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EFFECTS OF EXPOSURE DURATION AND RECOVERY TIME DURING PULSED EXPOSURES

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Abstract—In pulsed toxicant exposures, the concentration, duration, and frequency of pulses can change through time. However, the conventional median lethal concentration (LC50) method cannot adequately predict the effects of pulsed exposure, because it is associated with fixed exposure duration and constant concentration and does not include postexposure (latent) mortality. Many studies that tried to address the effects of pulsed exposure only provided qualitative or semiquantitative predictions. Survival time experiments conducted here quantified the effect of exposure duration on latent mortality, and the effect of recovery time between two pulses on mortality during a second pulse also was examined. This was done by exposing amphipods (*Hyalella azteca*) to two contrasting toxicants, copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP). In the exposure duration experiments, the amphipods were exposed to two toxicant concentrations for three durations. No significant effect of duration on latent mortality was detected within the experimental range; however, duration still may need to be considered under other conditions. In the recovery time experiments, the amphipods were provided four recovery times, and their survivals during the second exposure were modeled. Recovery time had a significant effect on the second-exposure mortality. Given enough time, the amphipods could recover to a state similar to their original toxicant resistance state. The complete recovery time for $CuSO_4$ was fivefold longer than that for NaPCP. It is important to quantify the effects of latent mortality, pulse duration and concentration, and recovery time for pulsed exposures. Survival analysis provides a better way to address these issues than does the conventional LC50 method.

Keywords—Survival analysis Pulsed exposure Latent mortality Amphipod Toxicity

INTRODUCTION

Increasing attention is being paid to pulsed toxicant exposures, which are characteristic of many spills, episodic runoff events, applications of agrochemicals, and industrial releases. Prediction of lethal consequences of pulsed exposures often is difficult, both because the conventional median lethal concentration is derived for an exposure of constant concentration and because mortality data associated with a fixed duration normally are used. It also does not routinely include mortality occurring after the exposure ends (latent mortality) [1]. For these reasons, it may be inadequate for prediction of the effects of pulsed exposure for which concentration, duration, and pulse frequency change through time.

Researchers have tried to address the effects of pulsed exposure in different ways. Some of these methods provide gross predictions of the influence of exposure duration. Sprague [2] described a method to predict the effects of exposure duration in which cumulative mortality and the corresponding exposure duration were plotted on logarithm-probability paper to generate a straight line for each concentration. In one pioneering investigation of both the duration and latent effects [3], *Gammarus pulex* were exposed to a range of Lindane concentrations for various periods of time and transferred to clean water. The toxicity curves were generated for concentration versus median survival time and for concentration versus the duration of exposure required to cause the eventual death of half the animals. The Acute-to-Chronic Estimation software [4] applies three methods (accelerated life testing, multifactor probit analysis, and linear regression analysis) to predict long-term toxicity based on data from short-term experiments. A moving window technique was used to approximate the 4- and 21-d average effect concentrations of atrazine [5]. Laboratory studies also have explored the latent effects after a single pulse [3,6–8], effects of pulse interval and frequency [9], and effects of different toxicants [10]. These studies provide semiquantitative predictions. Quantitative models are needed to better predict lethal consequences of pulsed exposures.

Our previous study [1] applied the methods of survival analysis to include both exposure duration and concentration in predictive models. We emphasized that it is important to address latent mortality, and the effects of pulse concentration and toxicant modes of action on latent mortality, to better predict the fate of field populations. In addition, other variables associated with a pulsed exposure, such as pulse duration and recovery time between pulses, can be important. Pulse duration could affect latent mortality, and recovery time could influence how an organism will respond to a subsequent exposure. Low latent mortality and long recovery time may result in better recovery; therefore, the effects of subsequent pulses will be less dependent on the previous exposure. To explore these issues, we conducted laboratory studies that quantified the influence of a subset of these variables. Specifically, we asked the following question: First, is there any effect of exposure duration on latent mortality? Second, can the complete recovery time (i.e., the time that an organism needs to return to its original level of toxicant resistance) be predicted? Third, what is the relationship between recovery time between pulses and the mortality during the second pulse? Copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) were used as model toxicants, because they have contrasting levels of latent mortality [1]. To address the first question, the amphipod *Hyalella azteca* was exposed to two toxicant concentrations for three

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durations. The effect of exposure duration on latent mortality was quantified with survival modeling. For the second and third questions, the amphipods were provided four different recovery times between two pulses of the same concentration and duration. Time-to-death data collected during the second exposure were modeled, the complete recovery times estimated, and the effect of recovery time quantified.

MATERIALS AND METHODS

Amphipod culture, maintenance, and general exposure design

The amphipods (*H. azteca*) came from a laboratory culture that had never experienced contaminant exposure. Well water was used as the culturing water and red maple (*Acer rubrum*) leaves as food. Test amphipods were one to two weeks old and ranged in length from 0.50 to 0.67 mm. They were maintained in reformulated, moderately hard reconstituted water (RMHRW) [11] with food at 23°C for 6 d before exposures began. During all exposures, amphipods were randomly assigned to a well of 12-well Costar 3513 Cell Culture Clusters (Corning, Corning, NY, USA) containing approximately 4 ml of toxicant solution. An amphipod was scored as dead if no appendage movement was discernible after repeated gentle proddings. All amphipods still alive at the end of the experiments were scored as right-censored (i.e., instead of the exact times-to-death, the only information known about them was that their times-to-death were beyond the time when the experiments ended). In each experiment, 36 amphipods were used as control animals and maintained in toxicant-free water. They were transferred to appropriate wells whenever treatment amphipods were transferred to ensure that the difference was not caused by excessive handling.

Effect of exposure duration on latent mortality

Amphipod exposure. The amphipods were exposed to two concentrations for three different durations for each toxicant (Table 1). Each well contained a piece of red maple leaf as food (weight, 0.6 ± 0.3 mg [mean ± SD] $n = 50$). After exposures, survivors were transferred to fresh RMHRW, and their latent mortality was observed every 3 to 6 h. The experiments ended when no more mortality was evident. All experiments were done twice.

Statistical analysis. Survival analysis, which recently was applied to environmental risk assessment and ecotoxicology [12,13], was used to analyze these data. The survival time approach involves exposing animals to a toxicant and then monitoring mortality through time. Here, a nonparametric log-rank hypothesis test initially was used to check for any significant difference between the survivals in the duplicates (Statistical Analysis Software [SAS] procedure LIFETEST; SAS Institute, Cary, NC, USA) [14]. The fully parametric, accelerated failure time method was then used to explore the effects of exposure duration. Exposure concentration and duration were fit as continuous variables (SAS procedure LIFEREG) [14].

Effect of recovery time on second-exposure mortality

Amphipod exposure. Approximately 350 amphipods were exposed to one nominal toxicant concentration for 12 h (Table 1). No food was provided during the exposures. Based on pilot studies, survivors were randomly assigned to four recovery time groups and allowed to recover to different degrees (Table 1). Red maple leaves (weight, 0.6 ± 0.3 mg; $n = 50$) were provided during recovery. After that, the survivors were exposed to toxicant solution with the same nominal concentration as the previous pulse for 12 h. The mortality was noted every half-hour or so. At the beginning and end of each experiment, two groups of naive amphipods of the same age as those in the treatment groups were established as reference animals and exposed to the same nominal toxicant concentration for 12 h to establish the background mortality. Because the mortality under identical exposure conditions could be different as amphipods age, these reference groups were used to check the potential confounding effect of age. Each experiment was repeated three times.

Table 1. Experimental design used to study effects of exposure duration (ED) and recovery time (RT) for copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP)

Toxicant	Concentration (mg/L)	Duration (h)	Pulse interval (h)
ED			
Dissolved Cu	0.8	20	—
	0.8	38	—
	0.8	61	—
	1.0	20	—
	1.0	38	—
	1.0	61	—
NaPCP	0.4	20	—
	0.4	40	—
	0.4	60	—
	0.6	20	—
	0.6	40	—
	0.6	60	—
RT			
Dissolved Cu	1.1	2 × 12	0
	1.1	2 × 12	24
	1.1	2 × 12	48
	1.1	2 × 12	72
NaPCP	1.5	2 × 12	0
	1.5	2 × 12	4
	1.5	2 × 12	8
	1.5	2 × 12	14

Statistical analysis. In each experiment, the time-to-death data of the two reference groups were compared with a log-rank test to check for any significant difference in mortality (SAS procedure LIFETEST) [14]. If no significant difference was found, the reference data were fit to the accelerated failure time model (SAS procedure LIFEREG) [14]:

$$\ln t_{Ri} = a_R + \varepsilon_{Ri} \quad (1)$$

where t_{Ri} is the time-to-death of the reference animals, a_R is the intercept, and ε_{Ri} is the error term, which equals σL for prediction, where σ is estimated in the model and L varies with the proportion dead for which a prediction is being made and can be obtained from Appendix 7 of Newman [15]. Next, the time-to-death data of amphipods with different recovery times were fit to the following model to quantify the effect of recovery time:

$$\ln t_{Ti} = a_T + b_T \times RT_i + \varepsilon_{Ti} \quad (2)$$

where t_{Ti} is the time-to-death of the treatment animals, a_T is the intercept, b_T is an estimated coefficient, RT_i is the recovery time, and ε_{Ti} is the error term. The t_{Ri} or t_{Ti} can be fit to a Weibull, exponential, log-logistic, or log-normal distribution; therefore, Akaike's information criterion (AIC) was used to select the best of these four candidate distributions. The pre-

Table 2. The pH values, dissolved oxygen (DO) concentrations, and water temperatures of the copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) exposure media for the exposure duration (ED) and recovery time (RT) experiments

Experiment	pH (median, range, *n*)	DO (mg/L, *n*)[a]	Water temp. (°C, *n*)[a]
ED			
$CuSO_4$	8.10, 7.95–8.21, 24	7.4 ± 0.5, 10	22.9 ± 0.7, 10
NaPCP	8.11, 8.02–8.28, 48	7.7 ± 0.1, 20	23.3 ± 0.2, 20
RT			
$CuSO_4$	7.94, 7.51–8.13, 60	7.5 ± 0.1, 30	23.1 ± 0.4, 30
NaPCP	8.14, 8.09–8.29, 60	7.7 ± 0.1, 30	23.2 ± 0.2, 30

[a] Values are presented as the mean ± standard deviation.

dicted complete recovery time, which is the value of RT_i at which, mathematically, the survival time of the treatment group equaled to that of the reference group at the specific reference mortality level, could be calculated by subtracting Equation 1 from Equation 2. The effect of recovery time can be statistically tested for significance with the coefficient b_T in Equation 2.

Water chemistry

The total alkalinity and pH of RMHRW were measured before exposures started to ensure that they were within the anticipated ranges. The solutions were renewed during the experiments every 12 to 20 h. Both newly prepared and exposed water samples were collected periodically for measurements of pH and toxicant concentration. The pH values were measured with an Accumet Model-15 pH Meter (Denver Instrument, Denver, CO, USA) and PerpHect ROSS Electrode Model 8256 (Orion Research, Boston, MA, USA). Water samples for dissolved copper measurement were acidified with Teflon®-distilled hydrochloric acid to pH less than 2, stored in glass bottles at 4°C, and analyzed with a Perkin-Elmer AAnalyst 800 atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT, USA). Samples for NaPCP analysis were collected in glass bottles, stored at 4°C, and analyzed with the method described by Carr et al. [16]. Each 25-ml water sample was mixed with 25 ml of deionized water and 0.5 ml of concentrated HCl. Ten milliliters of chloroform was added before the sample was shaken vigorously for 60 s. Five milliliters of the extract were collected in a polypropylene centrifuge tube. Two milliliters of 0.2 M NaOH were added to the extract, mixed vigorously for approximately 30 s, and centrifuged in an IEC HN-SII Centrifuge (International Equipment, Needham Heights, MA, USA) at 5,000 *g* for 5 min. The absorbance of the aqueous fractions was measured with a Beckman DU 650 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) at 320 nm. The temperature and dissolved oxygen were measured periodically with a Fisher Ever Read mercury thermometer (Fisher Scientific, Dubuque, IA, USA) and YSI Model 57 oxygen meter (YSI, Yellow Springs, OH, USA), respectively.

RESULTS

Water chemistry

The RMHRW for all solutions had an alkalinity of 56 ± 2 mg/L as $CaCO_3$ (mean ± standard deviation, $n = 8$) and median pH 8.18 (range, 7.82–8.23; $n = 14$). The pH values, dissolved oxygen concentrations, and water temperatures during the experiments are summarized in Table 2. Table 3 summarizes the measured dissolved copper and NaPCP concentrations for all the experiments. The toxicant concentrations for controls and water during the postexposure and recovery periods were less than the method detection limits (dissolved copper, 7 μg/L; NaPCP, 0.15 mg/L).

Effect of exposure duration on latent mortality

Higher concentration and longer duration resulted in higher mortality during all exposures. The log-rank test showed no significant difference between the latent mortalities of the duplicates for both toxicants ($\alpha = 0.05$); consequently, the data were pooled and the percentage latent mortalities plotted against time (Fig. 1). The latent mortalities of $CuSO_4$ (20–60% depending on concentration) were roughly one order of magnitude higher than those of NaPCP (<7%) and continued for a relatively long time. When the latent mortality data were fit to survival models, natural log transformations of duration and concentration were used (Table 4). For both toxicants, a log-normal distribution proved to be the best. Among all the coefficients, only that of ln(concentration) of $CuSO_4$ was sig-

Table 3. The dissolved copper and sodium pentachlorophenol (NaPCP) concentrations for the exposure duration (ED) and recovery time (RT) experiments[a]

Experiment		Toxicant concentration (mg/L)				
		Concentration 1	Concentration 2			
ED						
Dissolved Cu ($n = 8$)	1	0.66 ± 0.07	0.82 ± 0.12			
	2	0.63 ± 0.06	0.83 ± 0.08			
NaPCP ($n = 15$)	1	0.36 ± 0.06	0.62 ± 0.07			
	2	0.43 ± 0.01	0.68 ± 0.04			
		1st	2nd (1)	2nd (2)	2nd (3)	2nd (4)
RT						
Dissolved Cu ($n = 4$)	1	0.94 ± 0.12	0.93 ± 0.10	0.92 ± 0.05	0.89 ± 0.04	0.87 ± 0.03
	2	0.99 ± 0.10	0.99 ± 0.12	1.01 ± 0.07	1.02 ± 0.09	1.00 ± 0.09
	3	1.01 ± 0.09	1.02 ± 0.10	1.02 ± 0.11	1.05 ± 0.12	1.04 ± 0.13
NaPCP ($n = 12$)	1	1.54 ± 0.05	1.47 ± 0.04	1.45 ± 0.06	1.45 ± 0.02	1.45 ± 0.03
	2	1.51 ± 0.07	1.43 ± 0.05	1.49 ± 0.05	1.46 ± 0.02	1.47 ± 0.04
	3	1.39 ± 0.05	1.43 ± 0.02	1.48 ± 0.04	1.48 ± 0.09	1.41 ± 0.03

[a] Values are presented as the mean ± standard deviation.

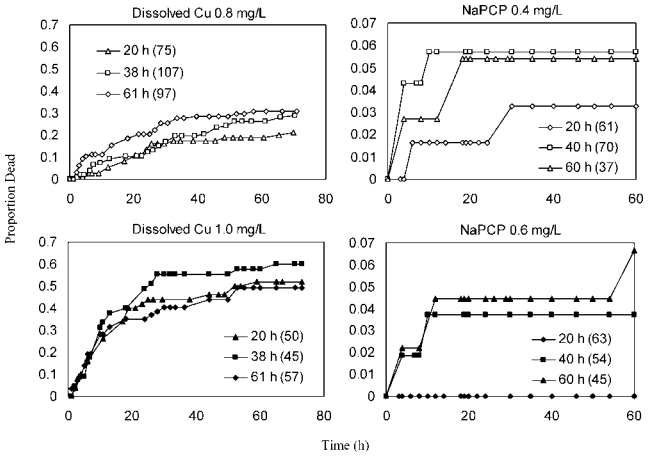

Fig. 1. Cumulative proportions of amphipods dead through time after the exposures in the copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) exposure duration experiments. The groups of lines indicate different exposure durations. The concentrations shown are nominal toxicant concentrations (for measured concentrations, see Table 3). The sample sizes are indicated in the brackets.

nificantly different from zero ($\alpha = 0.05$). The control mortalities were less than 5% in all experiments.

Effect of recovery time on second-exposure mortality

The log-rank test showed no significant difference in survival between the two reference groups of each experiment ($\alpha = 0.05$). Therefore, any age effect on mortality was judged to be insignificant, and the data of the reference groups were pooled for use as the background mortality of the exposure. The average background mortalities were 7, 11, and 12% for three $CuSO_4$ experiments and 18, 15, and 5% for three NaPCP experiments. The percentage mortalities during the second exposures are plotted in Figure 2. Recovery through time of the ability to resist the lethal effects of a second exposure was apparent for both toxicants. Longer recovery time resulted in less difference between the treatment and reference mortalities. Statistically, the mortalities with the shortest and second-shortest recovery times were significantly different from those of

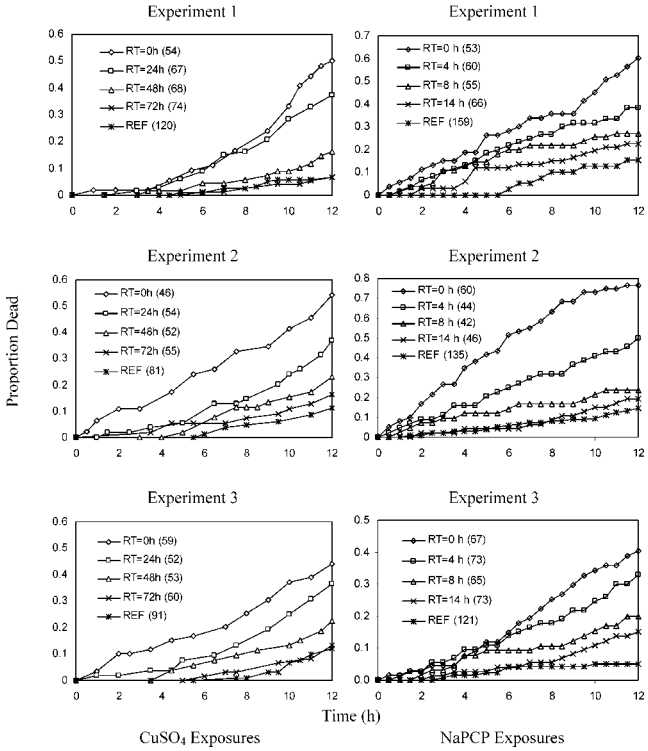

Fig. 2. Cumulative proportions of amphipods dead through time for the three copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) recovery time experiments. The groups of lines indicate treatments with different recovery times (RT) and reference (REF) groups. The sample sizes are indicated in the brackets.

the references, but those with the longest recovery times were not ($\alpha = 0.05$, log-rank test; one exception was the 14-h recovery time group in the third NaPCP experiment, in which $p = 0.02$). Weibull proved to be the best-fit model for the treatment groups of all experiments. The best-fit models were log-normal for the reference groups, except that Weibull has the lowest AIC for the second NaPCP experiment. Because the difference between the AIC values for the log-normal (135.7) and Weibull (135.3) models was small, we used a log-

Table 4. The best-fit models and the associated distributions for the latent periods of exposure duration (ED) experiments and for the treatment (TRT) and reference (REF) groups of recovery time (RT) experiments for copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP)[a]

Experiment			Model[b]	Distribution
ED				
$CuSO_4$			$\ln T = 4.65 - 5.83 \cdot \ln C - \mathit{0.46} \cdot \ln D + 2.13 \cdot L$	Log-normal
NaPCP			$\ln T = 22.26 + \mathit{1.87} \cdot \ln C - \mathit{2.32} \cdot \ln D + 4.73 \cdot L$	Log-normal
RT				
$CuSO_4$	1	TRT	$\ln T = 2.58 + 0.01 \cdot \mathrm{RT} + 0.35 \cdot L$	Weibull (81 h)
		REF	$\ln T = 3.73 + 0.83 \cdot L$	Log-normal
	2	TRT	$\ln T = 2.62 + 0.01 \cdot \mathrm{RT} + 0.51 \cdot L$	Weibull (87 h)
		REF	$\ln T = 3.21 + 0.59 \cdot L$	Log-normal
	3	TRT	$\ln T = 2.73 + 0.01 \cdot \mathrm{RT} + 0.50 \cdot L$	Weibull (81 h)
		REF	$\ln T = 2.88 + 0.34 \cdot L$	Log-normal
NaPCP	1	TRT	$\ln T = 2.67 + 0.07 \cdot \mathrm{RT} + 0.78 \cdot L$	Weibull (15 h)
		REF	$\ln T = 3.40 + 0.99 \cdot L$	Log-normal
	2	TRT	$\ln T = 2.17 + 0.13 \cdot \mathrm{RT} + 0.77 \cdot L$	Weibull (13 h)
		REF	$\ln T = 3.77 + 1.22 \cdot L$	Log-normal
	3	TRT	$\ln T = 2.88 + 0.05 \cdot \mathrm{RT} + 0.61 \cdot L$	Weibull (26 h)
		REF	$\ln T = 5.05 + 1.56 \cdot L$	Log-normal

[a] The calculated complete recovery time for each RT experiment is shown in the bracket following the distribution. Coefficients in italic denote that they were not significantly different from zero ($\alpha = 0.05$).

[b] T = time-to-death; C = concentration; D = duration; L varies with the proportion dead for which prediction is being made.

normal distribution as well (Table 4). The coefficients of recovery time were significantly different from zero ($\alpha = 0.05$). The three complete recovery times for each toxicant (Table 4) were then averaged to calculate the mean complete recovery time and the associated standard deviation ($CuSO_4$, 83 ± 3 h; NaPCP, 18 ± 7 h). Control mortalities were less than 5% in all experiments.

DISCUSSION

Effect of duration on latent mortality

The pattern of latent mortality is notionally related to the amount of damage caused during exposure, the length of time the chemical stays in the organism, and the extent of recovery of the organism after the exposure ends. The recovery of the exposed organism can be quick for toxicants such as NaPCP, which appears to cause less cumulative damage than $CuSO_4$. The toxicological mode of action of pentachlorophenol (PCP) is increased cellular oxidative metabolism resulting from the uncoupling of oxidative phosphorylation. It is conjugated directly by phase II reactions. Nuutinen et al. [17] quantified the *H. azteca* uptake, biotransformation, and elimination rates of PCP and found relatively short half-lives of 3.6 and 9.1 h for PCP and its metabolite, respectively. When removed from the environment, the toxicant effect is reversible, and the cumulative damage after exposure seems not to be as prominent as that of copper. Therefore, the latent mortality can be low and relatively independent of exposure concentration and duration. The results of NaPCP experiments demonstrated this point: Minimal latent mortality occurred, and neither concentration nor duration had any significant effect. In contrast, the recovery of the exposed organism can be slow for toxicants such as $CuSO_4$ that notionally cause significant cumulative damage. Copper inhibits Na^+/K^+–adenosine triphosphatase activity and induces cell necrosis and apoptosis [18]. The prevalence of lesions depends on the chemical concentration and exposure duration, and the tissue recovery depends on the severity of the damage and the environmental conditions [19]. Copper also bonds between heterocyclic bases of DNA, competes with the normal hydrogen binding, and destabilizes the DNA structure [20]. Because of this pervasive damage during copper exposure, organisms may need relatively long periods of time to recover. The latent mortality can be high and related to the cumulative damage, which is a function of both concentration and duration. (See Zhao and Newman [1] for detailed discussion of concentration effects on latent mortality and the toxicological mechanisms for $CuSO_4$ and NaPCP.) Pascoe and Shazili [21] found that longer cadmium exposure duration resulted in higher mortality after the exposure. Latent mortality of beetles increased as the original diatomaceous earth exposure interval increased [8]. The results of the $CuSO_4$ experiments showed a significant effect of concentration, with higher concentration resulting in higher latent mortality, and were consistent with those of our previous study [1]. However, the effect of exposure duration was not significant. One explanation is that although both concentration and duration affect latent mortality, duration plays a less prominent role than does concentration under the test conditions used here. Naddy et al. [22] found that the magnitude of concentration was a greater determining factor of chlorpyrifos toxicity to daphnids than was exposure duration. The explanation can be extended that the exposure duration–latent mortality curve is curvilinear. A critical duration exists beyond which the effect could be more manifest, whereas the selected durations fell in the range that the increase of exposure duration only resulted in a statistically insignificant increase of latent mortality. Therefore, the duration effect probably can be ignored within this experimental range. Regardless, we speculate that the effects of exposure duration on latent mortality would still need to be considered seriously under other concentrations and durations.

Effect of recovery time on second-exposure mortality

Under some circumstances, provided enough time is available between pulses, the organisms can recover from the previous pulse through processes such as detoxification, elimination, and/or healing of damaged tissues. Wang and Hanson [23] suggested that the pulsed-exposure toxicity was dependent on the time between pulses. Studies [9,23–26] showed that with the same total exposure duration and enough recovery time, the multiple-pulse exposures were less toxic than continuous ones. However, in some cases, the effects were irreversible, and recovery did not occur [6,9,27]. To explain this, Naddy and Klaine [9] suggested that the organisms accumulated an amount of toxicant that exceeded their critical body burden, or that the recovery period was not long enough for the organisms to recover from the previous exposure. In the present study, the mortalities of the groups with longest recovery times for both toxicants were not significantly different from the reference groups. This indicated that, provided sufficient recovery time is available between pulses, the amphipods could return to a state similar to their original toxicant resistance state (i.e., similar cumulative mortality at the end of the exposure and similar times-to-death during the exposure compared to the controls). Statistical analysis showed an insignificant difference between reference group mortalities of the two toxicants (MINITAB® [28], analysis of variance, $F = 0.40$, $p = 0.56$). However, to recover to similar background mortality levels (~10%), the complete recovery time for $CuSO_4$ was almost fivefold longer than that of NaPCP, suggesting a toxicant-dependent recovery process. A longer recovery time is needed for toxicants such as $CuSO_4$ that cause cumulative tissue damage and have significant latent effects. Although only mortality was measured in the experiments, sublethal effects after exposures also might be important. As with lethal effects, the pattern of sublethal effects is notionally a function of the cumulative damage, the depuration rate, and the extent of recovery, and it is toxicant-dependent as well. Reynaldi and Liess [29] found transient sublethal effects of fenvalerate in the 24-h pulse exposure of *Daphnia magna* Straus. After 21 d, the total neonates per female and population growth rate recovered to values close to those of the controls. Also, because many sublethal effects are more sensitive endpoints than lethal effects, the organisms may need a longer complete recovery time to go back to the original toxicant resistance state, or the effects could be nonreversible.

Although the issues regarding pulsed exposure have been addressed in several studies, none of them has fully quantified the effect of recovery time. With the accelerated failure time models in Table 4, one can predict the effect of recovery time on time-to-death during the second exposure. Taking the first NaPCP experiment as an example, the coefficient of 0.07 for recovery time indicates that for a certain proportion of organisms to die during the second exposure, an increase of the recovery time by 1 h will result in an increase of 1.07-fold in time-to-death. Figure 3 shows that for any recovery time within the experimental range, the time-to-death can be predicted for a certain proportion of animals dying during the second ex-

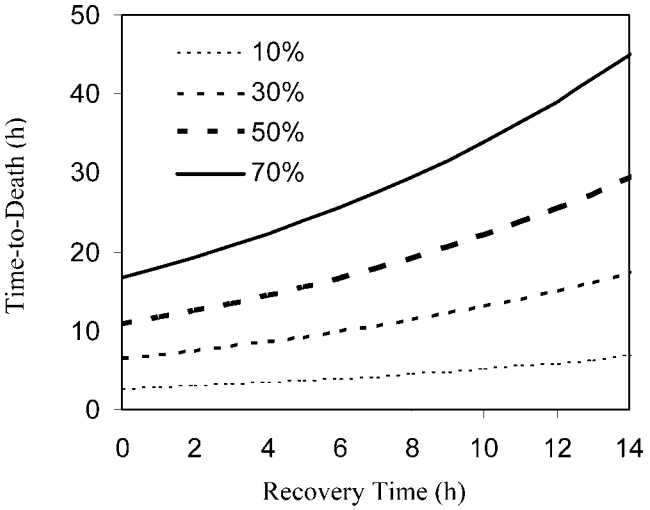

Fig. 3. The time-to-death for a certain proportion of animals to die during the second exposure for any recovery time within the experimental range.

posure. Figure 4 shows background time-to-death of the reference group and the observed and predicted times-to-death of the treatment groups. For the specific background level mortality, the times-to-death during the second exposure increase with the recovery time, converge with that of the reference group at the complete recovery time, and remain constant thereafter. If needed, variables such as concentration can

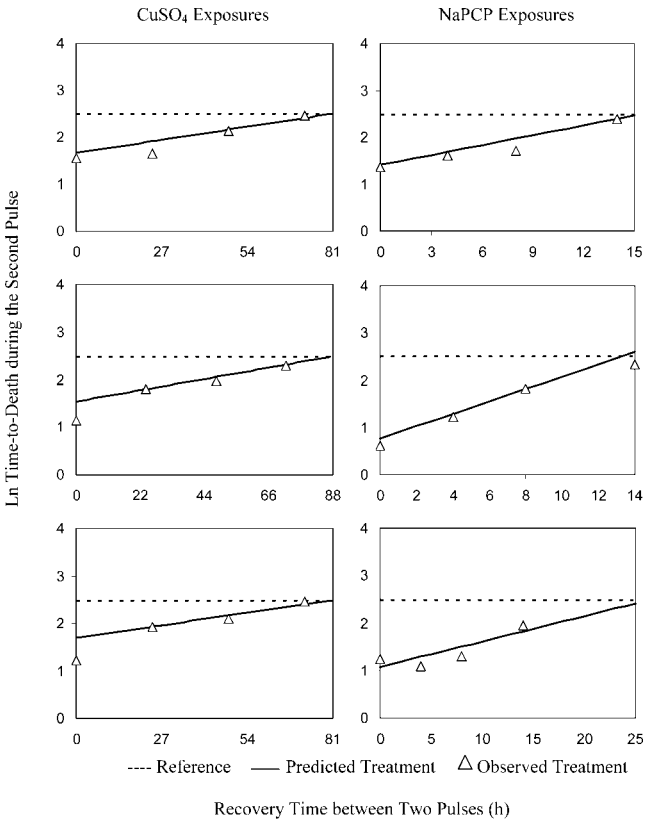

Fig. 4. The observed and predicted natural log (ln) times-to-death of the different recovery time groups, and the ln(times-to-death) of the reference groups for the copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) recovery time experiments. The points at which the two lines converge are the predicted complete recovery times.

be added to the model. We realize that some tolerance mechanism could have been induced during the first exposure, and acclimation could have played roles in the process [30,31]. Thus, the mortality of the completely recovered animals could have been less than that of the reference groups. The survival analysis method would be applicable to this case as well.

Importance of incorporating pulsed-exposure scenarios into current toxicity tests

The need to include latent mortality and exposure duration in current ecotoxicology studies has been discussed previously [1]. The results of the present study reinforce the points that latent lethal effect can be important and that more attention should be paid to assessing the effects of variables affecting latent mortality. Relatively short times are needed for the organisms to recover to background levels of resistance for NaPCP and toxicants that display a narcotic mode of action. Much longer times are required for $CuSO_4$ and similar toxicants. Thus, under similar exposure scenarios, the $CuSO_4$-exposed field population would be more likely to experience local extinction. Although pulsed exposures most often are associated with mortality, sublethal effects (e.g., effects on growth, reproduction, and development) also can be important when considering latent effects. A significant decrease in growth rates may [32] or may not [33,34] occur after short exposures. Handy [35] suggested that growth rates may not be reliable to indicate latent effect, because reproductive failures, such as reduced egg production and hatching success [33,36,37], are more sensitive endpoints. It also is reported that even very brief exposures to pesticides (i.e., 1 h) can have significant effects on development [38,39]. These sublethal latent effects might explain the decline in aquatic populations without apparent reason. Also, caution should be taken when extrapolating the current experimental results to much more intensive exposures of both toxicants, because under higher concentrations, the toxicological modes of action could change.

The episodic nature of the contaminants in aquatic ecosystems warrants the incorporation of pulsed-exposure scenarios into the current ecotoxicology framework. The nature of the toxicant, recovery times between pulses, previous exposure concentration, and exposure duration can affect the fate of organisms during subsequent exposures. It is important to characterize further the effects of these factors to better assess the consequences of pulsed exposure. By incorporating parameters such as duration, concentration, characteristics of organisms, and experimental conditions into the predictive models, survival analysis is a better approach than conventional toxicity tests to address these issues.

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