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THE THEORY UNDERLYING DOSE–RESPONSE MODELS INFLUENCES PREDICTIONS FOR INTERMITTENT EXPOSURES

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Abstract—Prediction of pulsed or intermittent exposure effects on populations is emerging as an important issue in ecotoxicology. However, the underlying theory of the dose–response models has not been tested rigorously enough to provide a true understanding for predicting the effects of pulsed exposures. Since its introduction, the individual effective dose (IED) theory has remained the dominant explanation in the literature. The present study tested whether the IED theory is the dominant explanation for the probit model (or similar models) for both copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP). Three groups of amphipods (*Hyalella azteca*) were first exposed to lethal, sublethal, and zero concentrations of each toxicant. After recovery, all survivors were exposed to lethal concentrations, and their mortalities during a second exposure were compared. Under the specific concentrations and durations used, stochastic processes were dominant for $CuSO_4$. Both stochasticity and IED appeared to be relevant for NaPCP, but stochasticity dominated the dynamics. Current ecotoxicology tests should include assessment of the underlying dynamics as one of the first steps of studying pulsed or intermittent exposure effects.

Keywords—Intermittent exposure Stochastic Individual effective dose Toxicity Survival analysis

INTRODUCTION

Traditionally, toxicity testing has focused on exposure to fixed concentrations of a single toxicant for a predetermined duration. In recent years, however, more attention has been paid to pulsed or intermittent exposure scenarios, which are typical of many spills, episodic runoff events, periodic agrochemical applications, and industrial releases. Aquatic organisms in the field often are exposed for long periods of time to background levels of toxicants, with lethal concentrations being reached only periodically. Consequently, effect prediction for intermittent exposures on populations is emerging as an important issue to be resolved.

Several features of pulsed exposure effects have been studied. The effect of exposure duration has been incorporated into predictive models [1–3]. Latent effects occurring after exposure stops also have been quantified [4–7]. Effects of pulse concentration, interpulse interval, frequency, and other factors of various toxicants on different species have begun to be studied [3,8–12]. The effects of tolerance induction from preexposure on effects during subsequent pulses have been addressed [13], as have sublethal effects during multiple pulses [14–16]. With the exception of Newman and McCloskey [6], none of the aforementioned studies attempted to clarify a key issue underlying the survival distributions that is critical in predicting population fate during pulsed exposures.

In toxicity testing, dose–response curves often are sigmoidal, and the probit approach (i.e., log-normal dose–response model) is most widely applied to fitting such data. Gaddum [17] postulated the individual effective dose (IED) theory to explain this log-normal model. According to this theory, every individual has a characteristic tolerance of a toxicant, and an individual will be dying only if its IED is exceeded. Furthermore, the IED notionally is log-normally distributed within populations. Early but unsuccessful supporters of the alternative log-logistic model questioned the IED concept, favoring a stochastic explanation instead. Notably, Berkson [18] performed a simple experiment, the result of which did not support the IED concept. He pointed out that individuals may vary from time to time in their response to chemicals and explained dose–response curves in stochastic terms.

Although the log-normal and similar models are applied pervasively to fit conventional dose–response curves, minimal testing of the generally accepted IED concept has been conducted. The IED concept remains the only explanation presented in most publications [19–21]. Yet, determination of the underlying mechanism is important in predicting consequences of pulsed exposures, as explained by Newman and McCloskey [6]. Suppose that a population were exposed to a toxicant periodically with concentrations equal to its 96-h median lethal concentration for durations of 96 h, with sufficient recovery times between pulses. The prediction from the stochastic theory is that every pulse would result in 50% mortality and, thus, that the population size would drop by 100% to 50% to 25% to 12.5%, and so on, of the original size during a series of such pulses. On the other hand, according to the IED theory, the animals with lower IEDs will be culled away during the first pulse, and all the survivors will have higher IEDs. Therefore, only a few individuals (or none) would die during subsequent pulses. In this case, the population size would drop by 100% to 50% to 50% to 50%, and so on. The IED theory therefore predicts that the population will persist much longer under a pulsed exposure scenario than the stochastic theory predicts.

We tested whether the IED theory was the dominant explanation for the survival distribution models of two toxicants, copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP). Amphipods (*Hyalella azteca*) were first exposed to lethal, sublethal, and zero concentrations of each toxicant so that few animals died in the sublethal and reference groups

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Table 1. The pH values, dissolved oxygen (DO) concentrations, and water temperatures of the copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) exposure media

	$CuSO_4$		NaPCP	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
pH[a]	8.03, 7.89–8.13, 12	7.93, 7.90–8.14, 12	8.17, 8.14–8.21, 16	8.16, 8.13–8.19, 16
DO (mg/L)[b]	7.6 ± 0.2	7.6 ± 0.3	7.5 ± 0.1	7.5 ± 0.2
Water temperature (°C)[b]	23.4 ± 0.2	23.1 ± 0.3	23.7 ± 0.7	23.2 ± 0.2

[a] The pH values are presented as the median, range, and *n*.
[b] Values are presented as the mean ± standard deviation ($n = 10$).

and significant numbers of animals died in the lethal group. Amphipods were given enough recovery time between the two exposures so that the previous exposures had no obvious effect on the animals' feeding or behavior. Then, all the survivors were challenged again with lethal concentrations, and their mortalities were compared using survival analysis methods. According to the IED theory, the IED is an intrinsic characteristic of an individual. If no induction of tolerance occurs, then the cumulative mortality curves of the sublethal and reference groups should be similar during the second exposure, because they reflect essentially the same population as the original one. For the lethal group, all the surviving animals from the first exposure would have relatively higher IEDs than those that had died. So, from the beginning of the second exposure to a certain time point, only a few of the animals would die, because the sensitive individuals had been eliminated. After that, more and more IEDs would be exceeded with time, and the animals would begin to die with higher rates. In contrast, the stochastic theory predicts no difference among the three curves, because the surviving amphipods of the lethal group would have no difference in resistance to the toxicant from those of the sublethal and reference groups. The results were interpreted in terms of the relative dominance of the two theories and the associated relevance to pulsed exposure issues. Although a log-normal survival distribution is assumed here, this reasoning is relevant to other distributions as well.

MATERIALS AND METHODS

Experimental design

The amphipods came from a laboratory population that had never experienced contaminant exposure. Well water was used as the culturing water, and red maple (*Acer rubrum*) leaves were used as food. Test amphipods were one to two weeks old (body length, 0.50–0.67 mm) and acclimated in reformulated, moderately hard reconstituted water [22] at 23°C for 6 d before exposures began.

Amphipods were haphazardly assigned to one of three treatments: Lethal ($n = 120$), sublethal ($n = 60$), and reference ($n = 60$). Nominal concentrations = 1.0, 0.4, and 0.0 mg/L of dissolved Cu, or 1.4, 0.4, and 0.0 mg/L of NaPCP. The exposure containers were 12-well Costar 3513 Cell Culture Clusters (Corning, NY, USA), with one amphipod and approximately 4 ml of toxicant solution per well. An amphipod was scored as dead if no appendage movement was discernible after repeated, gentle prodding. Because of the anticipated high and minimal latent (postexposure) mortalities caused by $CuSO_4$ and NaPCP, respectively, the amphipods were exposed until approximately 15 and 40% of them, respectively, had died in the lethal groups for the two toxicants. All survivors in the three treatments were then transferred to toxicant-free water and maintained for periods of time ($CuSO_4$, 14 d; NaPCP, 10 d) sufficient to allow apparent recovery from the first exposures. Red maple leaves were provided as food during the recovery periods. After that, all the survivors were transferred back to the toxicant solutions, with nominal concentrations identical to those of the first lethal exposures. The times-to-death during the second exposures were noted approximately every 1 h until the cumulative mortality approached 100%. Thirty-six amphipods were used as controls and maintained in toxicant-free water. These amphipods were transferred to appropriate wells any time treatment amphipods were transferred to ensure that the difference in mortalities was not the result of handling. The experiments were repeated twice for both toxicants. (The second experiment was performed after the first had been completed.)

Water chemistry

For the $CuSO_4$ experiments, the solutions were renewed every 12 h to compensate for any Cu loss as a result of sorption. The NaPCP solutions were not renewed, because preliminary experiments showed minimal loss of NaPCP. Both newly prepared and exposed water samples were collected for toxicant concentration and pH measurements. Water samples for dissolved Cu measurement were acidified to pH <2, stored in glass bottles at 4°C, and analyzed with an AAnalyst 800 atomic absorption spectrometer (PerkinElmer, Norwalk, CT, USA). Samples for NaPCP analysis were stored at 4°C and analyzed according to the method described by Carr et al. [23]. Temperature and dissolved oxygen also were measured periodically.

Data analysis

The mortality data from the second lethal exposures for the three treatments were plotted against time and checked visually for differences. The data also were formally analyzed using survival analysis methods (nonparametric product limit method, SAS® Procedure LIFETEST [24]). A log-rank test was used to determine whether any significant difference ($\alpha = 0.05$) existed among the three mortality curves. This method does not require the assumption of a specific underlying distribution for survival.

RESULTS

Water quality and the measured toxicant concentrations are summarized in Tables 1 and 2. During the first exposures for the two $CuSO_4$ experiments (terminated after 13.5 and 13 h), no animals died in the reference and sublethal treatments, but 12 and 10% mortality occurred in the lethal treatments. During the recovery periods, the additional mortality was 39% (51% total) and 32% (42% total) in the lethal, 21 and 7% in the sublethal, and 2 and 5% in the reference treatments. During

Table 2. Concentrations of dissolved Cu and sodium pentachlorophenol (NaPCP) during the sublethal, first lethal, and second lethal exposures[a]

		Toxicant concentrations (mg/L [*n*])		
	Experiment	Sublethal	Lethal (first)	Lethal (second)
Dissolved Cu	1	0.36 ± 0.02 (2)	0.88 ± 0.06 (2)	0.95 ± 0.04 (12)
	2	0.34 ± 0.01 (2)	0.84 ± 0.01 (2)	0.87 ± 0.05 (12)
NaPCP	1	0.32 ± 0.01 (2)	1.36 ± 0.01 (2)	1.39 ± 0.09 (2)
	2	0.40 ± 0.12 (2)	1.37 ± 0.06 (2)	1.49 ± 0.10 (2)

[a] The toxicant concentrations of the reference groups were all less than the detection limits of the methods (dissolved Cu, 7 μg/L; NaPCP, 0.15 mg/L).

the first exposures for the two NaPCP experiments (terminated after 10 and 12 h), no animals died in the reference and sublethal treatments, but 39 and 42% of animals died in the lethal treatments. During the recovery periods, the additional mortalities were 7% (46% total) and 10% (52% total) in the lethal, 2 and 2% in the sublethal, and 3 and 2% in the reference treatments. These results are consistent with our previous findings that Cu, but not NaPCP, can cause high levels of latent mortality [7]. Less than 5% of control amphipods died in all the experiments.

The plots of cumulative proportion dying during the second exposures (Fig. 1) showed that the shapes and trends of the curves of the three treatments were similar to one another for the two $CuSO_4$ experiments. Statistical analysis of log-rank test results also showed no significant difference in either experiment ($p = 0.26$ and 0.33). For the two NaPCP experiments, the reference and sublethal curves were similar. Initially, the lethal curves gradually diverged from the other two, indicating lower mortality rates. The lethal treatment mortality increased after approximately 15 h of exposure, and the three curves gradually converged at approximately 90% mortality by the end of the exposures. Log-rank tests, however, revealed no statistically significant difference among the three treatments in either experiment ($p = 0.24$ and 0.81).

DISCUSSION

Relative pertinence of the two theories in the experiments

For $CuSO_4$, because the amphipods were given 14 d to recover, there notionally was minimal cumulative damage remaining from the first exposure during the second exposure. The cumulative mortality curve of the lethal group appeared to be lower than the others, indicating some induced tolerance, but the curves showed no apparent divergence. Because formal statistical tests also showed no evidence of significant difference among the three, the stochastic theory is supported here.

For NaPCP, although the log-rank test showed no statistically significant difference, the cumulative mortality curve for the lethally exposed amphipods was visually different from the other two. To assess these data further, survival data for each experiment were divided into two parts. The first part included the data from the beginning of the second exposure to 14 or 16 h. At these two time points, the total proportion dying since the beginning of the first exposure in the reference and sublethal groups was equal to the total proportion dying in the lethal group right before the beginning of the second exposure. The second part included the mortality data of the survivors from 14 or 16 h until the end of the exposure. The data for the reference and sublethal groups were combined and

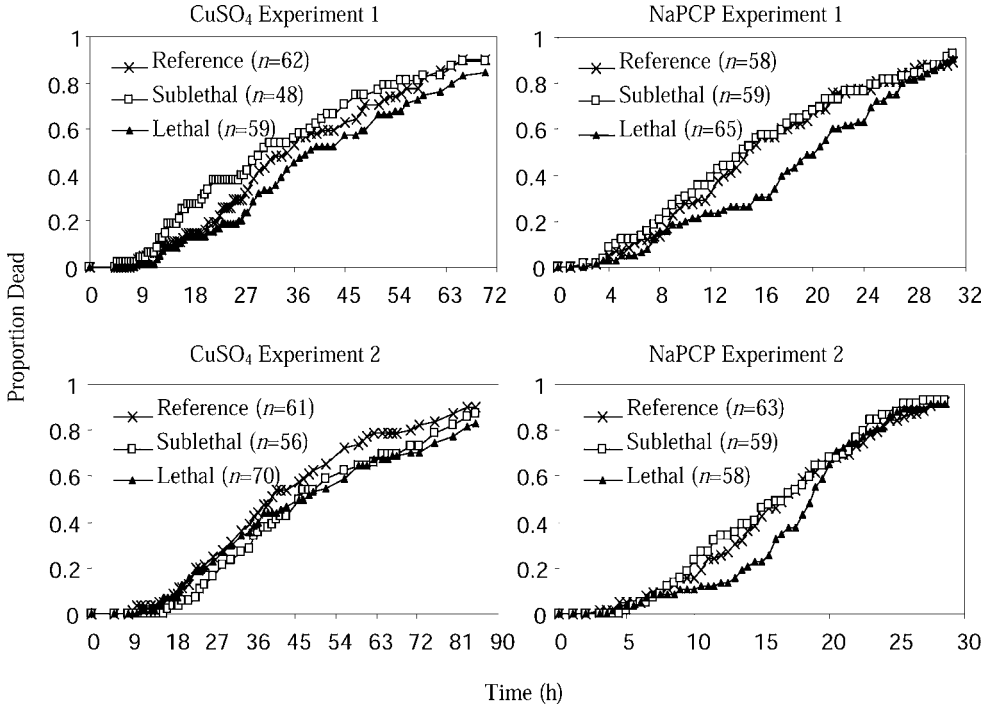

Fig. 1. Cumulative proportions dead through time in the reference, sublethal, and lethal treatments during the second exposures of two copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP) experiments. The sample sizes (*n*) are indicated in the brackets.

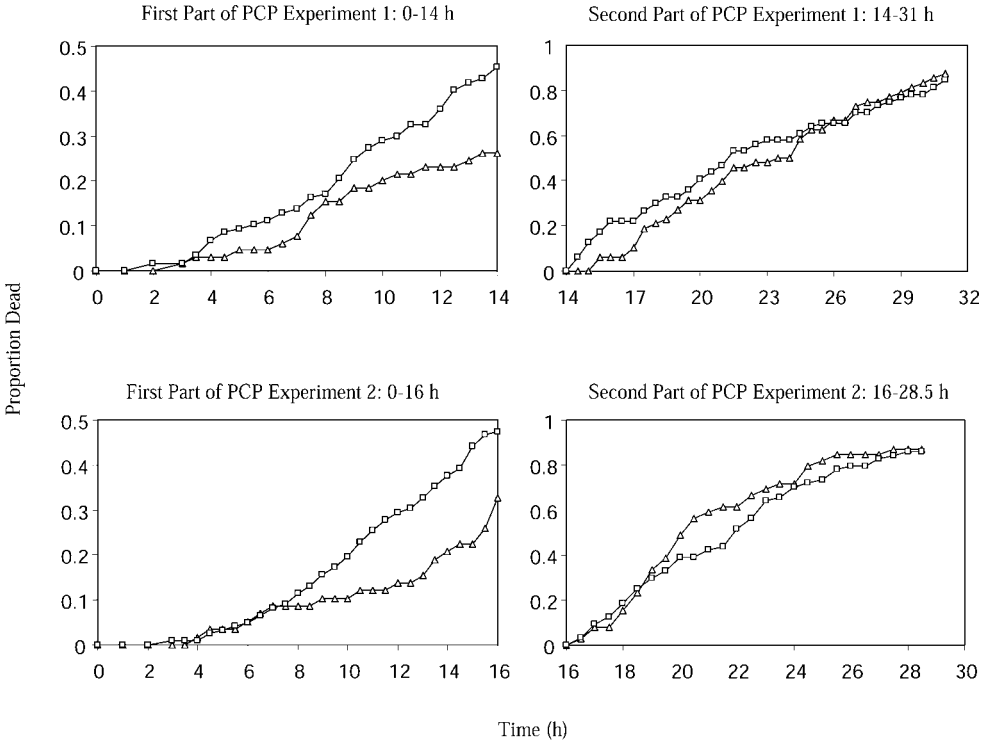

Fig. 2. Cumulative proportions dead through time during the first and second parts of the second lethal sodium pentachlorophenol (NaPCP) exposures. The data were divided into two parts at 14 and 16 h, respectively, for the two experiments. —△— = lethal group; —□— = reference plus sublethal groups.

compared with the lethal group, because no significant difference was found between them and more statistical power was gained by this means. Figure 2 shows the cumulative mortality curves after splitting the data. For the first components, the log-rank tests showed borderline significant difference ($p = 0.03$ and 0.06). For the second components, neither the mortality curves nor the log-rank tests showed any evidence of significant difference ($p = 0.90$ and 0.27). It was demonstrated that the latent effect of NaPCP was insignificant: The minimal amount of latent mortality stopped shortly after exposure ended [7]. Based on the half-lives of PCP and its conjugate in *H. azteca* of 3.6 and 9.1 h, respectively [25], no more than 0.000001% of the accumulated PCP and its conjugate remained in the amphipod body after 10 d of depuration—far less than the notionally defined complete depuration in toxicology (0.8%) [26]. Therefore, the statement can be made that the first NaPCP exposures had no demonstrable effect on the second exposure mortality: The animals appeared to have recovered completely. In addition, all three curves (Fig. 1) approached 100% mortality at approximately the same time, indicating no significant tolerance induction. Therefore, the most plausible explanation is that the IED theory might have played a minor role in the exposure. Relatively large proportions of lethal group animals (25–30%), however, died during the first components, suggesting that stochastic processes played the dominant role.

Studies have shown that under toxicant exposures, the survival times were different for organisms with different genetic qualities [27], providing one mechanism for the IED theory. Such genetic qualities, however, do not necessarily produce a log-normal distribution of IED values, nor do the clones (e.g., *Daphnia magna* assays) or cultures (e.g., algal assays) used in many tests possess such genetic differences among individuals. In a previous experiment, Newman and McCloskey [6] found some evidence of an IED effect for benzocaine but not for either sodium chloride or NaPCP. The present study showed that under the concentrations used in the experiments, the relative importance of the IED process for $CuSO_4$ and NaPCP could be different. This could be a result of the toxicological modes of action of the toxicants [7]: Copper causes oxidative damage, inhibits Na^+/K^+–adenosine triphosphatase activity, induces cell necrosis and apoptosis, and destabilizes the DNA structure, whereas PCP causes increased cellular oxidative metabolism, resulting from the uncoupling of oxidative phosphorylation. These findings suggest that the lethal response of a population to a toxicant might reflect elements of both the IED and stochasticity processes, and that the relative contributions might be different for different toxicants, organisms, and exposure intensities.

Application to pulsed or intermittent exposures

The significance of these experiments can be found in their application to pulsed or intermittent exposures in the field. Under the IED theory, the pulse resulting in the highest percentage mortality of the population will be the one that determines how much the population eventually will be affected after multiple exposures. The other pulses with lower intensities will not matter that much, because all the survivors of the highest-intensity pulse will have higher IED values and, thus, will not die during subsequent lower-intensity pulses. In contrast, every pulse is equally important under the stochastic theory. Therefore, the same population under the identical exposure scenario would have a higher possibility of becoming locally extinct if the stochastic hypothesis, rather than the IED hypothesis, were true.

In current ecotoxicological practices, the underling theory

of survival distributions needs more attention. Since its introduction, the IED theory has remained the dominant explanation in literature, but it has not been carefully tested. As more and more attention is being paid to lethal effects from multiple-pulse problems, the insufficiency of this state of understanding becomes manifest. For example, in a two-pulse experiment, if the first pulse results in a mortality that cannot be neglected and the stochastic mechanism dominates the survival distribution, then to compare control (naive) and treatment mortality during the second exposure for significant difference is justified. If the IED dominates, however, then the conclusion that the first exposure to the toxicant is a factor abating the toxicity during the second exposure (singly based on a significantly lower treatment mortality than in the control), or that after a certain recovery period the organisms have recovered completely from previous exposure (singly based on an insignificant difference between the control and treatment mortalities), will be invalid. In the first case, the IED theory predicts that until a certain point before 100% mortality, the treatment mortality will be much lower than the control without any induction of tolerance. In the second case, if the population has completely recovered, the treatment mortality still will be lower than the control mortality until a certain time. Therefore, we suggest that assessment of the dominant process be incorporated into and taken as one of the first steps of studying pulsed exposure effects.

CONCLUSION

In summary, the hypothesis that the IED theory is the dominant explanation for the dose–response model was rejected for both $CuSO_4$ and NaPCP. Under the specific concentrations and durations used in the present study, stochastic processes were dominant for $CuSO_4$ and NaPCP; both stochasticity and IED processes might have been relevant for NaPCP. Current ecotoxicology tests should take this ambiguity into consideration and assess the dominant process under specific exposure conditions (e.g., various concentrations or toxicants) to better predict the lethal consequences of pulsed exposures.

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