyrifos in treatment B, the subsequent pulse of carbaryl results in significantly higher mortality than without previous exposure to chlorpyrifos (first pulse in treatment A). The mortalities are 31% after the first pulse in treatment A vs 55% after the second pulse in treatment B (Figures 2 and 3). The difference is significant (31/100 vs 46/83, $p = 0.001$). In the opposite comparison, chlorpyrifos does not show significantly increased mortality after previous exposure to carbaryl. The mortalities are 12% (12/100) after the first pulse in treatment B vs 21% (13/61) after the second pulse in treatment A ($p = 0.13$).

There are two implications of this result. First, it does matter whether organisms are exposed to the other toxicant, even if it is 14 days earlier, and second it does make a difference to which compound they were previously exposed. We also assess the combined mortalities from both pulses in each treatment by calculating the percentage killed by both subsequent pulses from the product of the respective survival probabilities. The combined mortality is 45% {1 − (0.69 × 0.79)} in treatment A and 60% {1 − (0.88 × 0.45)} in treatment B. The combined doses of carbaryl and chlorpyrifos were the same in both treatments. Hence, the difference in the mortalities is attributed to the different sequence of exposure.

TABLE 2. Indicators of Model Performance

experiment	mean and maximum errors [%][a]		r^2	
	TDM	TWA pulse	TDM	TWA pulse
treatment A	4 (9)	7 (20)	0.94	0.85
treatment B	8 (16)	10 (25)	0.92	0.81

[a] In % of initial population, maximum error in parentheses.

Model Performances. The predicted survival is shown for both the TDM_{mix} and the TWA_{mix} model in Figure 1d. The prediction by the TDM shows a better agreement with the observed survival than the TWA model and this is supported by the statistical indicators (Table 2) which show a consistently better performance of the TDM. The TDM simulation of survival results in mean errors of 4 and 8% and maximum errors of 9 and 10% for treatments A and B, respectively. These are independent simulations that extrapolate from previous experiments, so the errors are partly due to interexperimental variability.

The graphs b and c in Figure 1 show intermediate calculation steps of the TDM, illustrating the use of the TDM for interpretation of experimental outcomes. Figure 1b shows the time course of the internal concentrations and illustrates that both compounds are almost completely depurated between pulses. Hence the increased mortalities from subsequent pulses in both treatments cannot be explained by residual internal concentrations of the previous compound. Figure 1c illustrates the time course of the damage ($D_{mix}(t)$) as simulated by the TDM. Together with the plotted threshold ($threshold_{mix}(t)$) it becomes very clear how the TDM predicts the increased mortality following the second pulse in treatment B, but not in A.

We observed a significant difference in the mortality following the two pulses of carbaryl. The TDM predicts a difference for both pulses and is in good agreement with the observations (Figures 2 and 3). There was no significant difference in the mortalities following chlorpyrifos exposure and again the TDM is in good agreement with this observation (Figures 2 and 3). The individual mortalities as predicted by the TDM are 22 vs 23% for chlorpyrifos (treatment B vs A, observed: 12 vs 21%, not significant, $p = 0.13$), and 33 vs 47% for carbaryl (treatment A vs B, observed: 31 vs 55%, significant difference, $p = 0.001$).

The TWA model also predicts no difference in the mortalities following exposure to chlorpyrifos (treatment B vs A; predicted: 22 vs 22%; observed: 12 vs 21%, not significant, $p = 0.13$). In contrast to the TDM and the observations, the TWA model also predicts only a minor difference in the mortalities following exposure to carbaryl (treatment A vs B; predicted: 26 vs 28%; observed: 31 vs 55%, significant difference, $p = 0.001$). The minor increase is attributed to the slightly larger initial concentration of carbaryl in the second pulse (26.5 μg/L in treatment A vs 27.5 μg/L in B).

The TDM outperforms the TWA model with respect to the prediction of the time course of survival (Figure 1, Table 2) and the prediction of the significant difference in mortalities following exposure to carbaryl, i.e., the TWA model does not simulate the effect of the sequence of exposure.

Process-Based Interpretation: Toxicodynamics. The TDM facilitates explanation of how previous exposure to chlorpyrifos leads to increased mortality from subsequent

pulses of carbaryl and why there is no such effect when the sequence is reversed. The damage following exposure to carbaryl (Figure 1c, treatment A) falls below threshold levels on day 8 due to fairly quick recovery processes, whereas the slower recovery for damage caused by chlorpyrifos results in damage levels above the threshold until day 15 when the second pulse starts (treatment B). Hence, our results are explained by the different toxicodynamic characteristics of chlorpyrifos and carbaryl. The difference in their toxicokinetics cannot explain our observations.

Activation of chlorpyrifos consists of desulfuration to yield the oxon, formation of a transient intermediate complex with AChE and subsequent rapid phosphorylation of the enzyme (*21*). The killing rate constant k_k lumps the kinetics of these steps, similarly to the overall AChE inhibition rate constant k_i in Legierse et al. (*21*), but also accounts for the proportionality to the hazard rate (*4*). Inhibition of AChE by chlorpyrifos is sometimes described as irreversible (*21*) because reactivation of the enzyme is very slow (*17*, *21*) and aging by dealkylation leads to an irreversibly inhibited enzyme (*17*, *21*). AChE inhibition by carbaryl does not require activation and aging does not occur. Measured rates of enzyme recovery from inhibition of acetylcholinesterase generally show faster reactivation after inhibition by carbamates (e.g., carbaryl) than that after inhibition by organophosphates (e.g., chlorpyrifos) (*17*). In the TDM the recovery from enzyme inhibition is described by the recovery rate constant k_r, which is smaller for chlorpyrifos (0.169 day^{-1}) than for carbaryl (0.97 day^{-1}). Hence the biochemical evidence, i.e., the different speed of recovery from inhibition of AChE, supports the different toxicodynamics of carbaryl and chlorpyrifos in the TDM (i.e., the different values for k_r).

In some cases, such as the two compounds used in this study, damage is likely to be linked to the same target, e.g., inhibition of AChE. Summing up of individual damage values is clearly well justified and it can be expect that toxicity from sequential pulsed exposure is sensitive to the sequence of exposure if the compounds show different speed of recovery.

The level of inhibited AChE required for effects on organisms is variable (*22*, *23*) which is accommodated in the TDM concept, where the parameters killing rate constant, recovery rate constant, and threshold are estimated by inverse modeling from survival data. The term damage is not directly associated with a specific mechanism of action and its attributed measure of toxic action (e.g., inhibition of acetylcholinesterase). Rather, the term damage is a generic measure for the overall reduction in fitness of the organisms just as the recovery rate constant k_r includes any possible recovery and compensating mechanisms of the organism, not only those on the biochemical level. The TDM is not restricted to toxicants that act through a specific mechanism but is designed to suit different mechanisms of action. The TDM for multiple toxicants (TDM_{mix}) might be applicable to independently acting toxicants as well. Further research is necessary to test whether the sequence of exposure also matters for compounds that act on different target sites.

Implications For Risk Assessment. It is important to establish the duration, following an initial pulse, over which effects from subsequent pulses are still affected by the previous exposure. This applies whether subsequent pulses are from the same or different compounds and the TDM could serve as a tool to establish this duration. When the internal damage has declined sufficiently it will not contribute to the effects of subsequent pulses anymore. If we define that it is sufficient for damage to fall below 5% of its maximum, then the relevant durations would be 3 days following a pulse of pentachlorophenol, 15 days after exposure to carbaryl and 25 days after exposure to chlorpyrifos (*5*).

Aquatic risk assessment could be improved by quantitatively predicting the effects resulting from realistic exposure patterns including sequential pulsed exposure to multiple compounds. Here, two approaches were compared in an independent model test, with the semimechanistic TDM performing best. The experimental results of this study can be predicted by the TDM when parametrized using independent experiments and the simulations reveal how slow recovery from internal damage caused by chlorpyrifos and faster recovery for carbaryl have consequences for sequential exposure to the two different toxicants. This study demonstrates interactions between chemical pulses even after depuration, but more research is necessary to investigate whether similar interactions as well as an effect of the sequence of exposure also exist for other organisms, compounds and exposure patterns.

Acknowledgments

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