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Application of survival analysis methods to pulsed exposures: Exposure duration, latent mortality, recovery time, and the underlying theory of survival distribution models

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APPLICATION OF SURVIVAL ANALYSIS METHODS TO PULSED EXPOSURES: EXPOSURE DURATION, LATENT MORTALITY, RECOVERY TIME, AND THE UNDERLYING THEORY OF SURVIVAL DISTRIBUTION MODELS

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天涯是客，霞落花飞皆颜色。为君逐远，夜半窗前剪灯花。老了秋水，抛却胭脂眉黛罢。清泪数行，梦里乡关月夜花。

——闲来成于十三花

二〇〇五年十一月于VIMS

DEDICATION

To my grandparents, parents, and husband, Taiping

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ABSTRACT

Ecotoxicologists adopted median lethal concentration (LC50) methods from mammalian toxicology. This conventional LC50 approach has shortcomings in spite of its expediency and convenience. Fixing the exposure duration and selecting the 50% mortality level result in loss of ecologically relevant information generated at all other times. It also ignores latent mortality that can manifest after exposure ends. As a result, the conventional LC50 approach cannot adequately predict pulsed exposure effects in which concentration, duration, and frequency of pulses change through time. Furthermore, the underlying theory of the dose-response models used to calculate LC50 values, stochastic versus individual effective dose (IED) theory, has not been tested rigorously, while a better understanding of it is needed in order to better predict the effects of pulsed exposures.

In this study, the effects of exposure duration and concentration on mortality during and after exposures, and the effects of recovery time between two pulses on mortality during the second pulse were quantified. The influences of toxicant modes of action on latent mortality were discussed. The underlying explanation for survival distribution models was further explored. Survival analysis methods were used to incorporate these factors affecting mortality during pulsed exposures into predictive models and to circumvent some of the shortcomings of the conventional LC50 method. The experiments were done with two notionally contrasting toxicants, copper sulfate ($CuSO_4$) and sodium pentachlorophenol (NaPCP). The amphipod, *Hyalella azteca*, was used as the model organism.

Latent mortality is an integral part of the lethal effects of some toxicants that cause cumulative damage. Exposure concentration has a significant effect on latent mortality. For toxicants that cause minimal damage during the exposure, the latent mortality is not significant and can be potentially ignored. Exposure duration did not show any significant effect on latent mortality within the experimental ranges for either toxicant. It is recommended that for other experimental conditions the effect still needs to be considered. Recovery time between two pulses had significant effect on mortality during the second pulse for both toxicants. However, to recover to a similar background level mortality, the time an exposed organism needed to return to a stage similar to its original resistance was much longer for $CuSO_4$ than for NaPCP. The hypothesis that individual effective dose is the dominant explanation for the dose-response models was rejected for both toxicants. By effectively incorporating exposure duration and other factors into the models, the application of survival analysis methods better predicted pulsed exposure consequences than did the conventional LC50 method. It is important for current ecotoxicology and environmental risk assessment to consider the factors potentially affecting pulsed exposure consequences. The survival analysis provides a better way to address the issue.

APPLICATION OF SURVIVAL ANALYSIS METHODS TO PULSED EXPOSURES: EXPOSURE DURATION, LATENT MORTALITY, RECOVERY TIME, AND THE UNDERLYING THEORY OF SURVIVAL DISTRIBUTION MODELS

CHAPTER I. INTRODUCTION

A. Current Metric of Lethality in Ecotoxicology

The current use of the median lethal concentration or dose approach (LC50/LD50) to measure chemical toxicity in ecotoxicology has its roots in classic mammalian toxicology. Historically, researchers quantified toxicity by defining threshold concentrations for toxicants using a fixed exposure duration but found that the high uncertainty in predictions at the lower tail of the dose-response curve made estimation too imprecise. Finney (1947) argued that the 50% response level should be used instead because the associated estimates of lethal concentrations at 50% exhibit less variability than those at higher or lower percentiles. The LC50/LD50 became the metric of toxicity in mammalian toxicology.

From the mid-1940s, environmental toxicologists began to adopt this approach for their use in laboratory bioassays, using the results to imply environmentally safe concentrations (Cairns and Pratt 1989). Experimental organisms are exposed to a series of toxicant concentrations and their percentage mortalities are recorded at the end of an experiment. Exposure durations are grossly defined as acute (e.g., 48 h or 96 h) or chronic (e.g., 10% or more of the species' life span). These time-endpoint methods are used to evaluate the chemical concentration producing a specific level of effect such as the 96 h LC50. This method is fast and simple to perform, and insensitive to violations of statistical assumptions, e.g., assuming either a log-normal or log-logistic distributed

model will result in similar LC50 values (Dixon and Newman 1991). During early applications of these methods, people adequately predicted toxicity associated with point sources and compared the relative toxicities of different toxicants or different species of the same toxicant. However, there was and still is a tendency to uncritically apply these routine toxicity test protocols to situations in which they have important shortcomings.

Toxic effect is a function of both exposure duration and intensity (concentration or dose). In general, the higher the exposure concentration or the longer the duration, the more damage that is caused. However, environmental regulators tend to focus on concentration while considering duration peripherally. The duration of a LC50 test is set based on convenience, e.g., 96 h fits conveniently within a work week, not ecological relevance. Exposure durations differing from 96 h are likely and the 96 h LC50 will imperfectly predict mortality for these other durations. Scoring mortality only at the end of the exposure period results in lose of valuable information generated for other ecologically relevant times. For example, with the 96 h LC50 of dissolved Cu of a fish species, we can neither quantitatively predict the proportion of fish dead at 48 h, nor if the fish population will be viable after the 96 h Cu exposure.

In the context of predicting ecological consequences, the statistically most precise 50% point has questionable biological significance. In many situations, concentrations resulting in lower or higher percentage mortalities can be meaningful to determine risk upon toxicant released and helpful for regulators while setting toxicant standards in the environment.

Conventional endpoint tests also do not address lethal effects that happen after exposure ends (latent mortality). In mammalian toxicology, the LC50 method is designed to measure toxic effects on individuals; in ecotoxicology, the primary interest is to predict the fate of a field population after exposure. Latent mortality could be an integral component of the exposure consequences (e.g., Newman and McCloskey 2000, Arthur 2001, Cerqueira and Fernandes 2002). To quantify latent mortality, observation must continue after the exposure ends. Noting mortality only at the end of exposure compromises the usefulness of the associated lethality predictions.

In reality, organisms experience pulsed exposures in which exposure concentration, duration, and frequency change through time. The conventional toxicity tests are derived for an exposure scenario of fixed durations and constant concentrations, and cannot accurately model pulsed exposure. Pulsed exposures normally occur after events such as multiple pesticide sprayings, snowmelt or rainstorms following dry periods, repeated discharges, or tidal fluctuation in an estuary. Chemicals, especially agrochemicals, occur at different sub-lethal concentrations for most of the time with potentially lethal concentrations being reached periodically. The assessment of these pollutant effects requires more than the conventional concentration-response methods provide.

Finally, people tend to use the LC50 metric as a measure of toxicity without paying sufficient attention to its underlying mechanism. A probit (log-normal) model is assumed in most analyses of dose-response data. The dominant explanation of this log-normal distribution is the individual effective dose (IED) theory: each individual has a characteristic tolerance for the toxicant and will die if the exposure dose or

concentration exceeds its IED (Gaddum 1953). The IED values are thought to be log-normally distributed within a population. This IED concept permeates interpretations of other models of acute lethality as well. However, it has not been rigorously tested before Newman and McCloskey (2000) and may not be a sufficient explanation for all, or even most, applications of the model. A plausible alternative explanation is a stochastic one: at a specific concentration, all exposed animals have the same chance of dying and which specific individual will die is determined by a random process defined commonly by a log-normal (or skewed) model (Berkson 1951). To determine the correct explanation for the probit and similar models is important in predicting population fate after pulsed exposures. Under the IED theory, the survivors of the exposures are more robust than the dead ones but, under the stochastic theory, there is no difference between the dead and the survivors. With a same exposure scenario of repeated pulses, a population would persist much longer if the IED theory were true rather than if the stochastic theory were.

B. Survival Analysis Method

The survival analysis method was applied in this dissertation to overcome several shortcomings of the LC50 methods mentioned above. The survival analysis method is also called time-to-event, survival time, or failure time analysis. It was initially developed in medical sciences and engineering. In agricultural sciences, it has been applied to insect pathology. This includes the study of mortality when insects are challenged by pathogenic microorganisms (e.g., Fenlon 2002). In engineering, it has been used in reliability testing of components and systems where the event of interest is

usually failure of the component or degradation of system performance (e.g., Kimber 2002). Fully parametric models are usually used in engineering because good predictive models for the failure time distribution are valued. In the medical field, it is used to predict the effects of various variables on survival of animals or humans. Semi-parametric models are often used if researchers are more interested in relative effects of different treatments, not the exact failure time. For example, in the classic Stanford Heart Transplant experiment (Crowley and Hu 1977), the Cox semi-parametric model was used to study whether transplantation raised or lowered the risk of death with covariates such as date of birth and age of acceptance included in the study. Other applications in medical, engineering, and other fields can be found in text books (e.g., Crowder et al. 1991, Ansell and Phillips 1994, Collett 1994, Allison 1995, Cantor 2003).

Only recently has survival analysis been applied to environmental risk assessment and ecotoxicology. Survival time modeling was applied to acute NaCl toxicity data for the mosquitofish, *Gambusia holbrooki* (Newman and Aplin 1992). Both concentration and fish wet weight were included in the model. The authors suggested consideration of this method as an adjunct to conventional toxicity testing endpoints. A population of rainbow trout was challenged with viral haemorrhagic septicaemia (VHS) and the survival function was estimated by assuming a Weibull distribution (Henryon et al. 2002). Honeybee (*Apis mellifera ligustica*) survival after chronic exposure to the insecticides, deltamethrin and imidacloprid, has been studied with survival models (Moncharmont et al. 2003). The time required for an individual amphipod (*Leptocheirus plumulosus*) to burrow below the sediment-water interface was recorded and analyzed with survival modeling (Vogt 2003). The results suggested that

amphipod burrowing behavior is sensitive to sediment contamination. Survival analysis was used to evaluate the effect of the number of infected fish and acute exposure on the development of branchial xenomas during a *Loma salmonae* infection (Becker et al. 2005).

The general approach of survival analysis involves exposing organisms to toxicant solutions and monitoring the effects (e.g., mortality, lose of equilibrium, hatch, sexual maturity, or spawn) through time. Non-responders (e.g., survivors) are treated as statistically censored because their exact times-to-event are unknown. Maximum likelihood estimations (MLE) are conventionally used to analyze the data because of this censoring.

Some terminologies are important and frequently used in survival analysis. The mortality of individuals can be described with a cumulative distribution function (cdf), $F(t)$, or probability density function (pdf), $f(t)$. The cdf at a certain time T is a function of the probability that the variable will be less than or equal to any value t that we choose: $F(t) = P(T \leq t)$. An estimate of the $F(t)$ would be the total number of individuals dead at time, t, divided by the total number of individuals exposed to the toxicant:

$$F(t) = \frac{Number\ Dead(t)}{Total\ Number\ Exposed} \tag{1.1}$$

The pdf is the derivative or slope of the cdf curve:

$$f(t) = \frac{dF(t)}{dt} \tag{1.2}$$

The survival function $S(t)$ describes the probability of surviving beyond time t. It is estimated by the number of individuals surviving to time t divided by the total number of individuals exposed to the toxicant:

$$S(t) = P(T > t) = 1 - F(t) = \frac{Number\ Survived(t)}{Total\ Number\ Exposed} \tag{1.3}$$

The hazard function $h(t)$ is used to describe the instantaneous death rate at time t conditioned on the organism's survival to time t:

$$h(t) = \lim_{\Delta t \to 0} \frac{P\langle t \le T < t + \Delta t \,|\, T \ge t \rangle}{\Delta t} = \frac{f(t)}{S(t)} \tag{1.4}$$

The cumulative hazard function $H(t)$ is the integral of the hazard function:

$$H(t) = \int_0^t h(t)dt = -\ln(1 - F(t)) \tag{1.5}$$

Nonparametric, semi-parametric, and fully parametric methods are available for analyzing these data. Nonparametric methods include product-limit and life-table methods, and do not require a specific distribution for the survival curve. Survival for different groups of exposed individuals can be tested for equality with the nonparametric log-rank or Wilcoxon test. The general form of the semi-parametric and parametric models is:

$$h\,(t, x_i) = e^{\,f(x_i)}\; h_0\,(t, e^{\,f(x_i)}) \tag{1.6}$$

where $h\,(t, x_i)$ is the hazard function for group x_i; $h_0\,(t, e^{\,f(x_i)})$ is the baseline hazard at time t for group x_i; $e^{\,f(x_i)}$ is a function that relates the hazard to the baseline hazard; and $f\,(x_i)$ is a function of either continuous variables such as concentration, or class variables such as sex. For a semi-parametric proportional hazards model, the distribution of baseline hazard $h_0\,(t, e^{\,f(x_i)})$ does not need to be specified. The hazard of a treatment group is some multiple of the baseline hazard. For a parametric model, Equation 1.6 can be rearranged to the form of an accelerated failure time model:

$$\ln t_i = f(x_i) + \varepsilon_i \tag{1.7}$$

where t_i is the time-to-event, $f(x_i)$ is a function that relates the covariates to t_i, and ε_i is the error term, which for prediction purposes is $\sigma \times L$. The L varies with the proportion dead for which prediction is being made and can be obtained from the Table 7 of Newman (1995). The scale parameter σ defines the scale of the hazard curve. The t_i will have a Weibull, exponential, log-logistic, or log-normal distribution if ε_i is assumed to have either the distribution of extreme value with two parameters, extreme value with one parameter, logistic, or normal, respectively (Cox and Oakes 1984). If the numbers of parameters for the four candidate distributions above were the same, the log likelihood statistic associated with each distribution could be applied directly to select the best-fit model. Larger log likelihood value indicates better fit. If the numbers of parameters differ among candidate models, Akaike's information critierion (AIC) can be used (Atkinson 1980):

$$AIC = -2 \times (\textit{log likelihood statistic}) + 2 \times (\textit{number of parameters}) \qquad (1.8)$$

It favors parsimony in selecting among models. Lowest AIC values indicate the best-fitting and parsimonious model, i.e., the model with the most information per estimated parameter.

Survival analysis has many advantages relative to the conventional LC50 method, including:

1) Survival analysis can include exposure duration, a crucial determinant of toxic effect, into the model.

2) More data can be produced from it than from the conventional LC50 method. Therefore, statistical power is greatly enhanced (Newman and Dixon 1996, Dixon 2002).

3) Because of the increase of statistical power, important covariates associated with the exposure such as concentration, temperature, and pH value can be included in the models and their effects quantified. Also, because times-to-death are recorded for individuals, covariates associated with individuals such as sex, age, and body weight can be included in predictive models more effectively.

4) Time-to-event results can be applied directly in ecological, demographic, and epidemiological models. For example, if the time-to-death and time-to-reproduce are monitored for a fish population exposed to a toxicant, the number of young born (m_x) and proportion of individuals dying (l_x) can be generated. With m_x, l_x, and the original number of a fish population, the Leslie matrix approach (Leslie 1945, Caswell 1996) can be used to predict population qualities and fate through time (Newman and McCloskey 2002).

5) The conventional endpoint estimates, such as LC50, and the associated confidence intervals can be estimated as well from survival models. More precise prediction can result in many (but not all) cases (Dixon 2002).

The advantages of survival analysis allow us to potentially overcome many of the shortcomings of conventional LC50 methods, especially to predict pulsed exposure effects more effectively. Firstly, both exposure duration and concentration can be included in the model and their effects predicted. Secondly, latent mortality and the variables affecting latent mortality can be quantified. Thirdly, lethal effect of pulsed exposure may be quantitatively predicted by incorporating former exposure concentration and duration, recovery duration, and current exposure time into the

model. Last, the underlying mechanism of log-normal and similar models can be further explored.

C. Model Chemicals: Copper Sulfate ($CuSO_4$) and Sodium Pentachlorophenol (NaPCP)

Copper sulfate and NaPCP were selected because their different modes of action on aquatic animals and anticipated contrasting latent effects. Copper can cause cumulative damage to gills and the exposed animals will likely need a long period of time to recover and the latent mortality might be high. For NaPCP which causes less cumulative damage, the exposed animals have a good chance of recovery after exposure ends and the latent mortality might be low. Because latent mortality is an integral, albeit often ignored, consequence of pulsed exposure, the effects of the two toxicants during pulsed exposure could be different as well. Furthermore, the two toxicants can be representative of two contrasting modes of action, and the underlying dominant theory (IED versus stochastic) for probit and similar models might be different. Their uses, environmental fates, and toxicities are briefly discussed below.

1. Copper and copper sulfate

Copper sulfate is used to control bacterial and fungal diseases of fruits, vegetables, nuts, and field crops. It is also used as an algaecide and herbicide, and to kill slugs and snails in irrigation and municipal water treatment systems.

Copper can be bound or adsorbed to organic material, and to clay and mineral surfaces. The degree of adsorption to soils depends on soil acidity or alkalinity. It is considered one of the more mobile metals in soils. Because of its binding capacity, its

leaching potential is low in all but sandy soils. Applied with irrigation water, $CuSO_4$ does not accumulate in the surrounding soils. About 60% deposits in the sediments at the bottom of the irrigation ditch, and becomes adsorbed to clay, mineral, and organic particles (U.S. National Library of Medicine 1995).

Copper sulfate is highly toxic to fish. Even at recommended rates of application, this material may be poisonous, especially in soft or acid waters. Its toxicity generally decreases as water hardness increases. It is toxic to aquatic invertebrates too. Some amphipod species are especially sensitive to it. Copper inhibits Na^+/K^+ ATPase activity and induces chloride cell necrosis and apoptosis. It increases gill membrane permeability and chloride cell dysfunction. In the end, the osmotic and ionic functions of gills are disrupted (Cerqueira and Fernandes 2002). 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 (Poleksic and Mitrovic-Tutundzic 1994). Copper also bonds between heterocyclic bases of DNA, competes with the normal hydrogen binding, and destabilized the DNA structure (Eichhorn 1975). Therefore, organisms may need relatively long periods of time to recover depending on the cumulative damage caused during exposure. Studies (e.g., Icely and Nott 1980, Caparis 1989, Eriksson and Weeks 1994) have shown that amphipods can accumulate significant amount of Cu by storing it in granules of midgut. Metallothioneins and metallothionein-like proteins can be induced by Cu, thus reduce the amount available to cause a toxic effect on amphipods.

2. Pentachlorophenol and sodium pentachlorophenol

Sodium pentachlorophenol's greatest use is as a wood preservative. It was banned for herbicide use in 1987. Other uses include soil fumigation for termites, seed treatment for beans, antibacterial agent in disinfectants/cleaners, and preservative for glues, starches, and photographic papers (EPA fact sheet 2003).

Pentachlorophenol and NaPCP may be released to the environment as a result of its production, storage, transport, or usage. In air, PCP will be degraded through photolysis. Any PCP released to soils will slowly biodegrade and leach into groundwater. It tends to adsorb to soil and sediment, and the adsorption is stronger under acid conditions. Evaporation from water is slow, especially at natural pH values. It does not appear to oxidize or hydrolyze under environmental conditions (EPA fact sheet 2003).

Hedtke et al. (1986) conducted PCP acute freshwater toxicity tests on the amphipod, *Crangonyx pseudogracilis*, obtaining 96 h LC50 values of 0.32, 0.22, and 1.55 mg/L in different periods during the summer. Juvenile amphipods, *Gammarus psuedolimnaeus* and *Crangonyx pseudogracilis*, were exposed to PCP at different pH values and PCP toxicity decreased with increased pH (Spehar et al. 1985). Pentachlorophenol is expected to bioconcentrate in organisms and the bioconcentration factor (BCF) will be dependent upon the pH of the water because PCP will be more dissociated at higher pH values (EPA fact sheet 2003). The toxicological mode of action of PCP is increased cellular oxidative metabolism resulting from the uncoupling of oxidative phosphorylation. It tends to diffuse across the gill from the amphipod's body

(Spacie and Hamelink 1985). It was found that the clearance rate increases as salinity increases (Tachikawa and Sawamura 1994). It can be converted directly by phase II conjugation reactions at a faster rate than contaminants that are transformed by oxidative metabolism with cytochrome P450. Therefore, after it is removed from the environment, the toxic effect is reversible and the cumulative damage after the exposure may not be as prominent as that of Cu. Organisms exposed to PCP could acquire increased tolerance through acclimation (e.g., Norup 1972).

D. Model Organism: Amphipod *Hyalella azteca*

The freshwater amphipods *H. azteca* were used as experimental animals. Amphipods are one of the orders in the subphylum Crustacea and class Malacostraca. The genus *Hyalella* belongs to the order Amphipoda and family Hyalellidae (Voshell 2002). They are bottom dwellers in small spaces, such as cool streams, springs, and ponds, coarse detritus, or upper layer of soft sediment.

The normal body length of adult *H. azteca* is 8 mm for males and 6 mm for females. Body color is a creamy light gray and somewhat translucent. It is omnivorous but its most common food is detritus. It also grazes on algae, fungi, and bacteria. It completes its life cycle in 27 days or longer depending on the temperature and die within one year. Individuals that live in waters where temperatures change seasonally usually reproduce in spring and summer. Bovee (1950) and Sprague (1963) found temperatures tolerated by *H. azteca* range from 0 to 33 °C.

Amphipods are nearly ubiquitous in permanent fresh waters of the New World. They are important items in the diet of many invertebrates, fish, amphibians, and water

birds. They are important in the breakdown of particulate organic matter. The amphipod *H. azteca* has many desirable characteristics as an experimental organism including short generation time, ease of culture, relative sensitivity to contaminants and tolerance of varying physical-chemical properties of environments, and an easily identifiable mortality end point. Therefore, it became one of the EPA recommended species for assessing acute toxicity of freshwater sediment (EPA 2000). However, in a recent study, Wang et al. (2004) suggested that because of the gap between laboratory and nature, it is probably a suitable surrogate species for determining sediments that are likely not toxic to field populations, while not suitable for determining sediments that are likely toxic to field populations.

In a study conducted on *H. azteca* (De March, 1978), higher temperatures produced smaller animals. Higher temperatures and longer photoperiods increased reproductive activity of the species (Kruschwitz 1978). The survival, growth, and reproduction of *H. azteca* were determined under various test conditions (Borgmann et al. 1989). There have been numerous studies with *H. azteca* on acidification (e.g., Grapentine and Rosenberg 1992, France 1996), and toxicity (e.g., Morris et al. 2003, Borgmann et al. 2005) and bioaccumulation (e.g., Jessiman and Qadir 1983, Burton et al. 2005) of various toxicants.

E. Hypotheses to be Tested

Based on the limitations of current LC50 method, the advantages of survival analysis method, and the need to predict lethal consequences of pulsed exposures, I established the following four hypotheses for my dissertation:

Hypothesis 1: Survival analysis better predicts lethal effects than does the conventional LC50 method. It more efficiently includes time, concentration, and other important covariates into predictive models.

Hypothesis 2: Survival analysis allows statistical testing for and effective quantification of latent mortality.

- For $CuSO_4$, cumulative damage to gills and other tissues causes high latent mortality and the mortality is a function of previous exposure concentration.
- For NaPCP, reversible and less pervasive cumulative damage causes insignificant latent mortality and the mortality is relatively independent of previous exposure concentration.

Hypothesis 3: Survival analysis permits more effective prediction of lethal effects from pulsed exposure.

After the preliminary experiments and the results of Hypothesis 2, the following sub-hypotheses of Hypothesis 3 were established:

- For $CuSO_4$, there is significant effect of previous pulse duration on latent mortality, and the effect can be quantified; for NaPCP, there is not significant effect of pulse duration on latent mortality.
- For both $CuSO_4$ and NaPCP, there are significant effects of recovery time between the two pulses on mortality during the second pulse.

Hypothesis 4: The IED theory is the sole or dominant explanation for the survival distribution model for both $CuSO_4$ and NaPCP.

CHAPTER II. INCORPORATION OF EXPOSURE DURATION INTO SURVIVAL ANALYSIS MODELS AND APPLICATION OF THE MODELS TO LATENT MORTALITY

A. Introduction

In a variety of fields such as medical sciences and engineering, duration is commonly included as one of the variables in the statistical models described in Chapter I. In ecotoxicology, scientists began including exposure duration in their models from the beginning of the last century. One of the first applications was a rectangular hyperbola-like survival curve for *Daphnia* (Warren 1900). The times-to-death and percent mortality data of tuberculoid mice were fit to log-probability paper, and a median lethal time (LT50, the predicted exposure time needed to get 50% mortality at a preset concentration) of 46 d was calculated (Litchfield 1949). Herbert and Merkens (1952) generated a log concentration versus log survival time graph for rainbow trout in potassium cyanide solutions. The linear range was expressed as $C^n \times T = k$, where C was concentration, T was survival time, and n and k were estimated parameters. Burdick (1957) expanded the model to $(C-a)^n \times (T-b) = K$, where a and b were the threshold concentration and time, respectively. Jones (1964) found that survival time increased as concentration decreased until a point was reached where it became indefinitely long (the threshold concentration), and as concentration increased, a stage could be reached when further increases in the concentration would not materially shorten the survival time (the

threshold reaction time). In order to normalize discrete atrazine exposure data for irregular sampling, a crude moving window technique was used more recently to approximate the 4-d and 21-d average atrazine effect concentrations (Solomon et al. 1995). Mayer et al. (1994) described a two-step linear regression approach that uses acute lethal data to estimate chronic toxicity with exposure time as an independent variable. The predicted no-observed-effect concentration (PNOEC) was estimated from the regression. Based on this and another two methods (accelerated life testing and multifactor probit analysis), the Acute-to-Chronic Estimation software (Ellersieck et al. 2003) was developed to predict long-term toxicity with data from short-term experiments. All of the methods described in this paragraph provide gross predictions of the influence of exposure duration.

Though many scientists made efforts to include time into their toxicological models, most have restricted their attention to times during the exposure. Latent mortality is not routinely quantified and reported in lethality studies. Pascoe and Shazili (1986) observed significant post exposure mortality resulted from brief cadmium exposure. The term median post exposure lethal time (peLT50) was proposed as a means of assessing and comparing the results of brief exposure to a pollutant. Reinert et al. (2002) suggested that, in order to demonstrate latency (or lack of latency), observation intervals should be continued after the exposure ends.

Latent mortality could be affected by several variables such as life stage of the exposed organisms, the toxicant to which the organisms are exposed, exposure concentration, exposure interval, and temperature. Guadagnolo et al. (2000) found that rainbow trout eggs were more sensitive after silver exposures during certain

development periods than during other periods. Arthur (2001) found grain beetle mortality after the initial exposures to wheat treated with diatomaceous earth increased as exposure interval and temperature increased. Newman and McCloskey (2000) observed mosquitofish latent mortality extended for 3 to 4 weeks after NaCl exposure, but only 8 h after PCP exposure. Naddy et al. (2000) found daphnids did not experience any delayed effects from chlorpyrifo exposures up to 20 d after exposure. The difference may be because the effects are reversible unless death has occurred for substances that display a baseline or narcotic mode of action (Reinert et al. 2002). None of these studies formally quantified the relationship between the degree of latent mortality and variables that could potentially affect it.

In the current study, the amphipod, *H. azteca*, was exposed to different $CuSO_4$ and NaPCP concentrations. Time-to-death data taken during and after the exposures were fit to survival models. By including exposure duration and concentration as covariates in the models, the proportion dead was predicted at any concentration and any exposure time within the experimental range. By comparing the conventional 48 h LC50 and the complete LC50 values (defined as the LC50 values calculated by including mortalities during and after exposure ends) and contrasting the latent effects of the two toxicants, the importance of including latent mortality into ecotoxicological models was demonstrated.

B. Methods

1. Amphipod culture and maintenance

The amphipods, *H. azteca*, came from a population that had been maintained in our laboratory for more than two years and 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 were obtained by gently siphoning water from the cultures onto screens. The amphipods that passed through a 0.67 mm sieve but were retained by a 0.50 mm sieve were used as test organisms. They were maintained in the reformulated moderately hard reconstituted water (RMHRW) (Smith et al. 1997) with food at 23°C for at least 72 h before the exposures began. The chemicals needed to prepare RMHRW and the expected alkalinity and pH ranges are listed in Appendix 1.

2. Exposure procedure

Several range-finding tests were conducted to determine the concentrations to be used in the following formal experiments. Three $CuSO_4$ exposures were conducted in January, February, and July 2003, respectively. Copper sulfate was dissolved in RMHRW to make five solutions with nominal dissolved Cu concentrations of 0.0, 0.2, 0.3, 0.4, 0.6 mg/L. Each solution was delivered to four 12-well COSTAR 3513 Cell Culture Clusters (Corning, Corning, NY, USA) with approximately 4 ml in each well. Two hundred and forty amphipods were then randomly assigned to the wells with one animal per well. Each well contained a piece of red maple leaf as food (leaf weight in each well: 0.61 ± 0.32 mg, mean ± standard deviation, $n = 40$). Every amphipod exposed to the same concentration was considered a replicate. The cluster plates were then placed in a LAB-LINE AMBI-HI-LO Chamber (Lab-Line Instruments, Melrose

Park, IL, USA). Mortality was checked at approximately 4 h intervals. An amphipod was scored as dead and removed from the well if no sign of appendage movement was discernible after gentle prodding. All the amphipods alive after 48 h were carefully transferred to fresh RMHRW. Latent mortality was noted approximately every 4 h. The experiment ended at 112 h when no more mortality was evident. All the survivors after that time were noted as right-censored. During all the experiments, 48 amphipods were established as control animals and maintained in water free of toxicant.

Three NaPCP exposures were conducted in early June, mid-June, and late July of 2003, respectively. Sodium pentachlorophenol was dissolved in RMHRW to make solutions with nominal NaPCP concentrations of 0.0, 0.2, 0.3, 0.5, 0.8 mg/L. The exposure and post exposure procedures were the same as those of $CuSO_4$. The only difference was that the experiments ended at 85 h, when no more latent mortality was evident. Forty eight amphipods were established as control animals as well.

The total alkalinity and pH of RMHRW were measured before exposures started to ensure that they were within the expected ranges. The solutions were renewed during the experiments every 12 h. Both newly prepared and 12 h-exposed water samples were collected for pH and toxicant concentration measurements. 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 Cu measurement were acidified, stored 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 with glass bottles, stored in 4°C, and analyzed with the method of Carr et al. (1982). Each 25 ml

water sample was mixed with 25 ml de-ionized water and 0.5 ml of concentrated HCl. Ten ml of chloroform was added before the sample was shaken vigorously for 60 s. Five ml of the extract was collected in a polypropylene centrifuge tube. Two ml of 0.2 M NaOH were added to the extract, mixed vigorously for about 30 s, and centrifuged in an IEC HN-SII Centrifuge (International Equipment, Needham Heights, MA, USA) at 5000 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. Samples for temperature and dissolved oxygen (DO) were taken periodically and measured with a Fisher mercury thermometer and YSI Model 57 oxygen meter (YSI, Yellow Springs, OH, USA), respectively.

3. Data analysis

The exposure concentration, total number of exposed amphipods, and number of dead amphipods were fit to a probit model with $\log_{10}$ transformation of concentration to calculate the conventional 48 h LC50 and complete LC50 values. The associated 95% fiducial limits were calculated as well (TOXSTAT® 1989).

The parametric accelerated failure time model was used to analyze the survival data with toxicant concentration as the independent variable:

$$\ln t_i = f(\textit{concentration}) + \varepsilon_i. \qquad (2.1)$$

In order to predict the mortality during and immediately after the exposures ended, and to determine if there was any significant effect of former exposure concentration on the latent mortality, the survival data of exposure, post exposure, and complete (exposure + post exposure) were fit to the accelerated failure time models

separately (SAS® Procedure LIFEREG, SAS 1999). The best-fit model selection criteria were the same as those described in Chapter I.

C. Results

The RMHRW for all solutions had an alkalinity of 59 ± 4 mg/L as $CaCO_3$ (n = 10) and a median pH of 8.15 (range: 8.12 – 8.16, n = 30), which were within the anticipated normal ranges. The pH, DO concentration, and water temperature during the experiments are summarized in Table 2-1. The treatments with higher dissolved Cu concentration had lower pH values likely due to the hydrolysis of the Cu^{2+}. They have a relatively broader range because both newly prepared and 12-h exposed water pH values were measured. Table 2-2 summarizes the dissolved Cu and NaPCP concentrations during the 48 h exposures. The toxicant concentrations for controls and water during the post exposure period were below the detection limits of the methods (7 µg/L for dissolved Cu, 0.15 mg/L for NaPCP).

The control mortalities were less than 5% in all experiments. The amphipod mortality through time of all the experiments can be found in Appendix 2 and 3. The cumulative proportions of dead amphipods at each observation time were plotted for the $CuSO_4$ and NaPCP experiments (Fig. 2-1). There was minimal mortality during the first several hours of exposure. After the $CuSO_4$ exposure ended, a large number of amphipods continued to die for a relatively long time. For NaPCP, only a few animals died during the post exposure period and most of their deaths occurred soon after the exposure ended.

The conventional and complete LC50 values with their 95% fiducial limits are shown in Figure 2-2. For $CuSO_4$, the conventional LC50 values were manifestly higher than the complete ones. In experiments 1 and 2, their 95% fiducial limits did not overlap and there was only about an 11% overlap in experiment 3. For NaPCP, the complete LC50 values were only a little lower than the conventional LC50 values, and more than 60% of their 95% fudicial limits overlapped. The data were then fit to the log-normal model (SAS® Procedure PROBIT, SAS 1999) with conventional/complete as an independent categorical variable. The results of a χ^2 statistic showed that for all three $CuSO_4$ experiments, the conventional and complete LC50 values were significantly different from each other ($p<0.0001$, $p<0.0001$, and $p=0.031$), but they were not for the three NaPCP experiments ($p=0.205$, $p=0.386$, and $p=0.690$).

The 112 h survival data for $CuSO_4$ and 85 h survival data for NaPCP were first fit to the accelerated failure time models with the candidate survival time distributions of exponential, Weibull, log-normal, and log-logistic (Table 2-3 and Table 2-4). Natural log transformation of the concentration was used because this is the most common concentration metameter (Newman 1995) and the associated AIC values were lower than those without this transformation. For all the data sets, log-normal distributions proved to be the best based on the AIC. For data generated during the exposures, either the Weibull or log-normal distribution displayed best fit. If only the post exposure data were used, the best-fit models for $CuSO_4$ were log-normal, while coefficients of concentration were not significantly different from 0 for NaPCP exposures ($\alpha=0.05$).

D. Discussion

1. Effects of the nature of toxicant on latent mortality

To illustrate the extent of latent mortality, the predicted proportion dead at the conventional LC50 concentrations and that after including latent mortality were plotted in Figure 2-3. When latent mortality for $CuSO_4$ was considered, 65% to 85% of exposed animals died at the LC50 concentration, not 50%. Any prediction of field population mortality based on the conventional LC50 method would underestimate mortality by 15% to 35%. In contrast, only 5% or fewer additional animals died for NaPCP. The amphipod displayed contrasting latent mortalities after the $CuSO_4$ and NaPCP exposures, mainly because these two chemicals have different modes of action. Gills are likely the primary target organ of Cu due to their high surface area in contact with the external medium. The damage of Cu is cumulative and needs a long period to recover. Changes in gill tissue of the tropical fish, *Prochilodus scrofa*, were investigated after 96 h Cu exposure (Cerqueira and Fernandes 2002). The restoration of gill structure (epithelial lifting, cell swelling and proliferation, and blood vessel anomalies) was not completed until the forth-fifth day post exposure. Red blood cells and hemoglobin concentration remained high until the seventh day. Plasma Na^+ and Cl^- decreased, K^+ increased significantly until the seventh day. In contrast to Cu, PCP toxicant effect is considered to be reversible and causes less cumulative damage. Nuutinen et al. (2003) quantified the *H. azteca* uptake, biotransformation, and elimination rates of PCP and got relatively short half-lives of 3.6 h and 9.1 h for PCP and its metabolite, respectively. Pentachlorophenol was converted directly by phase II conjugation reactions at a faster

rate than contaminants that are transformed by oxidative metabolism with cytochrome P450. Therefore, animals have a good chance of rapid recovery after exposure ends.

2. Effects of previous exposure concentration on latent mortality

For certain toxicants, previous exposure concentration can affect latent mortality. In the post exposure models of $CuSO_4$, the effects of former exposure concentration were significant: the higher the concentration, the less time needed for a certain proportion of animals to die during the post exposure period. Because in experiment 3 the dissolved Cu concentration for each treatment was the lowest among all the three experiments, the cumulative gill damage caused by Cu might not have been so extensive, and accordingly, the latent mortality was not as evident as in the other two experiments. For the post exposure models of NaPCP, the coefficients of log concentration were not significantly different from 0, indicating no significant effect of former exposure concentration on latent mortality. It was likely due to less cumulative damage occurring during the exposure, even at the highest concentration.

3. Incorporating exposure duration and latent mortality into survival models

The accelerated failure time models were generated for three different time periods (exposure only, immediately post exposure, and exposure plus post exposure). For most of the time, the best-fit-models were either log-normal or Weibull distributed. The log-normal model is among the most widespread models used for toxicity testing and has traditionally been used extensively for determination of acute lethality and other dichotomous responses. Furthermore, the log-normal distribution aspect of the model is

biologically plausible and accounts for some degree of inter-individual variability (Rees and Hattis 1994). The Weibull distribution includes the exponential distribution as a special case and has an extreme value justification. During a toxicant exposure, a series of biological processes of bioaccumulation, biotransformation, detoxification, elimination, etc., are involved in. If the exposed organism is overwhelmed in one weakest process, the toxic effect such as mortality will manifest. Thus, one can regard the series of processes as a large number of links and its failure is determined by the lowest strength of all the links. Results from probability theory indicate that the distribution of the minimum of a set of quantities has a particular limiting form. The Weibull distribution satisfies this limiting form for minima.

With these models, the relationship among time, concentration, and percent mortality was constructed, and if the values of any two variables were given, the third could be estimated. For the purpose of illustration, the response contours combining these three factors based on the models are shown in Figure 2-4. The conventional 48 h LC50 values and their 95% fiducial limits are also shown there. Compared with the single LC50 value, the response surface allows estimation of the concentration killing a certain proportion of amphipods at any time within the experimental range. As for the post exposure models, not only the effect of recovery duration, but also the effect of former Cu exposure concentration can be quantified.

4. The importance of incorporating latent mortality and exposure duration into current ecotoxicology studies

The conventional LC50 methods tend to minimize the effects of covariates by controlling all the experimental conditions except concentration. Exposure duration is considered peripherally and is often fixed. Consequently, information generated for all other times is lost, limiting the ability to predict toxicant effects on field populations. The survival analysis used in this study is a better approach than point estimation for avoiding this shortcoming. Predictions from survival models are also more useful than those from the conventional LC50 method because effects of other covariates such as exposure time, and effects of latent mortality and pulsed exposures, can be quantified more efficiently.

When the LC50 metric was introduced into mammalian toxicology, the primary interest was quantifying relative chemical toxicity. When the method was adopted by ecotoxicologists, the toxic inferences should have been put into a broader, ecological context. It is inappropriate for ecotoxicologists to focus on lethal effects during the exposures only. Latent mortality should be taken into consideration, especially when relating laboratory effects to those occurring in the field. For two chemicals whose 48 h LC50 values are the same for a certain species, their effects on a field population may be quite different because of the different levels of latent mortality. Copper sulfate and NaPCP results shown here illustrate this point. Recovery can be slow for toxicants like Cu that cause cumulative damage or have slow elimination. Therefore, if the Cu concentration was high enough to cause pronounced latent mortality, the proportion of exposed individuals dying will be much higher than the proportion projected with the LC50 value, and the species population may be at a higher risk of local extinction than suggested by the LC50 value. For toxicants with no significant latent mortality effect,

such as NaPCP, there is a trivial difference between the conventional and the complete metric of mortality. There will be less possibility for a population going locally extinct and less attention could be paid to its latent lethal effects. Therefore, we suggest that observation should be continued after exposure ends for some toxicants and that latent mortality information should be included in estimates of lethal consequences to field populations. Survival analysis is a useful means of quantifying mortality during and after exposure ends.

E. Conclusion

Different levels of latent mortality occurred after 48 h of $CuSO_4$ or NaPCP exposures. Because the nature of toxicant and exposure concentration affect latent effects, it is important to include latent mortality when comparing toxicities of chemicals and relating laboratory-derived metrics of toxicity to mortality in field populations. Survival analysis efficiently models such latent mortalities. Use of survival analysis to model both exposure and post exposure effects does not exclude calculating the conventional LC50. Furthermore, it can include several covariates in the model and consequently enhance our predictive capabilities for field populations. The current bioassay protocols could be extended to better include both exposure duration and latent mortality.

Table 2-1. The pH values, dissolved oxygen (DO) concentrations, and water temperatures of the $CuSO_4$ and NaPCP exposure media. The pH values were measured for both newly prepared and 12-h exposed water

	$CuSO_4$	NaPCP
pH (median)	8.10 (range=7.89 – 8.27, *n*=154)	8.19 (range=8.13 – 8.28, *n*=100)
DO (mean ± standard deviation, *n*=20, mg/L)	7.47 ± 0.15	7.57 ± 0.10
Water Temperature (mean ± standard deviation, *n*=30, °C)	22.97 ± 0.09	23.10 ± 0.29

Table 2-2. Measured concentrations of total dissolved Cu and NaPCP during the 48 h exposures

	Experiment #	Toxicant concentrations (mg/L, mean ± standard deviation, n = 8)			
		Treatment 1	Treatment 2	Treatment 3	Treatment 4
Dissolved Cu	1	0.19±0.02	0.28±0.03	0.35±0.04	0.53±0.06
	2	0.13±0.02	0.22±0.03	0.30±0.04	0.47±0.06
	3	0.13±0.02	0.21±0.03	0.29±0.04	0.45±0.05
NaPCP	1	0.20±0.08	0.36±0.04	0.51±0.03	0.77±0.07
	2	0.20±0.05	0.33±0.03	0.51±0.05	0.81±0.03
	3	0.19±0.02	0.32±0.06	0.50±0.02	0.79±0.05

Table 2-3. The Akaike's information criterion (AIC) values and the best-fit accelerated failure time models for $CuSO_4$ experiments. All the listed coefficients were significantly different from 0 ($p<0.001$)

Experiment		AIC values for each distribution [a]				Model [b]
		Exponential	Log-logistic	Log-normal	Weibull	
1	General [c]	315.6	282.0	**279.0**	287.2	$\ln T^{[d]}=3.33-1.39\times \ln C^{[e]}+0.73\times L$
	During [f]	173.2	123.0	124.6	**121.6**	$\ln T=3.64-0.58\times \ln C+0.21\times L$
	After [g]	232.6	231.0	**230.6**	232.2	$\ln T=2.65-1.99\times \ln C+1.28\times L$
2	General	395.2	361.0	**358.6**	369.6	$\ln T=3.33-0.98\times \ln C+0.80\times L$
	During	211.8	186.4	187.6	**186.2**	$\ln T=3.37-0.89\times \ln C+0.40\times L$
	After	320.2	320.8	**319.4**	322.2	$\ln T=2.92-1.17\times \ln C+1.50\times L$
3	General	421.2	411.2	**404.6**	423.0	$\ln T=2.64-1.54\times \ln C+1.24\times L$
	During	309.0	280.4	**275.4**	285.6	$\ln T=2.92-0.90\times \ln C+0.76\times L$
	After	151.2	152.0	**150.8**	152.4	$\ln T=3.70-1.82\times \ln C+2.19\times L$

[a] The values in bold denote the smallest AIC and the best-fitting distribution, [b] The best-fit model is indicated by the AIC values, [c] Model produced by fitting all data, including post exposure mortality, [d] Time-to-death, [e] Concentration, [f] Model produced by fitting only data from the exposure phase of the experiments, [g] Model produced by fitting only the data generated after the exposure ended

Table 2-4. The Akaike's information criterion (AIC) values and the best-fit accelerated failure time models for NaPCP experiments. All the listed coefficients were significantly different from 0 (p<0.001)

Experiment		AIC Values for Each Distribution [a]				Model [b]
		Exponential	Log-logistic	Log-normal	Weibull	
1	General [c]	382.7	359.6	**358.6**	367.7	$\ln T^{d}=3.51-1.11\times \ln C^{e}+0.90\times L$
	During [f]	309.5	248.7	259.7	**238.3**	$\ln T=3.67-0.56\times \ln C+0.34\times L$
	After [g]	112.0	110.3	**108.8**	110.6	/ [h]
2	General	343.7	317.7	**313.2**	332.7	$\ln T=3.18-1.53\times \ln C+0.83\times L$
	During	286.2	224.0	**218.0**	229.1	$\ln T=3.20-0.99\times \ln C+0.53\times L$
	After	**72.5**	73.5	72.7	73.7	/
3	General	289.0	280.4	**276.1**	285.2	$\ln T=3.89-1.29\times \ln C+1.04\times L$
	During	240.1	190.7	191.2	**189.0**	$\ln T=3.79-0.59\times \ln C+0.32\times L$
	After	**36.7**	38.7	39.4	38.6	/

[a] The values in bold denote the smallest AIC and the best-fitting distribution, [b] The best-fit model is indicated by the AIC values, [c] Model produced by fitting all data, including post exposure mortality, [d] Time-to-death, [e] Concentration, [f] Model produced by fitting only data from the exposure phase of the experiments, [g] Model produced by fitting only the data generated after the exposure ended, [h] The best-fit models were not listed because the coefficients of the natural log of concentration were not significantly different from 0 (α=0.05)

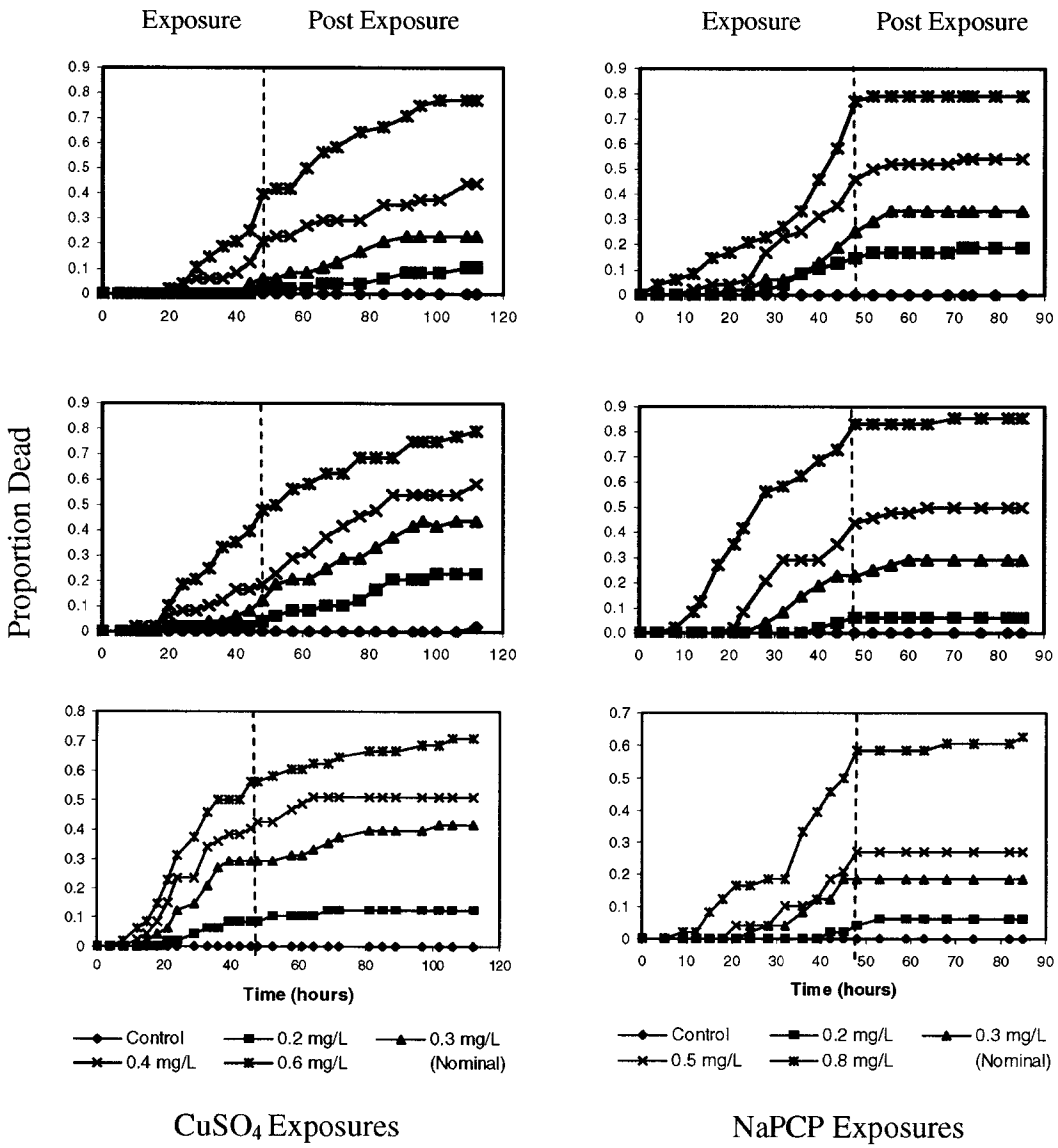


Figure 2-1. The cumulative proportions of amphipods dead through time for the $CuSO_4$ and NaPCP exposures. The groups of lines indicate different nominal toxicant concentrations. (Refer to Table 2-2 for measured toxicant concentrations.) The dashed lines at 48 h separate exposure and post exposure periods.

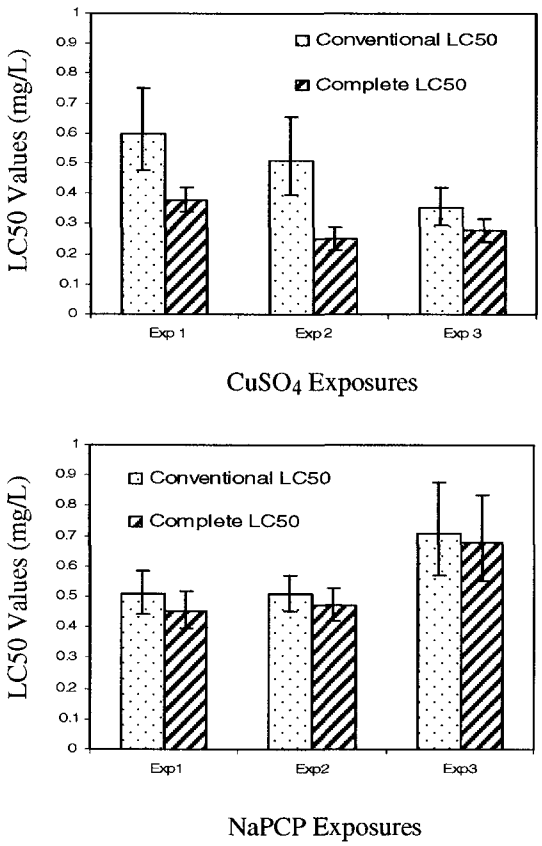


Figure 2-2. Conventional (during the exposure) and complete (exposure plus post exposure) LC50 values for the $CuSO_4$ and NaPCP experiments. The error bars indicate their 95% fiducial limits and Exp 1, 2, and 3 denote the first, second, and third experiment, respectively.

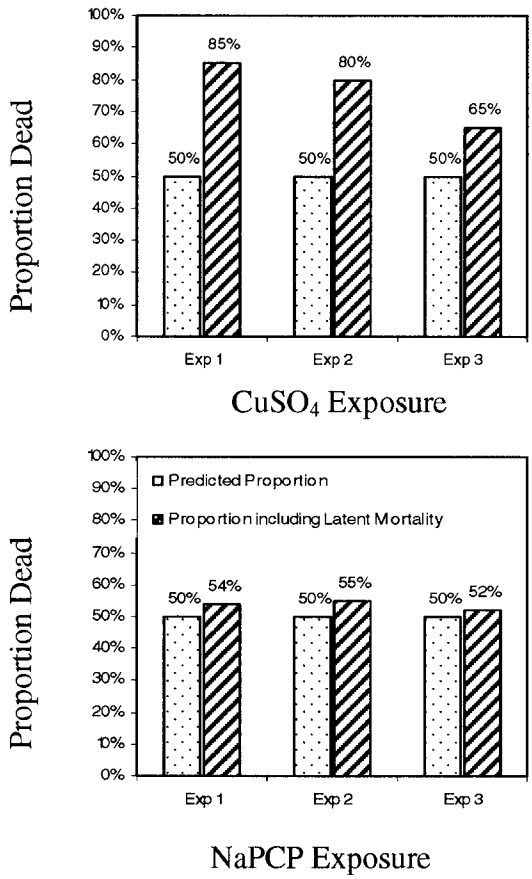


Figure 2-3. The predicted proportion dead at the conventional LC50 values and the proportion dead after including latent mortality for the $CuSO_4$ and NaPCP experiments. Exp 1, 2, and 3 denote the first, second, and third experiment, respectively.

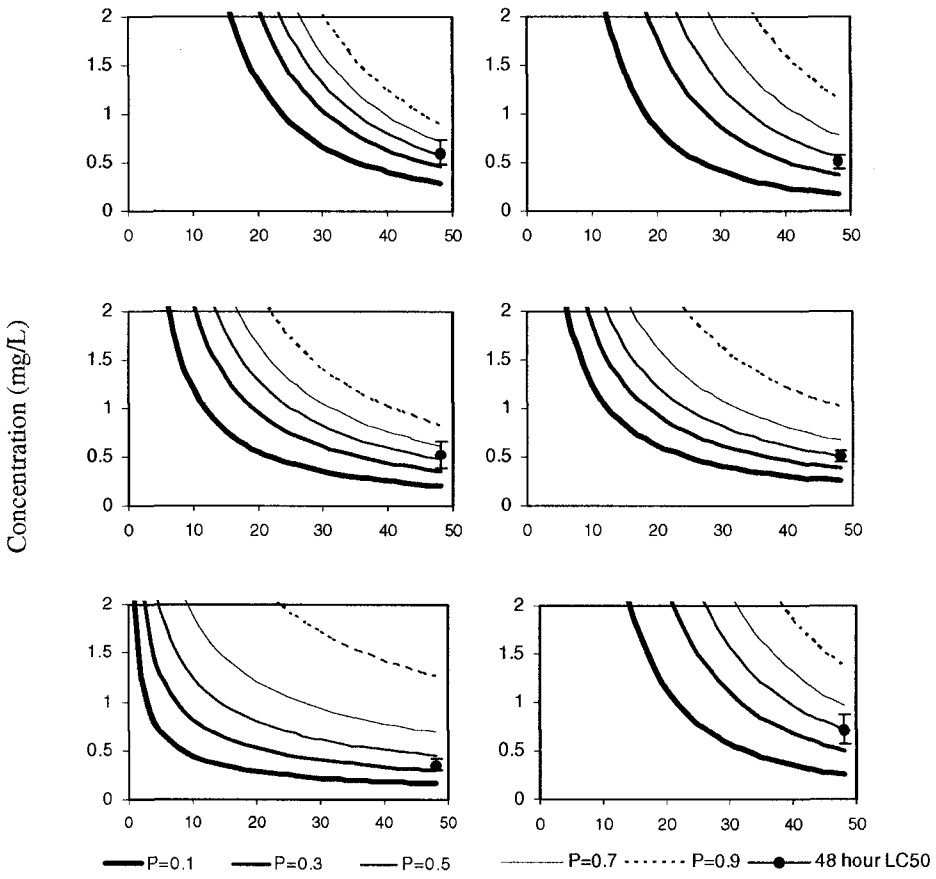


Figure 2-4. Response contours generated from survival models of 48 h exposures to $CuSO_4$ and NaPCP. The lines indicate different proportions dead. The 48 h LC50 values and their 95% fiducial limits were also shown.

CHAPTER III. PULSED EXPOSURE: EFFECTS OF PULSE DURATION AND RECOVERY TIME BETWEEN PULSES

A. Introduction

Although most pulsed exposure studies have been conducted on mammals, increasing attention is being paid to effects of pulsed toxicant exposures characteristic of spills, episodic surface runoff, applications of agrochemicals, and many industrial discharges. For most of the time, aquatic organisms are exposed to background levels of toxicants with lethal concentrations being reached periodically. Prediction of lethal consequences of pulsed exposure through conventional LC50 methods is often difficult because conventional methods are associated with a fixed duration and constant concentration, as was discussed in Chapter II. They also do not routinely include latent mortality. For these reasons, they may not be adequate for prediction of pulsed exposure effects for which concentration, duration, and pulse frequency change through time.

Some researchers realize the inadequacy of the conventional methods and have begun exploring pulsed exposure effects in different ways. Allin and Wilson (2000) investigated the sub-lethal acclimation effect on the 4 d lethal pulsed exposure to aluminum (Al) with juvenile rainbow trout, *Oncorhynchus mykiss*. The swimming behavior, mortality, and hematological changes were compared between the Al acclimated and naive groups. The results suggested that previous exposure to sub-lethal levels of Al in the natural environment could be an important factor abating Al impact.

The effects of pulse frequency and interval among multiple chlorpyrifos exposures on mortality, mobility, and natality of *Daphnia magna* were evaluated (Naddy and Klaine 2001). It was suggested that the organism could recover from the previous lethal chlorpyrifos exposure if there was adequate time for recovery between exposures. Clark et al. (1986) found that multiple-pulse exposures of substances were less toxic to aquatic organisms than continuous exposures of equal total duration. The reasons may be that some detoxification, repair, or elimination occurring during the non-toxic period can reduce the toxic effects of the earlier exposures (Parsons and Surgeoner 1991) and is dependent on the length of time between pulses (Wang and Hanson 1985). Reinert et al. (2002) discussed the tools for deciding whether time-varying exposures are relevant in a particular risk assessment and approaches for laboratory toxicity testing, modeling, and risk characterization. However, most of these studies either made the predictions semi-quantitatively with statistical method such as conventional analysis of variance (ANOVA), or did not incorporate duration effectively. Quantitative models that incorporate duration effect are needed to make better predictions.

In addition to exposure concentration and toxicant modes of action as discussed in Chapter II, other variables such as pulse duration and recovery time between pulses can be important in order to better predict field population fate in pulsed exposures. 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 time for recovery could result in better recovery, and therefore, the effects of subsequent pulses will be less dependent on the previous exposure. To explore these hypotheses, I conducted laboratory studies that quantify effects of a subset of these factors.

Specifically, I asked: (1) is there any effect of exposure duration on latent mortality, (2) can the complete recovery time (i.e., the time that an organism needs to return to its original level of toxicant resistance) be predicted, and (3) what is the relationship between recovery time between pulses and mortality during the second pulse. Copper sulfate and NaPCP were used because they have contrasting levels of latent mortality. To address the first question, *H. azteca* was exposed to two toxicant concentrations for three 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.

B. Methods

1. Effect of exposure duration on latent mortality

The amphipod culture, maintenance, and general exposure design were similar to those described before. One to two weeks old amphipods were sieved and acclimated in the RMHRW for 6 d before the exposures began. Experimental concentrations, durations, and animal sample sizes were found based on preliminary experiments and the test results of Chapter II. In the formal experiments, the $CuSO_4$ and NaPCP were dissolved in RMHRW to make two nominal concentrations for each (dissolved Cu: 0.8 and 1.0 mg/L, NaPCP: 0.4 and 0.6 mg/L). The amphipods were exposed 20, 38, and 61 h to $CuSO_4$, and 20, 40, and 60 h to NaPCP. Both newly prepared and exposed water samples were collected for toxicant concentration and pH measurement. Samples for

temperature and DO were taken periodically. After exposures, survivors were transferred to fresh RMHRW and their latent mortality observed every 3 to 6 h. The experiments ended when no more mortality was evident. Red maple leaf was provided throughout the experiment as food. Both NaPCP and $CuSO_4$ experiments were done twice. In each experiment, 36 amphipods were used as control animals and maintained in toxicant-free water. They were transferred to appropriate wells the same way the treatment amphipods were transferred to ensure that the difference was not due to handling.

The nonparametric method log-rank test (SAS® procedure LIFETEST, SAS 1999) was used initially to test whether there was any significant difference between the survivals of the replicates. The fully parametric, accelerated failure time model was then used to explore the effects of exposure duration. Exposure concentration and duration were fit as continuous variables (SAS® procedure LIFEREG, SAS 1999):

$$\ln t_i = f(\textit{concentration}) + g(\textit{duration}) + \varepsilon_i, \qquad (3.1)$$

where t_i is the time-to-death during the post-exposure period, f (*concentration*) and g (*duration*) are some functions of the former exposure concentration and duration, and ε_i is the error term.

2. Effect of recovery time on mortality during a second exposure

The amphipods of 1 to 2 weeks old were sieved and acclimated in the RMHRW for 6 d before beginning the exposures to ensure enough acclimation to the experimental water. Several preliminary experiments were conducted to find the adequate exposure concentrations, durations, and recovery intervals. In the experiments,

approximately 350 amphipods were exposed to nominal toxicant concentrations (dissolved Cu: 1.1 mg/L, NaPCP: 1.5 mg/L) for 12 h, when about 10% of the exposed animals were dead. No food was provided during the exposures. After that, all survivors were randomly assigned to different recovery time groups and allowed to recover. Based on preliminary experimental results, 0, 24, 48, and 72 h for $CuSO_4$, and 0, 4, 8, and 14 h for NaPCP, were chosen. Red maple leaves were provided during recovery. Immediately after those recovery times, the survivors were exposed to the toxicant solution with the same nominal concentration as the first exposure for another 12 h. Their mortalities were checked every 1 h or so. At the beginning and end of each experiment, two groups of naive amphipods of the same age as treatment groups were set as reference animals and exposed to the same toxicant concentration for 12 h to establish the background level mortality. Because the mortality under identical exposure conditions could be different as amphipods age, these similar-age reference groups were used to check the potential confounding effect of age. All the animals in the reference groups were handled the same way as those in the treatment groups to ensure the similar handling stress. Throughout the experiment, 36 amphipods were set as the control group and maintained in toxicant-free water. They were transferred to appropriate wells the same way treatment amphipods were transferred to ensure that the difference was not due to handling. Both NaPCP and $CuSO_4$ experiments were repeated three times.

In each experiment, the survival data for the two reference groups were first compared with nonparametric log-rank test to check whether there is significant difference in mortality (SAS® procedure LIFETEST, SAS 1999). If it resulted in

insignificant difference, the reference data were combined and fit to the accelerated failure time model (SAS® procedure LIFEREG, SAS 1999):

$$\ln t_{Ri} = a_R + \varepsilon_{Ri} \quad (3.2)$$

where t_{Ri} is the time-to-death of the reference animals, a_R is the intercept, and ε_{Ri} is the error term. 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:

$$\ln t_{Ti} = a_T + b_T \times rt_i + \varepsilon_{Ti} \quad (3.3)$$

where t_{Ti} is the time-to-death of the treatment animals, a_T is the intercept, b_T is 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 predicted complete recovery time, the value of rt_i at which mathematically the survival time of the treatment group was equal to that of the reference group at the specific reference mortality level, could be calculated by subtracting (3.2) from (3.3). The effect of recovery time can be statistically tested with the coefficient b_T in (3.3).

C. Results

1. Water chemistry

The RMHRW for all solutions had an alkalinity of 56 ± 2 mg/L as $CaCO_3$ (mean ± standard deviation, n = 8) and a median pH of 8.18 (range: 7.82 – 8.23, n = 14). The pH values, DO concentrations, and water temperatures during the experiments are summarized in Table 3-1. Tables 3-2 and 3-3 show the measured dissolved Cu and

NaPCP concentrations. The toxicant concentrations for controls and water during the post exposure and recovery periods were below the method detection limits (Cu: 7 μg/L, NaPCP: 0.15 mg/L).

2. Effect of exposure duration on latent mortality

The raw mortality data of all the exposure duration experiments are shown in Appendix 4 and 5. The total proportions of amphipods dead during the exposures for each treatment group are shown in Table 3-4. Predictably, higher concentration and longer duration resulted in higher mortality during all exposures. The log-rank test showed no statistically significant difference between the duplicates for both toxicants (α=0.05); therefore, the data from the duplicates were pooled for the following statistical analysis. The cumulative mortality of amphipods at each observation time during the post exposure periods were plotted in Figure 3-1. The total latent mortality of NaPCP experiments did not exceed 7%, as opposed to 20% to 60% for the $CuSO_4$ experiments depending on concentration. The NaPCP latent mortality happened soon after the exposures ended, but that of $CuSO_4$ continued for longer than 40 h. This reinforced the conclusion reached in Chapter II about different latent mortality effects of $CuSO_4$ and NaPCP. If the latent mortality data were fit to accelerated failure time models, natural log transformations of duration and concentration were used because the associated AIC values were smaller than those without transformation and they are the most frequently used metameters in the literature (Table 3-5). For both toxicants, a log-normal distribution proved to be the best. Only the coefficient of ln (concentration)

of $CuSO_4$ was significantly different from zero (α=0.05). In all the experiments, the control mortalities were less than 5%.

3. Effect of recovery time on mortality during a second exposure

The raw mortality data of all the recovery time experiments are shown in Appendix 6 through Appendix 11. Within each experiment, the reference survivals showed no significant difference (log-rank test, α=0.05), indicating no detectable age effect on mortality. This allowed the combination of the two reference group data within one experiment as background mortality of the exposure. The average mortalities of the reference groups were 7%, 11%, and 12% for 3 $CuSO_4$ experiments and 18%, 15%, and 5% for 3 NaPCP experiments. During the recovery periods, only a minimal number of amphipods died for NaPCP but many died for $CuSO_4$, again reinforcing the conclusion of Chapter II.

The percent mortalities during the second exposures are plotted in Figure 3-2. Recovery of the ability to resist the lethal effects through time 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 the references, but those with longest recovery times were not (α=0.05, log-rank test; one exception was the 14 h recovery time group in the third NaPCP experiment, for which p=0.02). Weibull proved to be the best-fit model for the treatment groups of all experiments. For the reference groups, the best-fit models were log-normal, except that Weibull has the lowest AIC for the second NaPCP experiment. Because the difference between the AICs of log-normal (135.7) and

Weibull (135.3) was small, I used a log-normal distribution as well. The coefficients of recovery time were significantly different from zero (α=0.05) (Table 3-6 and 3-7). The three complete recovery times for each toxicant were then averaged to calculate the mean complete recovery time and the associated standard deviation ($CuSO_4$: 86 ± 9 h, NaPCP: 19 ± 8 h). The control mortalities were less than 5% in all experiments.

D. Discussion

1. Effect of exposure duration on latent mortality

The pattern of latent mortality is notionally related to the amount of damage caused during exposure and the extent of recovery after exposure ends. The recovery of the exposed organism can be quick for toxicants such as NaPCP, which appears to cause less cumulative damage than $CuSO_4$. Therefore, the latent mortality can be low and relatively independent of exposure concentration and duration. The results of NaPCP experiments of this study 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. The latent mortality can be high and related to the cumulative damage, which is a function of both concentration and duration. (See Chapter II for detailed discussion of concentration effects on latent mortality and the toxicological mechanisms for $CuSO_4$ and NaPCP.) Pascoe and Shazili (1986) found longer cadmium exposure duration resulted in higher mortality after the exposure. Latent mortality of beetles increased as the original diatomaceous earth exposure interval increased (Arthur 2001). The results of the $CuSO_4$ experiments

showed significant effect of concentration, with higher concentration resulting in higher latent mortality, and were consistent with the previous study (Chapter II). However, exposure duration effect was not significant. One explanation is that, although both concentration and duration do affect latent mortality, duration plays a less prominent role than concentration under the test conditions used here. The explanation can be furthered that the exposure duration-latent mortality curve is curvilinear. The selected durations fell in the range that the increase of exposure duration resulted in a statistically insignificant increase of latent mortality. Therefore, the duration effect can probably be ignored if predictions were being made within this experimental range. Regardless, I speculate that the effects of exposure duration on latent mortality might still need to be considered under other concentrations and durations.

2. Effect of recovery time on mortality during a second exposure

Under some circumstances, provided enough time between pulses, the organisms can recover from the previous pulse through processes such as detoxification, elimination, and healing of damaged tissue. Wang and Hanson (1985) suggested that the pulsed exposure toxicity was dependent on the time between pulses. Studies (Mancini 1983, Wang and Hanson 1985, Clark et al. 1986, Kallander et al. 1997, Naddy and Klaine 2001) showed that with the same total exposure duration and enough recovery time, the multiple pulsed exposures were less toxic than continuous ones. However, in some cases, the effects were irreversible and recovery did not occur (Parson and Surgeoner 1991, Van der Hoeven and Gerritsen 1997, Naddy and Klaine 2001). To explain this, Naddy and Klaine (2001) suggested that the organisms accumulated an

amount of toxicant that exceeded the organism's critical body burden, or the recovery period was not long enough. In this study, the mortalities of the groups with longest recovery times for both toxicants were not significantly different from the reference groups. This indicated that the amphipods could return to a state similar to their original toxicant resistance state provided sufficient recovery time between pulses. Statistical analysis showed no significant difference between reference group mortalities of the two toxicants (MINITAB® release 14.1, ANOVA, F=0.40, p=0.56). However, to recover to similar background mortality levels (about 10%), the complete recovery time for $CuSO_4$ was almost 5 times that of NaPCP, suggesting a toxicant-dependent recovery process. Longer recovery time is needed for toxicants such as $CuSO_4$ that notionally cause considerate cumulative tissue damage and have significant latent effects. The discussion can be furthered based on the results and discussion of Chapter II. For toxicants that do not have significant concentration effects on latent mortality, the increased background mortality level associated with increasing the exposure concentration during the first exposure will not change the complete recovery time much. However, for toxicants that have significant concentration effects on latent mortality, the increase of background mortality level associated with increasing the exposure concentration will even prolong the complete recovery time.

Though the pulsed exposure issues have been addressed in several studies, none of them fully quantified the effect of recovery time. With the accelerated failure time models in Table 3-6 and 3-7, one can predict the effect of recovery time on time-to-death during the second exposure. Taking the first NaPCP experiment as example, Figure 3-3 shows that the time-to-death for a certain proportion of animals to die during

the second exposure can be predicted for any recovery time within the experimental range. Figure 3-4 shows the 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. Variables such as concentration can be added to the model if needed. It is realized that some tolerance mechanism could have been induced during the first exposure, e.g., production of metallothionein or glutathione, and acclimation could have played roles in the process. 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 too.

3. The importance of incorporating pulsed exposure scenarios into current toxicity tests

The need to include latent mortality and exposure duration into current ecotoxicology studies was discussed in Chapter II. The results of this study reinforced the point that latent lethal effect should not be ignored, and more attention should be paid to assessing the effects of variables affecting latent mortality. For NaPCP and similar toxicants, relatively short times are needed for the organisms to recover to background levels of resistance. Much longer times are required for $CuSO_4$ and similar toxicants. Thus, under similar exposure scenarios, the exposed field population would be more likely to experience local extinction. Also, the episodic nature of the contaminants in aquatic ecosystems warrants the incorporation of pulsed exposure

scenarios into current ecotoxicology framework. The nature of the toxicant, recovery times between pulses, and previous exposure concentration and duration can affect the fate of organisms during subsequent exposures. It is important to further characterize the effects of these factors in order to better assess the consequences of pulsed exposure.

E. Conclusion

The effects of both exposure duration and recovery time during pulsed exposures were tested in this study. No significant effect of exposure duration on latent mortality was found but more powerful tests under other experimental conditions are warranted to fully explore this issue fully. Significant effect of recovery time on mortality during a second exposure was found for both toxicants. For different toxicants that cause different cumulative damage, the complete recovery time needed was different. Environmental risk assessment involved with pulsed exposures should take these effects into consideration.

Table 3-1. The pH values, dissolved oxygen (DO) concentrations, and water temperatures of the $CuSO_4$ and NaPCP exposure media for the exposure duration (ED) and recovery time (RT) experiments.

Experiment		pH (median, range, *n*)	DO (mean ± standard deviation, mg/L, *n*)	Water Temp. (mean ± standard deviation, °C, *n*)
ED	$CuSO_4$	8.10, 7.95 – 8.21, 24	7.4 ± 0.5, 10	22.8 ± 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	7.6 ± 0.1, 30	23.2 ± 0.2, 30

Table 3-2. The dissolved Cu and NaPCP concentrations for the exposure duration experiments. The concentrations of the treatments with the same nominal concentration but different durations were averaged.

Experiment		Toxicant concentration (mean ± standard deviation, mg/L)	
Dissolved Cu	1	0.66 ± 0.07	0.82 ± 0.12
(*n*=8)	2	0.63 ± 0.06	0.83 ± 0.08
NaPCP	1	0.36 ± 0.06	0.62 ± 0.07
(*n*=15)	2	0.43 ± 0.01	0.68 ± 0.04

Table 3-3. The dissolved Cu and NaPCP concentrations (mean ± standard deviation, mg/L) for the recovery time experiments. The concentrations for first exposures and second exposures with different recovery times were averaged separately.

		1st	2nd (1)	2nd (2)	2nd (3)	2nd (4)
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

Table 3-4. The percentages of amphipods dead (P) at the end of exposures for the exposure duration experiments.

$CuSO_4$	Treatment	P (%)	NaPCP	Treatment	P (%)
	20 h 0.8 mg/L	5.0		20 h 0.4 mg/L	0.0
	20 h 1.0 mg/L	16.7		20 h 0.6 mg/L	13.9
Replicate	38 h 0.8 mg/L	15.6	Replicate	40 h 0.4 mg/L	16.7
1	38 h 1.0 mg/L	66.7	1	40 h 0.6 mg/L	37.5
	61 h 0.8 mg/L	48.0		60 h 0.4 mg/L	60.4
	61 h 1.0 mg/L	77.5		60 h 0.6 mg/L	67.2
	20 h 0.8 mg/L	7.5		20 h 0.4 mg/L	0.0
	20 h 1.0 mg/L	16.7		20 h 0.6 mg/L	8.6
Replicate	38 h 0.8 mg/L	15.7	Replicate	40 h 0.4 mg/L	37.5
2	38 h 1.0 mg/L	58.3	2	40 h 0.6 mg/L	50.0
	61 h 0.8 mg/L	54.0		60 h 0.4 mg/L	62.5
	61 h 1.0 mg/L	75.0		60 h 0.6 mg/L	66.7

Table 3-5. The best-fit accelerated failure time models and the associated distributions during the latent periods of exposure duration experiments. The coefficients in bold denote that they were not significantly different from zero.

Experiment	Model	Distribution
$CuSO_4$	$\ln T$ [a]$=4.65-5.83\times\ln C$ [b]$\mathbf{-0.46}\times\ln D$ [c]$+2.13\times L$ [d]	Log-normal
NaPCP	$\ln T=22.26\mathbf{+1.87}\times\ln C\mathbf{-2.32}\times\ln D+4.73\times L$	Log-normal

[a] T=time-to-death (h), [b] C=concentration (mg/L), [c] D=duration (h), [d] L varies with the proportion dead for which prediction is being made

Table 3-6. The best-fit accelerated failure time models for the treatment (TRT) and reference (REF) groups, the associated distributions, and the calculated complete recovery time (complete RT) during the second exposures of the $CuSO_4$ recovery time experiments.

		Model	Distribution	Complete RT
1	TRT	$\ln T^{a} = 2.58 + 0.01 \times RT^{b} + 0.35 \times L^{c}$	Weibull	84 h
	REF	$\ln T = 3.73 + 0.83 \times L$	Log-normal	
2	TRT	$\ln T = 2.62 + 0.01 \times RT + 0.51 \times L$	Weibull	96 h
	REF	$\ln T = 3.21 + 0.59 \times L$	Log-normal	
3	TRT	$\ln T = 2.72 + 0.01 \times RT + 0.50 \times L$	Weibull	79 h
	REF	$\ln T = 2.88 + 0.34 \times L$	Log-normal	

[a] T=time-to-death (h), [b] RT=recovery time (h), [c] L varies with the proportion dead for which prediction is being made

Table 3-7. The best-fit accelerated failure time models for the treatment (TRT) and reference (REF) groups, the associated distributions, and the calculated complete recovery time (complete RT) during the second exposures of the NaPCP recovery time experiments.

		Model	Distribution	Complete RT
1	TRT	$\ln T^{a} = 2.67 + 0.07 \times RT^{b} + 0.78 \times L^{c}$	Weibull	16 h
	REF	$\ln T = 3.40 + 0.99 \times L$	Log-normal	
2	TRT	$\ln T = 2.17 + 0.13 \times RT + 0.77 \times L$	Weibull	13 h
	REF	$\ln T = 3.77 + 1.22 \times L$	Log-normal	
3	TRT	$\ln T = 2.88 + 0.05 \times RT + 0.61 \times L$	Weibull	28 h
	REF	$\ln T = 5.05 + 1.56 \times L$	Log-normal	

[a] T=time-to-death (h), [b] RT=recovery time (h), [c] L varies with the proportion dead for which prediction is being ma

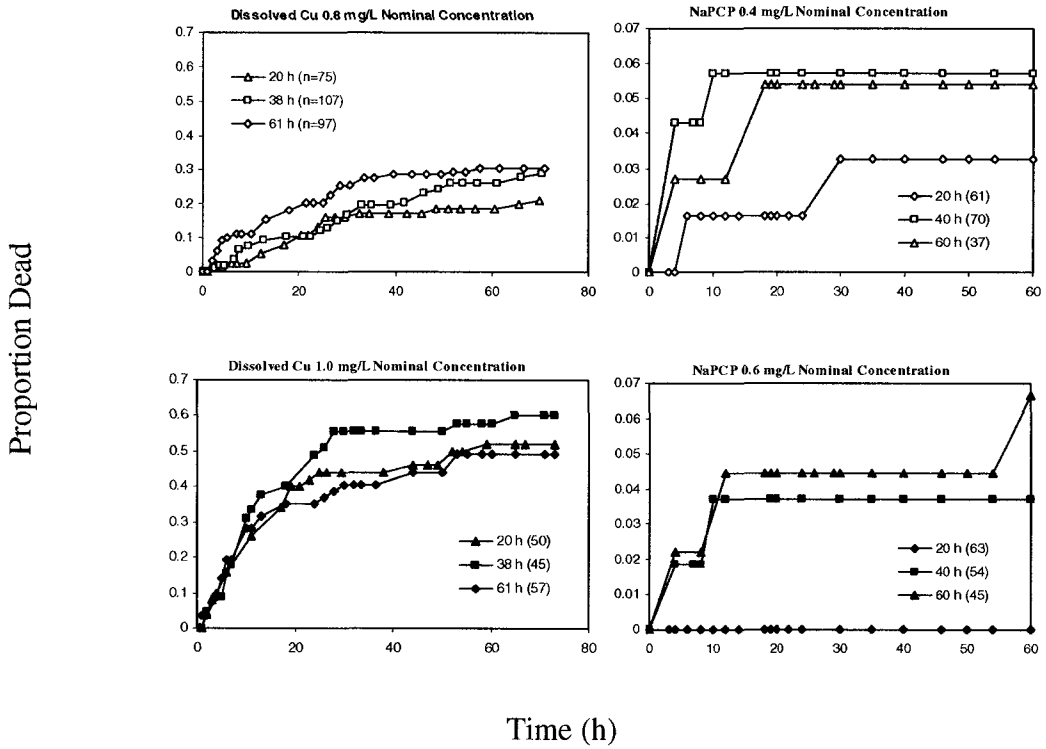


Figure 3-1. Cumulative proportions of amphipods dead through time after the $CuSO_4$ and NaPCP exposure duration experiments. The lines within each graph indicate different exposure durations. The concentrations denoted are nominal toxicant concentrations (for measured concentrations, see Table 3-2). The sample sizes were indicated in the brackets.

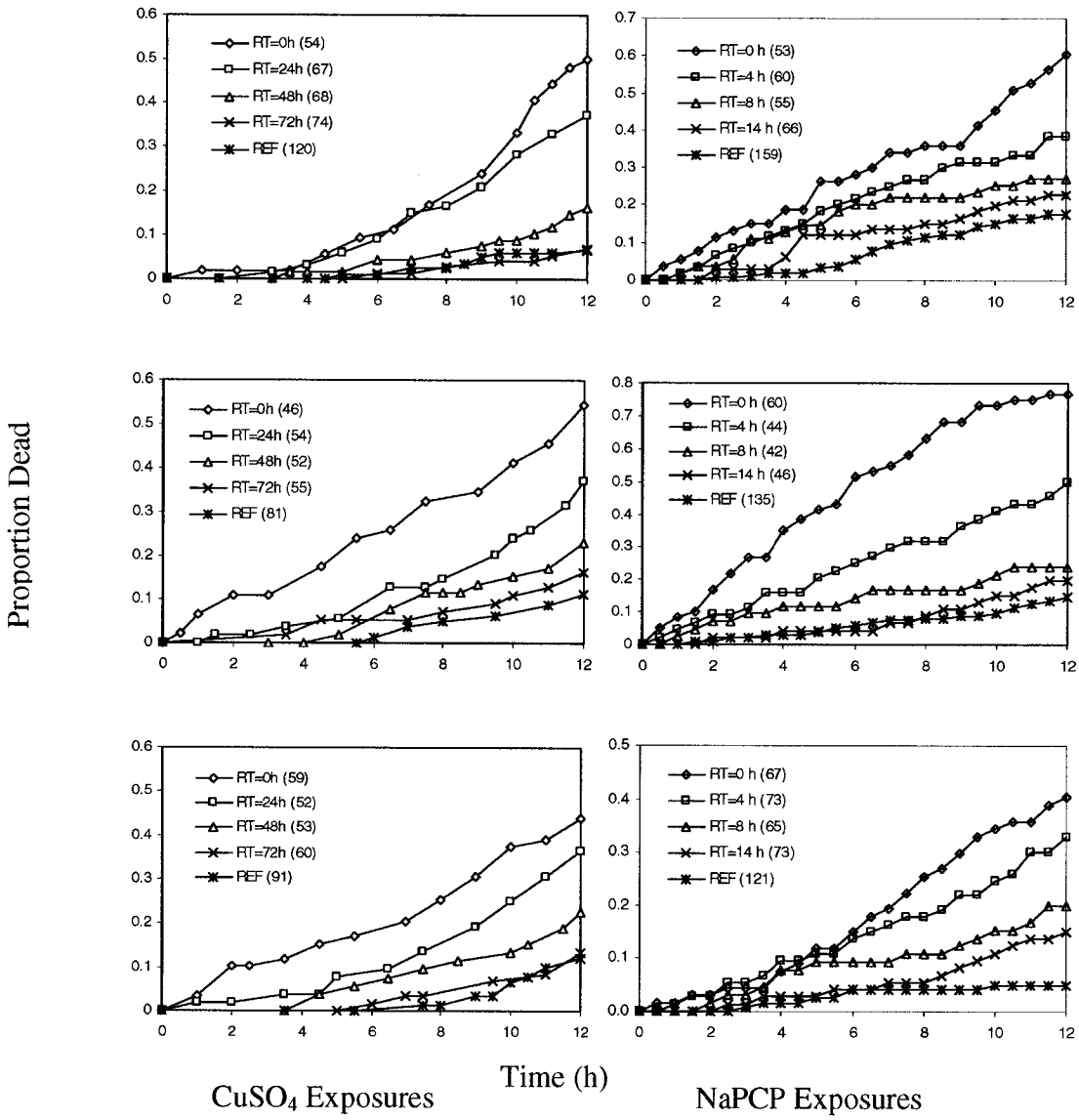


Figure 3-2. Cumulative proportions of amphipods dead through time for $CuSO_4$ and NaPCP recovery time experiments. The groups of lines indicate treatments with different recovery times (RT) and reference (REF) groups. The sample sizes were indicated in the brackets.

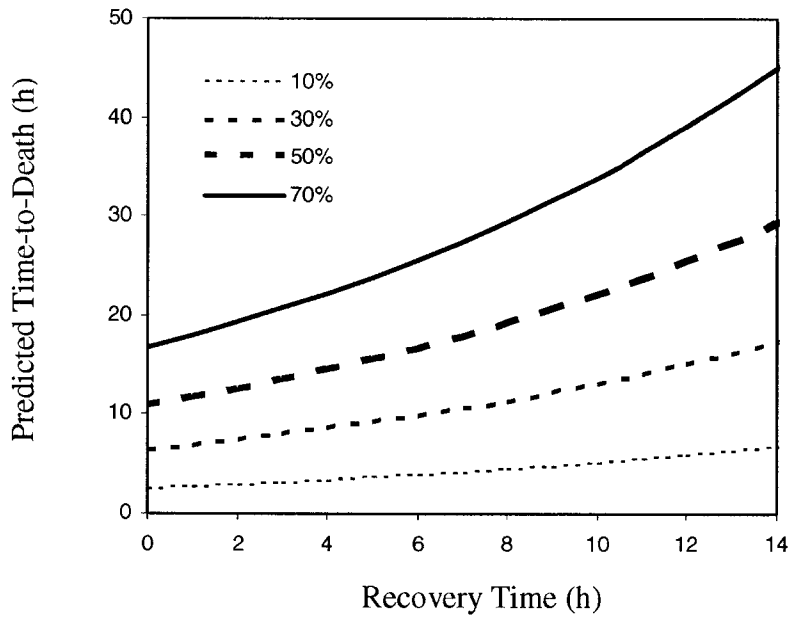


Figure 3-3. Taking the first NaPCP recovery time experiment as an example, the time-to-death for a certain proportion of animals to die during a second exposure for any recovery time within the experimental range can be predicted.

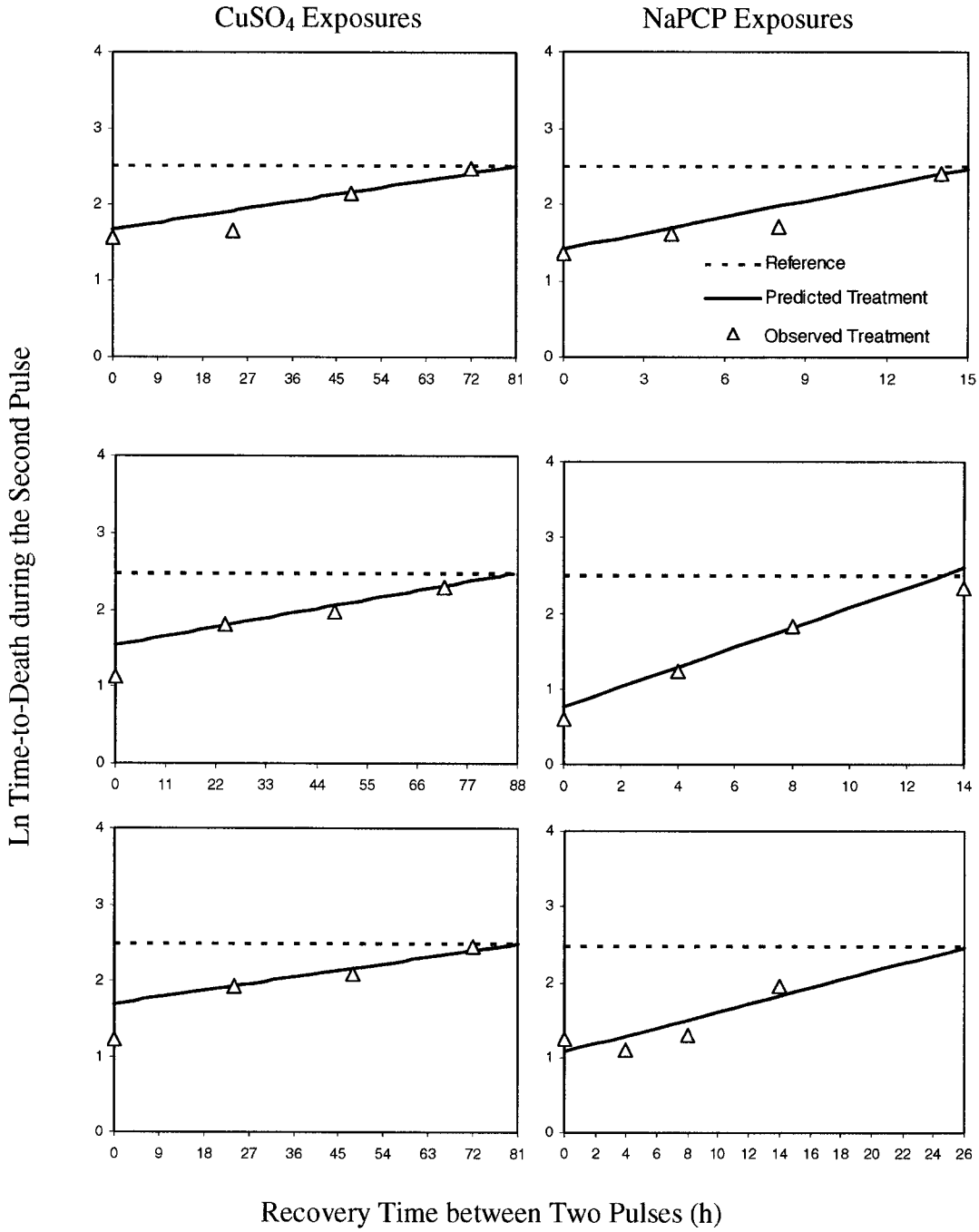


Figure 3-4. The observed and predicted times-to-death of the different recovery time groups, and the times-to-death of the reference groups for the $CuSO_4$ and NaPCP recovery time experiments. The points at which the two lines converge are the predicted complete recovery times.

CHAPTER IV. TEST THE UNDERLYING THEORY OF DOSE-RESPONSE MODEL WITH SURVIVAL ANALYSIS METHODS

A. Introduction

In mammalian toxicology and ecotoxicology, dose-response curves are often sigmoid. The probit approach is most widely applied to fitting such data. The dose or concentration is log transformed and the corresponding proportion affected is probit transformed:

$$\text{Probit (proportion affected)} = A \times \log (\text{concentration}) + B \qquad (4.1)$$

In 1933, Gaddum proposed to transform each proportion to its normal equivalent deviation (NED). The NED expresses a proportion responding in terms of standard deviations from the mean of a normal distribution (N: 0, 1). In 1934, Bliss (1935) proposed the division of the interval between 0.01% and 99.99% into units of normal deviation (probit) and modified the definition of the probit by adding 5 to the NED to avoid negative numbers:

$$\text{Probit} = \text{NED} + 5 \qquad (4.2)$$

Thus, the probit analysis assumes a log-normal dose-response curve. Soon after a probit-based model was introduced into toxicology, Gaddum postulated the individual effective dose (IED) theory to explain the log-normal model: every individual has a characteristic tolerance for the toxicant and an individual will be affected only if its IED is exceeded. The IED is log-normally distributed within a population (Bliss and Cattell

1943). That is, if λ is the intensity of the toxicant exposure, and dP is the proportion of the whole population consisting of individuals whose tolerances lie between λ and $\lambda+d\lambda$, and $f(\lambda)$ is the normal density function, then the distribution of tolerances can be expressed by

$$dP = f(\lambda)\, d\lambda \tag{4.3}$$

The proportion of all individuals responding to a given dose, λ_0 (tolerances less than λ_0) is

$$P = \int_0^{\lambda_0} f(\lambda)\, d\lambda \tag{4.4}$$

A common alternative dose-response transformation is based on a log-logistic model. Early supporters of this model questioned the IED concept, favoring a stochastic explanation instead. Notably, Berkson (1951) did a re-challenge experiment that did not support the IED concept. Barometric chamber experiments were conducted twice in which pilots were subjected to high-altitude conditions and their all-or-none responses of getting the "bends" were noted. Many individuals who fainted during the first challenge did not faint the second time, and many who did not faint the first time did faint the second time. Berkson pointed out that individuals may vary from time to time in their response to chemicals and tried to explain dose-mortality curves in terms of chance alone.

Though the log-normal and similar models have been repeatedly used to fit dose-response curves, there have been few careful testings of underlying mechanisms. The IED concept remains the only explanation presented in many publications for the log-normal model, e.g., Finney (1971). Only recently, Newman and McCloskey (2000) tested the hypothesis by exposing fish to benzocaine, PCP, or NaCl. They concluded

that neither the IED nor stochastic hypothesis alone was the sole or dominant explanation for the log-normal model; determination of the correct explanation is crucial to predicting consequences to populations after repeated or chronic exposures. Besides this, no other studies have been conducted. As will be seen in the discussion section of this chapter, resolution of this ambiguity is important in risk assessment involving pulsed exposures.

In this study, whether the IED theory is the dominant explanation for the probit model (or similar models such as the log-logistic) for both $CuSO_4$ and NaPCP was further tested. Three groups of amphipods were first exposed to lethal, sub-lethal, and zero concentrations of each toxicant, with enough recovery time between the two exposures. Then, all the survivors were exposed to lethal concentrations and their mortality functions during the second exposures compared with survival analysis method. The results were interpreted in terms of relative dominance of the two theories and the associated ecological relevance.

B. Methods

1. Exposure procedure

Amphipod culture and maintenance, toxicant solution preparation, exposure systems, and toxicant concentration measurement procedures were the same as those described in Chapter II and III. After several range-finding tests, the amphipods were randomly assigned to one of the three treatments: lethal, sub-lethal, and reference (nominal concentrations: 1.0, 0.4, and 0.0 mg/L of dissolved Cu; 1.4, 0.4, and 0.0 mg/L of NaPCP). In $CuSO_4$ experiments, because of the anticipated high latent mortality

caused by Cu, the amphipods were exposed only until approximately 15% of them died in the lethal group. In NaPCP experiments, the amphipods were exposed until about 40% of them died in the lethal group. All survivors were then transferred to water free of toxicant and maintained for periods of time ($CuSO_4$, 14 d; NaPCP, 10 d) that were considered sufficient to recover from the first exposures. During the recovery periods, the water was changed every other day. After that, all the survivors in the three treatments were transferred back to the toxicant solutions with nominal concentrations identical to those of the first lethal exposures. The mortalities during the second exposures were observed about every hour. Food (red maple leaves and commercial rabbit food) was only provided during the recovery periods. During all experiments, 36 amphipods were used as control animals and maintained in toxicant-free water. For the second lethal exposures of $CuSO_4$ experiments, the solutions were renewed every 12 h or so to compensate for any surface sorption of Cu. Both exposed and newly prepared water samples were collected for concentration measurement. Because previous experiments showed minimal loss of NaPCP during the exposures, the NaPCP solutions were not renewed and water samples were collected at the beginning and end of the exposures only. The exposure experiments of both toxicants were repeated twice.

2. Data analysis

The time-to-death data from the second lethal exposure for the three treatments were analyzed with the nonparametric, product limit method (SAS® Procedure LIFETEST, SAS 1999). A log-rank test was used to test whether there was any significant difference among the three mortality curves.

3. The expected results under the two hypotheses

According to the IED hypothesis, the IED of an individual is an intrinsic characteristic of that individual and the relative position of the individual's IED within a population of IED values is constant. Under the experimental design here, the IED of an amphipod can be interpreted as Σ (exposure concentration × exposure duration) until it dies. The two hypothetical plots assuming a log-normal survival distribution in Figure 4-1 illustrate the mortality curves of the three treatments during the second exposure under the IED and stochastic theories, respectively. Suppose that the lethal, sub-lethal, and reference exposures result in 50%, 5%, and 0% mortality (including latent mortality). The IED distributions of the amphipods in the sub-lethal and reference groups will not change because few animals died. Therefore, their cumulative mortality curves should be similar during the second exposure because they reflect essentially the same population. (The assumption is made in this example of no induction of any detoxifying or sequestration mechanisms.) The surviving animals in the lethal group all have IED values greater than the mean of the log-normally distributed pdf of the IED (IED_{50}). From the beginning of the second exposure until 50% mortality occurs in the reference group, no individual will die in the lethal group because all of them have IED values greater than the IED_{50}. The sensitive individuals were eliminated during the first exposure. After that, more and more amphipods' IED will be exceeded through time. The animals in the three groups will die with similar patterns and rates. If there were no change of population resistance to the toxicant (i.e., induced tolerance) in the lethal and sub-lethal groups after the first exposure, the three curves should approach 100%

mortality at approximately the same time. One assumption here is that if there were any resistance change because of age, only the IED_{50} of the population would change, not the relative position of each amphipod's IED. Therefore, Figure 4-1 would still apply.

In contrast, stochastic theory predicts no difference among the three mortality curves during the second exposure because the surviving amphipods of the lethal group should have no difference in resistance to the toxicant with those of the sub-lethal and reference groups.

C. Results

Two $CuSO_4$ and NaPCP experiments were conducted in the spring of 2004 and the mortality curves are shown in Appendix 14. However, the amphipods in the control groups had unusually high mortality (>15%) in some of the experiments, and the mortalities during the second exposures were much faster than the first lethal exposures under similar conditions. Therefore, the experimental results were judged to be unreliable. The possible confounding factor was the contamination by some bacteria in the container of deionized water used to make the RMHRW. The container was cleaned thoroughly and all experiments were redone in the fall of 2004.

The alkalinity and pH of RMHRW were within the normal range. The pH, DO concentration, and water temperature during the experiments are summarized in Table 4-1. The measured toxicant concentrations are shown in Table 4-2.

For the two $CuSO_4$ experiments, no animals died in the reference and sub-lethal groups after the first exposures (at 13.5 and 13 h, respectively), but 12% and 10% mortalities occurred in the lethal groups. During the 14 d recovery periods, the

additional mortalities were 39% (51% total) and 32% (42% total) in the lethal groups, 21% and 7% in the sub-lethal groups, and 2% and 5% in the reference groups. These results reinforced the conclusion of Chapter II that $CuSO_4$ exposure can cause significant latent mortality. The higher concentration and longer duration in the first experiment compared with those in the second experiment could explain its higher latent mortality. The time-to-death data during the second exposures are shown in Appendix 12. The cumulative proportions of dead amphipods at each observation time during the second lethal exposures were plotted in Figure 4-2. Visually the shapes and trends of the curves of lethal, sub-lethal, and reference groups were similar to each other. Formal statistical analysis of log-rank test results showed that there was no significant difference among these three curves in either experiment (α=0.05, Table 4-3). No amphipods died in the control group.

For the two NaPCP experiments, no animals died in the reference and sub-lethal groups after the first exposures (at 10 and 12 h, respectively). In the lethal groups, 39% and 42% of animals died. During the recovery periods (10 d), the additional mortalities were 7% (46% total) and 10% (52% total) in the lethal groups, 2% and 2% in the sub-lethal groups, and 3% and 2% in the reference groups. These results reinforced the conclusion of Chapter II that NaPCP exposure has modest levels of latent mortality. The time-to-death data during the second exposures are shown in Appendix 13. Figure 4-3 shows the cumulative proportions of dead amphipods through time during the second lethal exposures. The mortality curves of reference and sub-lethal groups are similar to each other. Originally, the curves of the lethal groups gradually diverged from the other two, indicating lower mortality rate. After about 15 hours of exposure, the lethal group

mortality rates increased and the three curves converged at the end of the exposures, at approximately 90% mortality. However, log-rank test revealed no significant difference among the three treatments in either experiment (α=0.05, Table 4-3). No amphipods died in the control group.

D. Discussion

1. The relative importance of IED and stochastic theories in $CuSO_4$ and NaPCP experiments

For $CuSO_4$, neither the cumulative mortality curves nor the formal statistical tests showed any evidence of significant difference among the 3 treatments. Because the amphipods were given 14 d to recover, it was assumed that during the second exposure there was no cumulative damage remaining from the first exposure in both the sub-lethal and lethal groups. There was no apparent evidence of induced tolerance either. Therefore, the stochastic theory is favored here as the dominant explanation.

For NaPCP, though the log-rank test showed no evidence of significant difference, the shape of the lethal group curve was visually different from the other two. To further test the hypothesis, the data for the second exposure for each experiment were divided into two parts. The first part included the data from the beginning of the second exposure to 14 and 16 h, respectively. At these two time points, the total proportions dead (including the mortality during and after the first exposure, and during the second exposure) in the reference groups were equal to the total proportion dead in the lethal groups from the beginning of the first exposure until the end of the recovery periods. All the survivors after these two time points were censored. The second parts

only included the mortality data of the survivors from 14 and 16 h onward until the end of the second exposures. Because there was no significant difference between the mortality of the reference and sub-lethal groups, the data were combined to gain more statistical power and compared with the lethal groups. Figure 4-4 and 4-5 show the cumulative mortalities through time after dividing the data. The log-rank test was applied to these two parts separately. For the first parts, both the mortality curves and log-rank test show borderline significant difference between the mortality of the two groups (Table 4-3 and Figure 4-4, α 0.05). For the second part, neither the curves nor log-rank test show any evidence of significant difference (Table 4-3 and Figure 4-5, α 0.05).

Based on the experimental results of Chapter II, the latent effect of NaPCP was insignificant: the minimal amount of latent mortality stopped shortly after exposure ended. Based on the half-lives of PCP and its conjugate in *H. azteca* of 3.6 and 9.1 h (Nuutinen et al. 2003), after 10 d (240 h) depuration, no more than 0.000001% of the accumulated PCP and its conjugate remained in the amphipod body, far less than the notionally defined complete depuration in toxicology (0.8%) (Medinsky and Klaassen 1996). 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 in Figure 4-3 approached 100% mortality at approximately the same time and the shapes of the sub-lethal and reference curves were similar, indicating no significant tolerance induction either. Therefore, the most plausible explanation is that the IED theory might have played some role in the exposures. However, relative large proportions of animals (25%-30%) died during the

first part in the lethal group, indicating that stochastic processes played a dominant role. Such a process could be a mixture of these two components.

Many studies showed that under acute toxicant exposures, the times-to-death were different for organisms with different genetic qualities (e.g., Duan et al. 2001), suggesting the intrinsic qualities may have played important roles, which favored the IED theory. Pena-Llopis et al. (2003) proposed an intermediate hypothesis between IED and stochastic theories: the tolerance to organophosphate dichlorvos of European eel (*Anguilla anguilla* L.) depends on maintaining and increasing the hepatic glutathione redox status. Regardless, our study showed that under the concentrations used in the experiments, the relative domination of IED versus stochasticity for $CuSO_4$ and NaPCP was different. This could be a result of the toxicological mechanisms of different toxicants as described in Chapter II. Also, Heagler et al. (1993) and Newman and McCloskey (2000) suggested that, at low toxicant concentrations, the innate differences among individuals could more manifest and the IED model may dominate during the exposure. At high concentrations, the stochastic model may become the dominant one.

2. Ecological relevance

The lethal response of a population to a toxicant could be a combination of both the IED and stochasticity models. The relative contributions of the two may be different for different toxicants, organisms, and exposure concentrations. The significance of these experimental results can be found in their application to pulsed exposures in the field. Suppose a fish population was exposed to a toxicant periodically with concentrations equal to its 96 h LC50 and durations of 96 h, and there are sufficient

recovery times between pulses. The prediction from the stochasticity theory is that every pulse will result in 50% mortality and the population size will be 100%→50%→25%→12.5% of the original size under 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 have higher IEDs. No or only few fish will die during the subsequent pulses. The change of the fish population size should be 100%→50%→50%→50%. Therefore, the same population under the identical exposure scenario will have a higher possibility of becoming locally extinct if the stochastic hypothesis was true than if the IED hypothesis was. The discussion can be extended to pulsed exposures with the nature (concentration, duration, or both) of the pulse changing through time. Under the IED theory, the pulse resulting in highest percent mortality of the population will be the one that determines how much the population will be affected eventually during these multiple exposures. The other pulses with lower intensities will not matter that much because all the survivors of the highest-intensity pulse have higher IEDs and will not die during subsequent lower-intensity pulses. In contrast, every pulse counts under stochastic theory. Predicting which model to apply is crucial to ecological risk assessment and environmental regulations concerning with pulsed exposure problems.

E. Conclusion

In this study, the hypothesis was rejected that the IED theory is the dominant explanation for the dose-response model for both $CuSO_4$ and NaPCP. Under the specific concentrations and durations used, stochastic processes were dominant for

$CuSO_4$. Both stochasticity and IED might be relevant for NaPCP, but stochasticity seemed to dominate the dynamics. Further experiments under different experimental conditions (e.g., concentrations or toxicants) are required to determine whether there is any relationship between the relative contributions of the two.

Table 4-1. The pH values, dissolved oxygen (DO) concentrations, and water temperatures of the $CuSO_4$ and NaPCP exposure media.

	$CuSO_4$ 1st experiment	$CuSO_4$ 2nd experiment	NaPCP 1st experiment	NaPCP 2nd experiment
pH (median, range, *n*)	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 (mean ± standard deviation, *n*=10, mg/L)	7.6 ± 0.2	7.6 ± 0.3	7.5 ± 0.1	7.5 ± 0.2
Water Temp. (mean ± standard deviation, *n*=10, °C)	23.4 ± 0.2	23.1 ± 0.3	23.7 ± 0.7	23.2 ± 0.2

Table 4-2. Concentrations of dissolved Cu and NaPCP during the sub-lethal, first lethal, and second lethal exposures. For the two $CuSO_4$ experiments, the solutions were renewed every 12 h or so. For NaPCP exposures, the solutions were not changed and water samples were collected at the beginnings and ends of the exposures.

		Toxicant concentrations (mg/L, mean ± standard deviation, *n*)		
	Experiment No.	Sub-lethal	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)

Table 4-3. The *p* values of log-rank test for the mortality data of the reference, sub-lethal, and lethal treatments during the second exposures for $CuSO_4$ and NaPCP experiments. The *p* values of the two NaPCP experiments when analyzed first and second parts of the data separately are also shown.

Experiment No.	$CuSO_4$	NaPCP		
		All	first Part	second Part
1	0.26	0.24	0.03	0.90
2	0.33	0.81	0.06	0.27

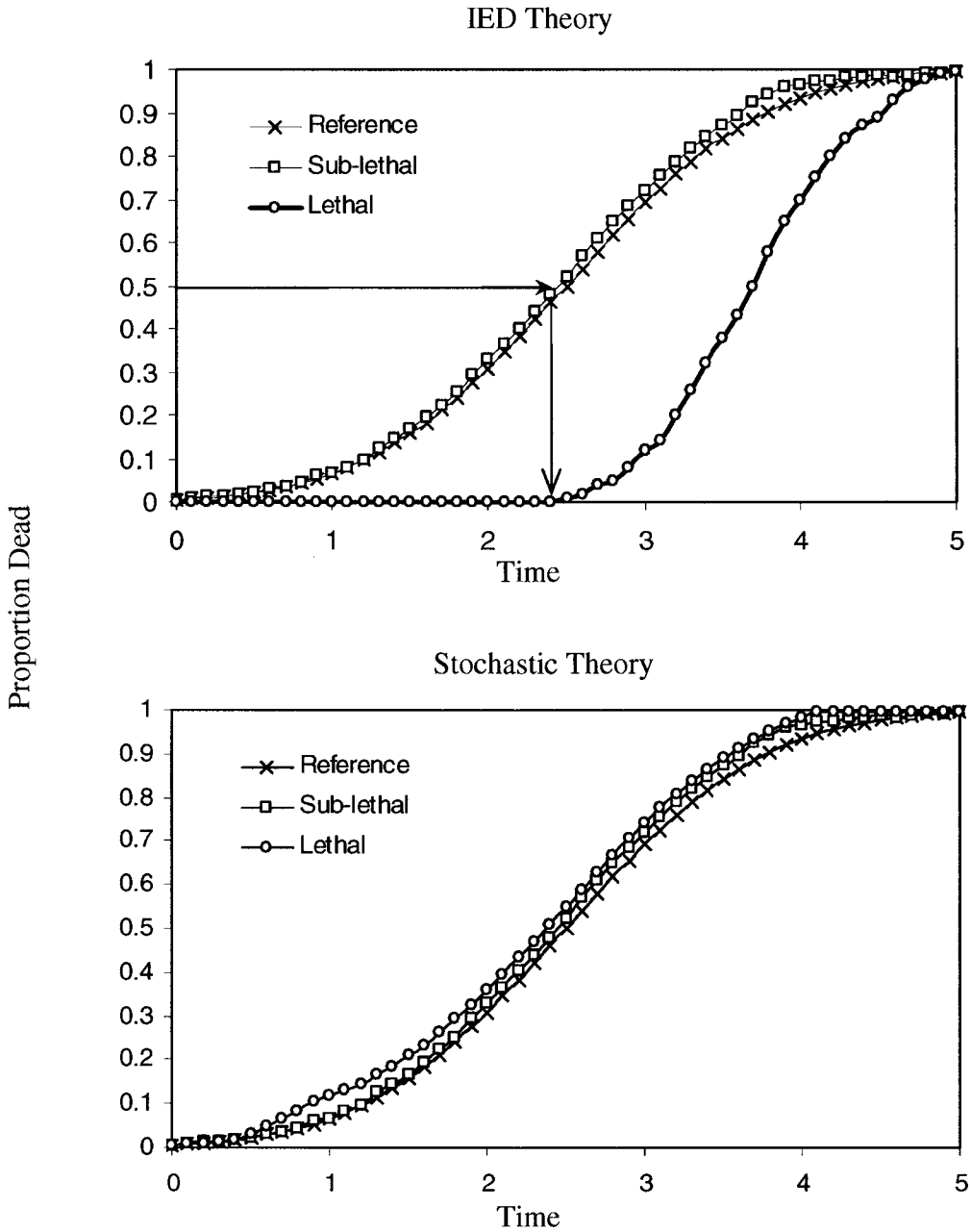


Figure 4-1. Plots of cumulative mortality of the hypothetical reference, lethal, and sub-lethal groups under IED (top panel) and stochastic (bottom panel) theory. Arrows indicate that, if 50% of the organisms died in the reference and sub-lethal groups, those in lethal group would begin to die.

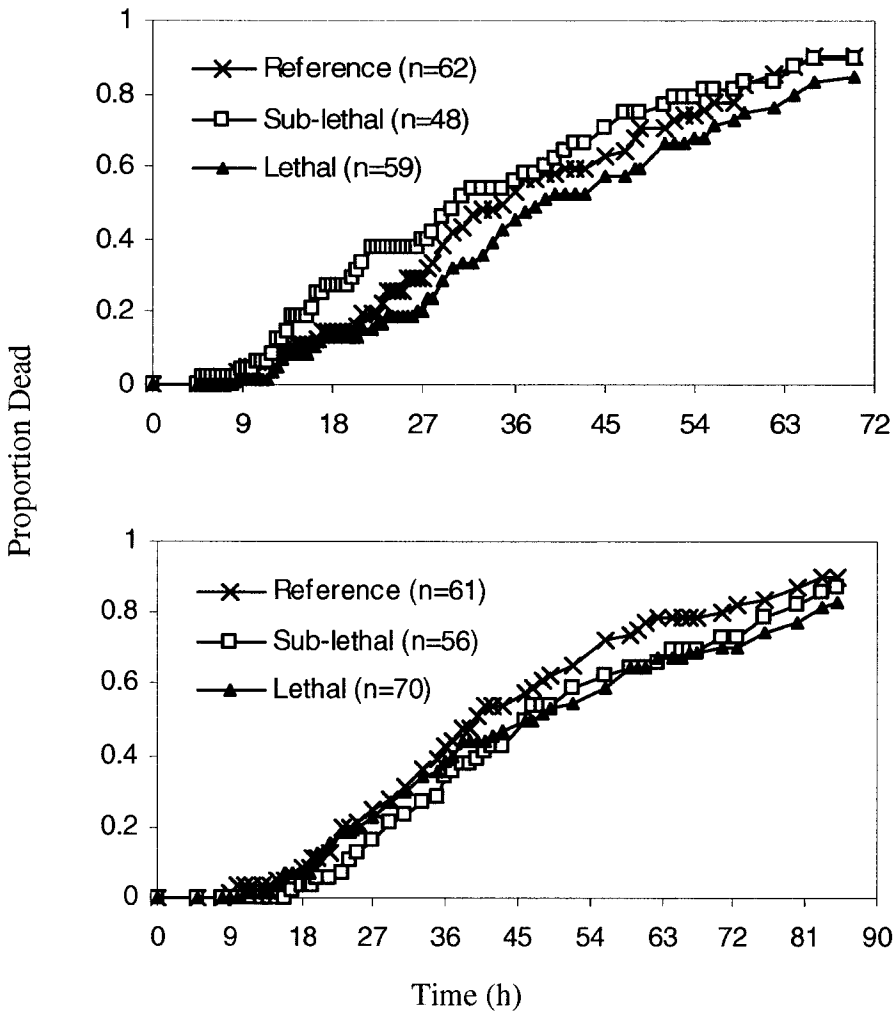


Figure 4-2. Cumulative mortalities through time during the second lethal exposures for the two $CuSO_4$ experiments. The sample sizes (n) are shown in the brackets.

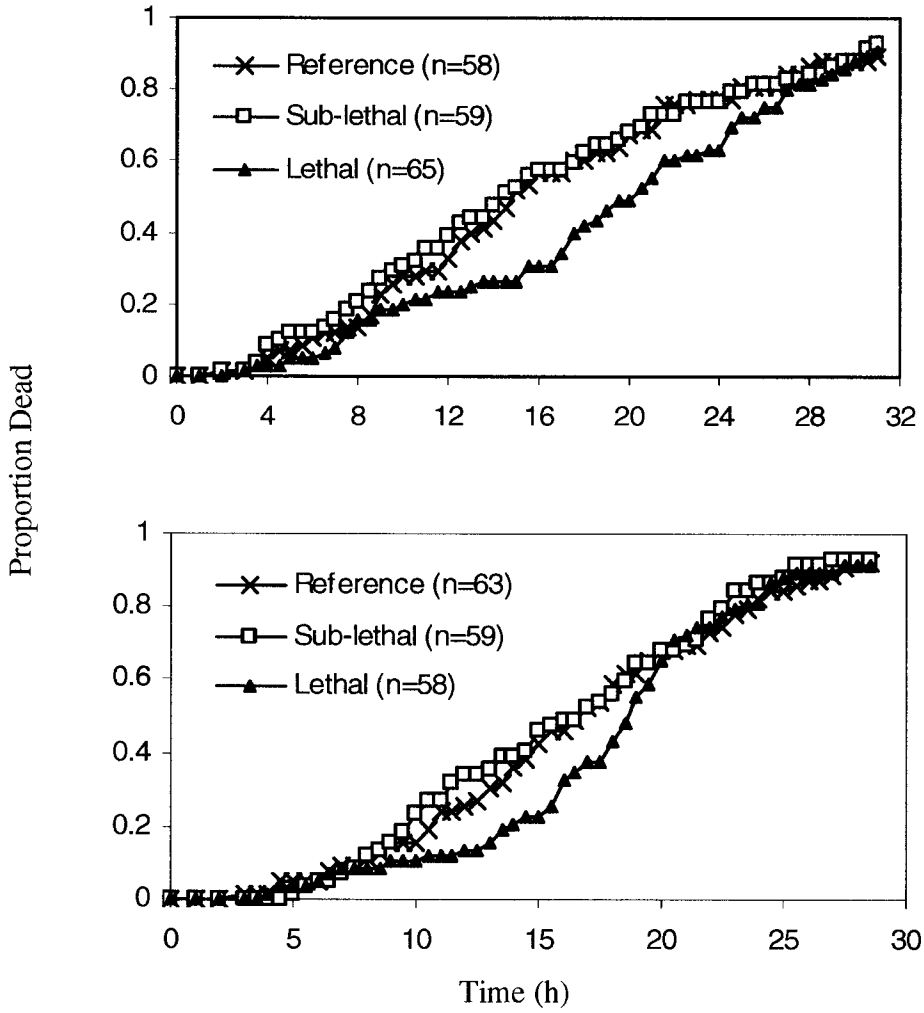


Figure 4-3. Cumulative proportion mortalities through time during the second lethal exposures for the two NaPCP experiments. The sample sizes (*n*) are shown in the brackets.

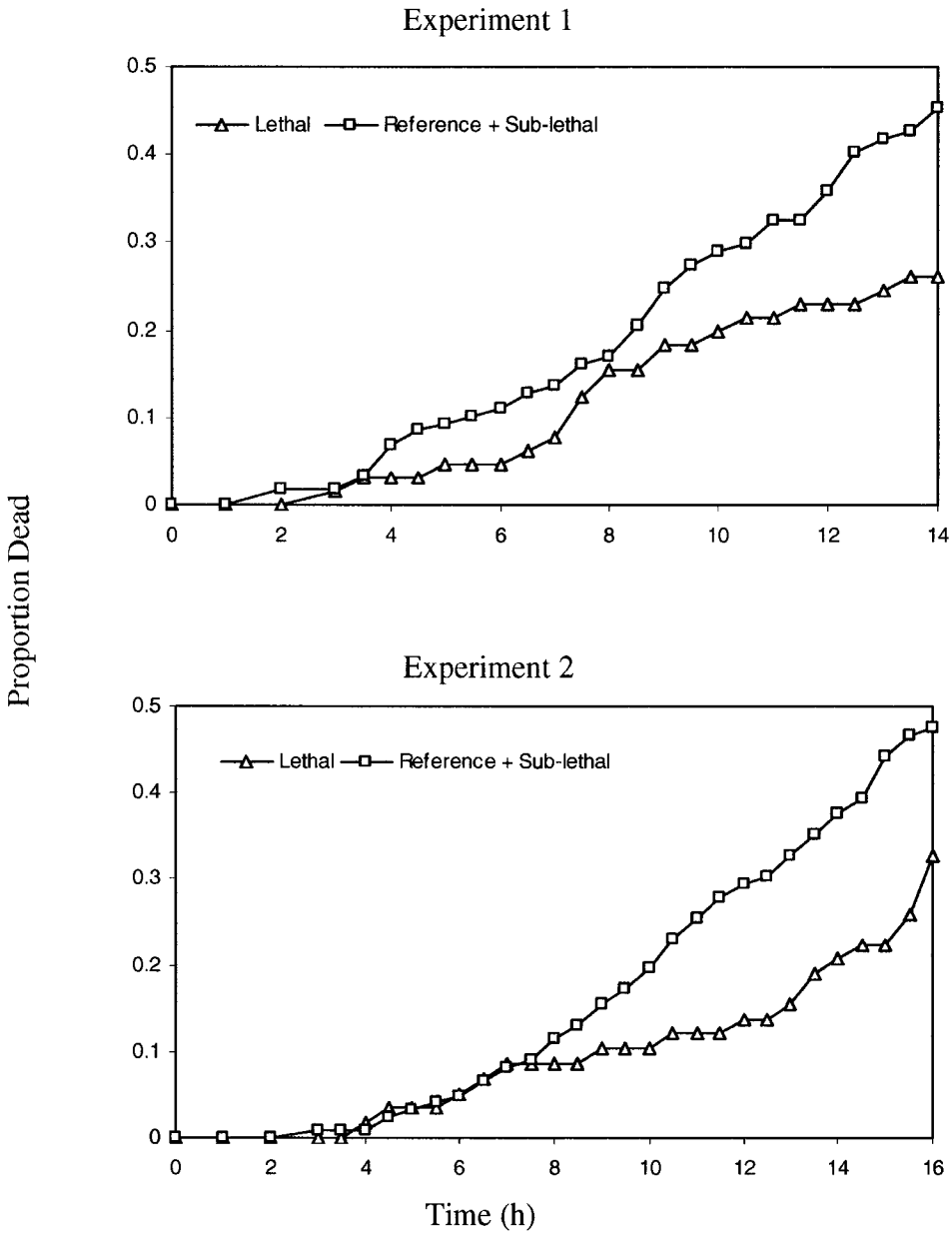


Figure 4-4. Cumulative mortalities through time during the first part of the second lethal NaPCP exposures. The data were divided into 2 parts at 14 h and 16 h for the two experiments.

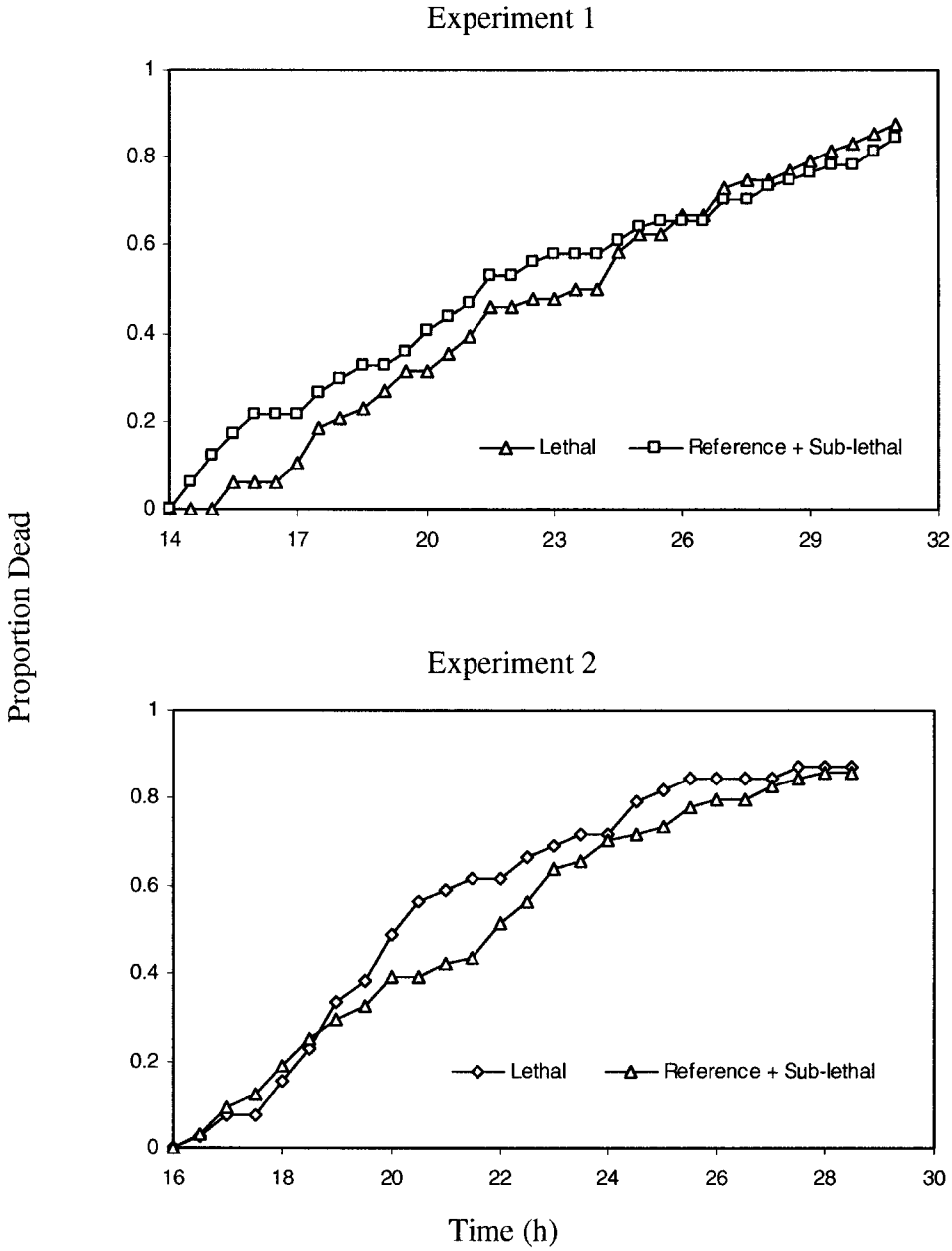


Figure 4-5. Cumulative mortalities through time during the second part of the second lethal NaPCP exposures. The data were divided into 2 parts at 14 h and 16 h for the two experiments.

CHAPTER V. SUMMARY AND CONCLUSION

The current widely used LC50 method in ecotoxicology has many advantages, such as being fast and simple to perform, and insensitive to violations of statistical assumptions. It is very useful in predicting point source contamination and comparing relative toxicities of different chemicals or one chemical under different conditions, e.g., to determine which form is the most toxic species of a metal.

Despite its many advantages, the LC50 method has serious shortcomings that cannot be ignored. This is especially true when considering environmental problems associated with varying exposure duration, concentration, and frequency. In my dissertation, survival analysis methods were applied to avoid these shortcomings while addressing issues associated with pulsed exposures. The amphipod, *H. azteca*, was used as the model organism, and $CuSO_4$ and NaPCP as model toxicants.

The exposure duration effect is often considered peripherally in the conventional LC50 approach. By applying a different experimental design and analyzing data with survival models, not only exposure duration but also concentration were effectively incorporated into the model and their effects on mortality quantified. Survival analysis methods better predict lethal effects than does the conventional LC50 method.

Researchers have been focused on mortality during exposure while what happens after the exposure ends is often ignored. This study brought up the concepts of complete LC50 and latent mortality. For certain toxicants, significant differences exist

between the conventional LC50 and complete LC50, and the latent mortality could be important relative to the fate of a population.

Among the variables that could affect mortality during pulsed exposures that frequently occur in the field, the effect of previous pulse duration on latent mortality and recovery time between pulses on mortality during the second pulse were addressed quantitatively. Within the exposure ranges used, the study did not find any significant effect of duration on latent mortality. However, the effect might still need to be considered under other exposure situations. For both toxicants, there were significant effects of recovery time on mortality during the second pulse. The toxicant modes of action also played important roles in how long the organisms took to recover to near their original resistant state. Survival analysis permits more effective prediction of lethal effects from pulsed exposure than the conventional method.

Although IED theory is cited by many as the underlying theory of dose-response models, especially the probit model, few rigorous testings of it have been conducted. In this study, by applying survival analysis experimental design and models, the hypothesis that the IED theory is the dominant explanation for the survival distribution model for both $CuSO_4$ and NaPCP was rejected. This conclusion is important in predicting the fate of a population under pulsed exposures.

APPENDIX 1

Chemical components needed to prepare 1 L of reformulated moderately hard reconstituted water (RMHRW) and the expected alkalinity and pH ranges.

Chemical Name	Amount
$CaSO_4 \cdot H_2O$	0.050 g
$CaCl_2$	0.050 g
$MgSO_4$	0.030 g
$NaHCO_3$	0.096 g
KCl	0.004 g

Alkalinity	50-70 mg/L $CaCO_3$
pH	7.6-8.2

APPENDIX 2

a. The times-to-death of amphipods during the 1st $CuSO_4$ latent mortality experiment.

		Time-to-death (hour)												
Treatment	**Total #**	**0**	**5**	**9**	**12**	**16**	**20**	**24**	**28**	**32**	**36**	**40**	**44**	**48**
0.2	**48**	0	0	0	0	0	0	0	0	0	0	0	0	1
0.3	**48**	0	0	0	0	0	0	0	0	0	0	0	2	3
0.4	**48**	0	0	0	0	0	1	2	3	3	3	4	6	10
0.6	**48**	0	0	0	0	0	1	2	5	7	7	10	12	19

		Time-to-death (hour)											
Treatment	**Total #**	**52**	**56**	**61**	**66**	**70**	**77**	**84**	**91**	**95**	**101**	**109**	**112**
0.2	**48**	1	1	1	2	2	2	3	4	4	4	5	5
0.3	**48**	3	4	4	5	6	8	10	11	11	11	11	11
0.4	**48**	11	11	13	14	14	14	17	17	18	18	21	21
0.6	**48**	20	20	24	27	28	31	32	34	36	37	37	37

b. The times-to-death of amphipods during the 2nd $CuSO_4$ latent mortality experiment.

		Time-to-death (hour)												
Treatment	**Total #**	**0**	**6**	**10**	**12**	**16**	**20**	**24**	**28**	**32**	**36**	**40**	**44**	**48**
0.2	**48**	0	0	0	0	0	1	1	1	1	1	1	1	2
0.3	**48**	0	0	0	0	0	1	1	1	2	2	3	4	6
0.4	**48**	0	0	1	1	1	3	4	4	5	6	8	8	9
0.6	**48**	0	0	0	0	1	5	9	10	12	16	17	19	23

		Time-to-death (hour)												
Treatment	**Total #**	**52**	**57**	**62**	**67**	**72**	**77**	**82**	**87**	**93**	**96**	**100**	**106**	**112**
0.2	**48**	3	4	4	5	5	6	8	10	10	10	11	11	11
0.3	**48**	9	10	10	12	14	14	16	18	20	21	21	21	21
0.4	**48**	11	14	15	18	20	22	23	26	26	26	26	26	28
0.6	**48**	24	27	28	30	30	33	33	33	36	36	36	37	38

c. The times-to-death of amphipods during the 3rd $CuSO_4$ latent mortality experiment.

		Time-to-death (hour)												
Treatment	**Total #**	**0**	**4**	**8**	**12**	**15**	**18**	**21**	**24**	**29**	**33**	**36**	**39.5**	**42.5**	**46**
0.2	**48**	0	0	0	0	0	0	1	1	2	3	3	4	4	4
0.3	**48**	0	0	0	0	1	2	3	6	7	10	13	14	14	14
0.4	**47**	0	0	0	1	2	4	7	11	11	16	17	18	18	19
0.6	**48**	0	0	1	3	4	7	11	15	18	22	24	24	24	27

		Time-to-death (hour)												
Treatment	**Total #**	**48**	**52.5**	**58**	**61**	**64.5**	**69**	**72**	**81**	**85**	**89**	**97**	**102**	**106**	**112**
0.2	**48**	4	5	5	5	5	6	6	6	6	6	6	6	6	6
0.3	**48**	14	14	15	15	16	17	18	19	19	19	19	20	20	20
0.4	**47**	20	20	22	23	24	24	24	24	24	24	24	24	24	24
0.6	**48**	27	28	29	29	30	30	31	32	32	32	33	33	34	34

APPENDIX 3

a. The times-to-death of amphipods during the 1st NaPCP latent mortality experiment.

		Time-to-death (hour)										
Treatment	**Total #**	**0**	**4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	**36**	**40**
0.2	**48**	0	0	0	0	0	0	0	1	2	4	5
0.3	**48**	0	0	0	0	0	1	1	3	3	4	6
0.5	**48**	0	0	0	1	2	2	3	8	11	12	15
0.8	**48**	0	2	3	4	7	8	10	11	13	16	22

		Time-to-death (hour)										
Treatment	**Total #**	**44**	**48**	**52**	**56**	**60**	**64**	**69**	**72**	**74**	**79**	**85**
0.2	**48**	6	7	8	8	8	8	8	9	9	9	9
0.3	**48**	9	12	14	16	16	16	16	16	16	16	16
0.5	**48**	17	22	24	25	25	25	25	26	26	26	26
0.8	**48**	28	37	38	38	38	38	38	38	38	38	38

b. The times-to-death of amphipods during the 2nd NaPCP latent mortality experiment.

		Time-to-death (hour)										
Treatment	**Total #**	**0**	**4**	**8**	**12**	**14**	**18**	**21**	**23**	**28**	**32**	**36**
0.2	**48**	0	0	0	0	0	0	0	0	0	0	0
0.3	**48**	0	0	0	0	0	0	0	0	2	4	7
0.5	**48**	0	0	0	0	0	0	1	4	10	14	14
0.8	**48**	0	0	1	4	6	13	17	20	27	28	30

		Time-to-death (hour)										
Treatment	**Total #**	**40**	**44**	**48**	**52**	**56**	**60**	**64**	**70**	**76**	**82**	**85**
0.2	**48**	1	2	3	3	3	3	3	3	3	3	3
0.3	**48**	9	11	11	12	13	14	14	14	14	14	14
0.5	**48**	14	17	21	22	23	23	24	24	24	24	24
0.8	**48**	33	35	40	40	40	40	40	41	41	41	41

c. The times-to-death of amphipods during the 3rd NaPCP latent mortality experiment.

		Time-to-death (hour)										
Treatment	**Total #**	**0**	**5**	**9**	**12**	**15**	**18**	**21**	**24**	**28**	**32**	**36**
0.2	**48**	0	0	0	0	0	0	0	0	0	0	0
0.3	**48**	0	0	0	0	0	0	0	1	2	2	4
0.5	**48**	0	0	0	0	0	0	2	2	2	5	5
0.8	**48**	0	0	1	1	4	6	8	8	9	9	16

		Time-to-death (hour)										
Treatment	**Total #**	**39**	**42**	**45**	**48**	**53**	**59**	**63**	**68**	**74**	**82**	**85**
0.2	**48**	0	1	1	2	3	3	3	3	3	3	3
0.3	**48**	6	6	9	9	9	9	9	9	9	9	9
0.5	**48**	6	9	10	13	13	13	13	13	13	13	13
0.8	**48**	19	22	24	28	28	28	28	29	29	29	30

APPENDIX 4

The times-to-death of amphipods in the $CuSO_4$ exposure duration experiment

a. Exposure duration=61 hour, measured Cu concentration=0.656 mg/L, total amphipod=51.

Time-to-death (hour)	**0**	**1**	**2**	**3**	**4**	**5**	**7**	**8**	**10**	**13**
Number Died	0	0	1	2	4	5	6	6	6	8
Time-to-death (hour)	**18**	**21.5**	**23**	**25**	**26.5**	**28.5**	**30.5**	**33.5**	**35.5**	**39.5**
Number Died	10	11	11	11	13	15	15	15	15	16
Time-to-death (hour)	**43.5**	**46.5**	**49.5**	**52**	**54.5**	**57.5**	**61.5**	**66.5**	**71**	
Number Died	16	16	16	17	17	17	17	17	17	

b. Exposure duration=38 hour, measured Cu concentration=0.656 mg/L, total amphipod=54.

Time-to-death (hour)	**0**	**1**	**2.5**	**3.5**	**4.5**	**6.5**	**7.5**	**9.5**	**12.5**
Number Died	0	0	1	1	1	2	3	4	5
Time-to-death (hour)	**17.5**	**21**	**22.5**	**24.5**	**26**	**28**	**30**	**33**	**35**
Number Died	5	5	5	6	6	7	8	11	11
Time-to-death (hour)	**39**	**42**	**46**	**49**	**51.5**	**54**	**57**	**61**	**66**	**70.5**
Number Died	11	12	14	15	15	15	15	15	16	17

c. Exposure duration=20 hour, measured Cu concentration=0.656 mg/L, total amphipod=38.

Time-to-death (hour)	**0**	**1**	**3**	**4**	**6**	**7**	**9**	**12**	**17**
Number Died	0	0	0	0	1	1	1	2	3
Time-to-death (hour)	**20.5**	**22**	**24**	**25.5**	**27.5**	**29.5**	**32.5**	**34.5**	**38.5**
Number Died	4	4	5	5	5	5	5	5	5
Time-to-death (hour)	**42.5**	**45.5**	**48.5**	**51**	**53.5**	**56.5**	**60.5**	**65.5**	**70**
Number Died	5	5	6	6	6	6	6	7	8

d. Exposure duration=61 hour, measured Cu concentration=0.632 mg/L, total amphipod=46.

Time-to-death (hour)	**0**	**1**	**2**	**3**	**4**	**5**	**7**	**8**	**10**	**13**
Number Died	0	0	2	4	5	5	5	5	5	7
Time-to-death (hour)	**18**	**21.5**	**23**	**25**	**26.5**	**28.5**	**30.5**	**33.5**	**35.5**	**39.5**
Number Died	8	9	9	9	9	10	10	12	12	12
Time-to-death (hour)	**43.5**	**46.5**	**49.5**	**52**	**54.5**	**57.5**	**61.5**	**66.5**	**71**	
Number Died	12	12	12	12	12	13	13	13	13	

e. Exposure duration=38 hour, measured Cu concentration=0.632 mg/L, total amphipod=53.

Time-to-death (hour)	**0**	**1**	**2.5**	**3.5**	**4.5**	**6.5**	**7.5**	**9.5**	**12.5**
Number Died	0	0	0	1	1	2	4	4	5
Time-to-death (hour)	**17.5**	**21**	**22.5**	**24.5**	**26**	**28**	**30**	**33**	**35**
Number Died	6	6	6	7	8	9	10	10	10
Time-to-death (hour)	**39**	**42**	**46**	**49**	**51.5**	**54**	**57**	**61**	**66**	**70.5**
Number Died	10	11	11	11	13	13	13	13	14	14

f. Exposure duration=20 hour, measured Cu concentration=0.632 mg/L, total amphipod=37.

Time-to-death (hour)	**0**	**1**	**3**	**4**	**6**	**7**	**9**	**12**	**17**
Number Died	0	0	1	1	1	1	1	2	3
Time-to-death (hour)	**20.5**	**22**	**24**	**25.5**	**27.5**	**29.5**	**32.5**	**34.5**	**38.5**
Number Died	4	4	5	7	7	7	8	8	8
Time-to-death (hour)	**42.5**	**45.5**	**48.5**	**51**	**53.5**	**56.5**	**60.5**	**65.5**	**70**
Number Died	8	8	8	8	8	8	8	8	8

g. Exposure duration=61 hour, measured Cu concentration=0.816 mg/L, total amphipod=27.

Time-to death (hour)	**1**	**2**	**4**	**5**	**6**	**7**	**10**	**11**	**13**
Number Died	1	1	2	4	5	5	7	7	9
Time-to death (hour)	**18**	**24**	**26**	**28**	**30**	**32**	**33.5**	**36.5**	
Number Died	10	10	11	12	12	12	12	12	
Time-to death (hour)	**44**	**50**	**53**	**55**	**58**	**60**	**65**	**71**	**73**
Number Died	13	13	14	14	14	14	14	14	14

h. Exposure duration=38 hour, measured Cu concentration=0.816 mg/L, total amphipod=20.

Time-to death (hour)	**1**	**2**	**4**	**5**	**6**	**7**	**10**	**11**
Number Died	0	1	2	2	3	4	6	7
Time-to death (hour)	**13**	**18**	**24**	**26**	**28**	**30**	**32**	**33.5**	**36.5**
Number Died	8	8	9	9	10	10	10	10	10
Time-to death (hour)	**44**	**50**	**53**	**55**	**58**	**60**	**65**	**71**	**73**
Number Died	10	10	10	10	10	10	11	11	11

i. Exposure duration=20 hour, measured Cu concentration=0.816 mg/L, total amphipod=25.

Time-to death (hour)	**1**	**2**	**3**	**4**	**6**	**11**	**17**	**19**
Number Died	0	1	2	3	4	7	9	10

Time-to death (hour)	**21**	**23**	**25**	**26.5**	**29.5**	**38**	**44**	**47**
Number Died	10	11	11	11	11	11	12	12

Time-to death (hour)	**49**	**52**	**54**	**59**	**65**	**67**	**73**
Number Died	12	12	12	13	13	13	13

j. Exposure duration=61 hour, measured Cu concentration=0.834 mg/L, total amphipod=30.

Time-to death (hour)	**1**	**2**	**4**	**5**	**6**	**7**	**10**	**11**	**13**
Number Died	1	1	3	4	6	6	9	9	9

Time-to death (hour)	**18**	**24**	**26**	**28**	**30**	**32**	**33.5**	**36.5**	**44**
Number Died	10	10	10	10	11	11	11	11	12

Time-to death (hour)	**50**	**53**	**55**	**58**	**60**	**65**	**71**	**73**
Number Died	12	14	14	14	14	14	14	14

k. Exposure duration=38 hour, measured Cu concentration=0.834 mg/L, total amphipod=25.

Time-to death (hour)	**1**	**2**	**4**	**5**	**6**	**7**	**10**	**11**
Number Died	0	1	2	2	4	4	8	8
Time-to death (hour)	**13**	**18**	**24**	**26**	**28**	**30**	**32**	**33.5**	**36.5**
Number Died	9	10	13	14	15	15	15	15	15
Time-to death (hour)	**44**	**50**	**53**	**55**	**58**	**60**	**65**	**71**	**73**
Number Died	15	15	16	16	16	16	16	16	16

l. Exposure duration=20 hour, measured Cu concentration=0.834 mg/L, total amphipod=25.

Time-to death (hour)	**1**	**2**	**3**	**4**	**6**	**11**	**17**	**19**
Number Died	0	1	2	2	4	6	8	10
Time-to death (hour)	**21**	**23**	**25**	**26.5**	**29.5**	**38**	**44**	**47**
Number Died	10	10	11	11	11	11	11	11
Time-to death (hour)	**49**	**52**	**54**	**59**	**65**	**67**	**73**
Number Died	11	13	13	13	13	13	13

APPENDIX 5

The times-to-death of amphipods in the NaPCP exposure duration experiment

a. Exposure duration=20 hour, measured NaPCP concentration=0.363 mg/L, total amphipod=36.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	0	0	0	0	0	1	1	1	1	1

b. Exposure duration=40 hour, measured NaPCP concentration=0.363 mg/L, total amphipod=40.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	1	1	1	1	1	1	1	1	1	1

c. Exposure duration=60 hour, measured NaPCP concentration=0.363 mg/L, total amphipod=19.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	1	1	1	1	1	1	1	1	1	1

d. Exposure duration=20 hour, measured NaPCP concentration=0.431 mg/L, total amphipod=25.

Time-to death (hour)	**0**	**3**	**4**	**6**	**10**	**14**	**18**	**20**	**22**	**40**	**50**	**60**
Number Died	0	0	0	1	1	1	1	1	1	1	1	1

e. Exposure duration=40 hour, measured NaPCP concentration=0.431 mg/L, total amphipod=30.

Time-to death (hour)	**0**	**4**	**7**	**10**	**12**	**20**	**30**	**40**	**60**
Number Died	0	2	2	3	3	3	3	3	3

f. Exposure duration=60 hour, measured NaPCP concentration=0.431 mg/L, total amphipod=18.

Time-to death (hour)	**0**	**4**	**8**	**12**	**18**	**20**	**26**	**29**	**40**	**50**	**60**
Number Died	0	0	0	0	1	1	1	1	1	1	1

g. Exposure duration=20 hour, measured NaPCP concentration=0.623 mg/L, total amphipod=31.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	0	0	0	0	0	0	0	0	0	0

h. Exposure duration=40 hour, measured NaPCP concentration=0.623 mg/L, total amphipod=30.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	0	0	0	0	0	0	0	0	0	0

i. Exposure duration=60 hour, measured NaPCP concentration=0.623 mg/L, total amphipod=25.

Time-to death (hour)	**0**	**4**	**8**	**12**	**19**	**24**	**30**	**35**	**46**	**54**	**60**
Number Died	0	1	1	1	1	1	1	1	1	1	1

j. Exposure duration=20 hour, measured NaPCP concentration=0.677 mg/L, total amphipod=32.

Time-to death (hour)	**0**	**3**	**4**	**6**	**10**	**14**	**18**	**20**	**22**	**40**	**50**	**60**
Number Died	0	0	0	0	0	0	0	0	0	0	0	0

k. Exposure duration=40 hour, measured NaPCP concentration=0.677 mg/L, total amphipod=24.

Time-to death (hour)	**0**	**4**	**7**	**10**	**12**	**20**	**30**	**40**	**60**
Number Died	0	1	1	2	2	2	2	2	2

l. Exposure duration=60 hour, measured NaPCP concentration=0.677 mg/L, total amphipod=20.

Time-to death (hour)	**0**	**4**	**8**	**12**	**18**	**20**	**26**	**29**	**40**	**50**	**60**
Number Died	0	0	0	1	1	1	1	1	1	1	1

APPENDIX 6. The times-to-death of amphipods during the 1st $CuSO_4$ pulsed exposure experiment.

a. RT=0 hour, total amphipod=54

Time-to death (hour)	**0**	**1**	**2**	**3.5**	**4.5**	**5.5**	**6.5**	**7.5**	**9**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	0	1	1	1	3	5	6	9	13	18	22	24	26	27

b. RT=24 hour, total amphipod=67

Time-to death (hour)	**0**	**1.5**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Number Died	0	0	1	2	4	6	10	11	14	19	22	25

c. RT=48 hour, total amphipod=68

Time-to death (hour)	**0**	**3**	**3.5**	**4**	**5**	**6**	**7**	**8**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	0	0	1	1	1	3	3	4	5	6	6	7	8	10	11

d. RT=72 hour, total amphipod=74

Time-to death (hour)	**0**	**4.5**	**5**	**6**	**7**	**8**	**9.5**	**10.5**	**11**	**12**
Number Died	0	0	0	1	1	2	3	3	4	5

e. Reference 1, n=59

Time-to death (hour)	**0**	**1.5**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Number Died	0	0	0	1	1	3	3	5	5	5	5

f. Reference 2, n=61

Time-to death (hour)	**0**	**3**	**4.5**	**5**	**6**	**7**	**8**	**8.5**	**9.5**	**10.5**	**12**
Number Died	0	0	0	0	0	0	0	1	2	2	3

APPENDIX 7. The times-to-death of amphipods during the 2nd $CuSO_4$ pulsed exposure experiment.

a. RT=0 hour, total amphipod=46

Time-to death (hour)	**0**	**0.5**	**1**	**2**	**3**	**4.5**	**5.5**	**6.5**	**7.5**	**9**	**10**	**11**	**12**
Number Died	0	1	3	5	5	8	11	12	15	16	19	21	25

b. RT=24 hour, total amphipod=54

Time-to death (hour)	**0**	**1**	**1.5**	**2.5**	**3.5**	**5**	**6.5**	**7.5**	**8**	**9.5**	**10**	**10.5**	**11.5**	**12**
Number Died	0	0	1	1	2	3	7	7	8	11	13	14	17	20

c. RT=48 hour, total amphipod=52

Time-to death (hour)	**0**	**3**	**4**	**5**	**6.5**	**7.5**	**8**	**8.5**	**9**	**10**	**11**	**12**
Number Died	0	0	0	1	4	6	6	6	7	8	9	12

d. RT=72 hour, total amphipod=55

Time-to death (hour)	**0**	**3.5**	**4.5**	**5.5**	**7**	**8**	**9.5**	**10**	**11**	**12**
Number Died	0	1	3	3	3	4	5	6	7	9

e. Reference 1, total amphipod=39

Time-to death (hour)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9.5**	**11**	**12**
Number Died	0	0	0	0	0	0	1	3	3	4	5

f. Reference 2, total amphipod=42

Time-to death (hour)	**0**	**4.5**	**5.5**	**7**	**8**	**9.5**	**11**	**12**
Number Died	0	0	0	0	1	2	3	4

APPENDIX 8 The times-to-death of amphipods during the 3rd $CuSO_4$ pulsed exposure experiment.

a. RT=0 hour, total amphipod=59

Time-to death (hour)	**0**	**1**	**2**	**2.5**	**3.5**	**4.5**	**5.5**	**7**	**8**	**9**	**10**	**11**	**12**
Number Died	0	2	6	6	7	9	10	12	15	18	22	23	26

b. RT=24 hour, total amphipod=52

Time-to death (hour)	**0**	**1**	**2**	**3.5**	**4.5**	**5**	**6.5**	**7.5**	**9**	**10**	**11**	**12**
Number Died	0	1	1	2	2	4	5	7	10	13	16	19

c. RT=48 hour, total amphipod=53

Time-to death (hour)	**0**	**3.5**	**4.5**	**5.5**	**6.5**	**7.5**	**8.5**	**10**	**10.5**	**11.5**	**12**
Number Died	0	0	2	3	4	5	6	7	8	10	12

d. RT=72 hour, total amphipod=60

Time-to death (hour)	**0**	**3.5**	**5**	**6**	**7**	**7.5**	**9.5**	**11**	**12**
Number Died	0	0	0	1	2	2	4	5	8

e. Reference 1, total amphipod=46

Time-to death (hour)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**6.5**	**7.5**	**9**	**10**	**11**	**12**
Number Died	0	0	0	0	0	0	0	0	1	3	5	5	7

f. Reference 2, total amphipod=45

Time-to death (hour)	**0**	**4.5**	**5.5**	**7**	**8**	**9.5**	**10**	**10.5**	**11**	**12**
Number Died	0	0	0	0	0	0	1	2	4	4

APPENDIX 9. The times-to-death of amphipods during the 1st NaPCP pulsed exposure experiment

a. RT=0 hour, total amphipod=53

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	2	3	4	6	7	8	8	10	10	14	14	15

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	16	18	18	19	19	19	22	24	27	28	30	32

b. RT=4 hour, total amphipod=60

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	1	2	4	5	6	7	8	9	11	12	13

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	14	15	16	16	18	19	19	19	20	20	23	23

c. RT=8 hour, total amphipod=55

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	1	2	2	3	6	6	7	8	8	10	11

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	11	12	12	12	12	12	13	14	14	15	15	15

d. RT=14 hour, total amphipod=66

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	2	2	2	2	4	8	8	8	8

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	9	9	9	10	10	11	12	13	14	14	15	15

e. Reference 1, total amphipod=80

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	1	1	2	2	2	2	3	3	5

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	6	8	9	9	10	10	12	12	14	14	15	15

f. Reference 2, total amphipod=79

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	0	0	0	1	1	1	2	3	4

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	6	7	8	9	9	9	11	12	12	12	13	13

APPENDIX 10. The times-to-death of amphipods during the 2nd NaPCP pulsed exposure experiment

a. RT=0 hour, total amphipod=60

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	3	5	6	10	13	16	16	21	23	25	26	31
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	32	33	35	38	41	41	44	44	45	45	46	46	

b. RT=4 hour, total amphipod=44

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	1	2	3	4	4	5	7	7	7	9	10	11
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	12	13	14	14	14	16	17	18	19	19	20	22	

c. RT=8 hour, total amphipod=42

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	1	2	3	3	4	4	5	5	5	5	6
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	7	7	7	7	7	7	8	9	10	10	10	10	

d. RT=14 hour, total amphipod=46

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	1	1	1	1	2	2	2	2	2

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	2	3	3	4	5	5	6	7	7	8	9	9

e. Reference 1, total amphipod=68

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	1	1	3	3	3	3	3	4	4	4

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	5	6	6	6	6	7	7	8	9	9	10	12

f. Reference 2, total amphipod=67

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	0	0	0	1	1	1	1	3	4

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	4	4	4	5	5	5	5	5	6	8	8	8

APPENDIX 11. The times-to-death of amphipods during the 3rd NaPCP pulsed exposure experiment

a. RT=0 hour, total amphipod=67

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	1	1	2	2	3	3	3	5	6	8	8	10
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	12	13	15	17	18	20	22	23	24	24	26	27	

b. RT=4 hour, total amphipod=73

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	1	2	2	4	4	5	7	7	8	8	10
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	11	12	13	13	14	16	16	18	19	22	22	24	

c. RT=8 hour, total amphipod=65

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	1	2	2	3	5	5	6	6	6
Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	
Number Died	6	6	7	7	7	8	9	10	11	11	13	13	

d. RT=14 hour, total amphipod=73

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	0	1	1	2	2	2	2	3	3

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	3	4	4	4	5	6	7	8	9	10	10	11

g. Reference 1, total amphipod=57

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	0	0	1	1	1	1	1	1	1

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	1	1	1	2	2	2	2	4	4	4	4	4

h. Reference 2, total amphipod=64

Time-to death (hour)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Number Died	0	0	0	0	0	0	0	1	1	1	1	1	2

Time-to death (hour)	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Number Died	2	2	2	2	2	2	2	2	2	2	2	2

APPENDIX 12

a. The times-to-death of amphipods during the 1st IED vs. stochastic $CuSO_4$ experiment.

		Time-to-death (hour)																
Treatment	**Total #**	**0**	**4.5**	**5**	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Reference	**62**	0	0	0	0	0	0	0	1	1	2	2	3	3	4	4	4	5
Sub-lethal	**48**	0	0	1	1	1	1	1	1	1	1	2	2	2	3	3	3	4
Lethal	**59**	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2

		Time-to-death (hour)																
Treatment	**Total #**	**12.5**	**13**	**13.5**	**14**	**14.5**	**15**	**15.5**	**16**	**16.5**	**17**	**17.5**	**18**	**18.5**	**19**	**19.5**	**20**	**20.5**
Reference	**62**	6	6	6	6	7	7	7	7	8	9	9	9	9	9	9	9	10
Sub-lethal	**48**	6	6	7	9	9	9	9	10	12	12	13	13	13	13	13	14	15
Lethal	**59**	3	4	5	5	5	5	5	6	7	8	8	8	8	8	8	8	8

		Time-to-death (hour)																
Treatment	**Total #**	**21**	**22**	**22.5**	**23**	**23.5**	**24**	**24.5**	**25**	**25.5**	**26**	**26.5**	**27**	**27.5**	**28**	**29**	**30**	**31**
Reference	**62**	12	12	12	14	16	16	16	16	18	18	18	18	20	21	24	26	27
Sub-lethal	**48**	16	18	18	18	18	18	18	18	18	18	18	19	19	20	22	23	25
Lethal	**59**	9	9	10	10	11	11	11	11	11	11	12	12	14	14	17	19	20

		Time-to-death (hour)													
Treatment	**Total #**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**45**	**47**
Reference	**62**	29	30	30	31	33	35	35	36	36	37	37	37	39	40
Sub-lethal	**48**	26	26	26	26	27	28	28	29	30	31	32	32	34	36
Lethal	**59**	20	21	23	25	27	28	29	30	31	31	31	31	34	34

		Time-to-death (hour)													
Treatment	**Total #**	**48**	**48.5**	**51**	**52**	**53**	**54**	**55**	**56**	**58**	**59**	**62**	**64**	**66**	**70**
Reference	**62**	42	44	44	45	46	46	47	48	48	51	53	54	56	56
Sub-lethal	**48**	36	36	37	38	38	38	39	39	39	40	40	42	43	43
Lethal	**59**	35	35	39	39	39	40	40	42	43	44	45	47	49	50

b. The times-to-death of amphipods during the 2nd IED vs. stochastic $CuSO_4$ experiment.

		Time-to-death (hour)													
Treatment	**Total #**	**0**	**5**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Reference	**61**	0	0	0	1	2	2	2	2	2	3	3	4	5	5
sub-lethal	**56**	0	0	0	0	0	0	0	0	0	0	0	1	2	2
Lethal	**70**	0	0	0	0	0	1	1	1	1	3	5	5	5	5

		Time-to-death (hour)													
Treatment	**Total #**	**19.5**	**20**	**21.5**	**23**	**24**	**25**	**27**	**29**	**31**	**33**	**35**	**36**	**37**	**38**
Reference	**61**	7	7	8	12	12	13	15	17	19	22	24	26	27	29
sub-lethal	**56**	2	3	3	4	6	7	9	12	13	15	16	19	20	21
Lethal	**70**	7	9	11	13	13	14	16	19	21	24	25	27	28	31

		Time-to-death (hour)													
Treatment	**Total #**	**39**	**40**	**41**	**42**	**43**	**46**	**47**	**48**	**49**	**52**	**56**	**59**	**60**	**61**
Reference	**61**	29	31	33	33	33	35	36	37	38	40	44	45	46	47
sub-lethal	**56**	21	22	23	24	24	28	30	30	30	33	35	36	36	36
Lethal	**70**	31	31	31	32	33	35	35	36	37	38	41	45	45	45

		Time-to-death (hour)										
Treatment	**Total #**	**62.5**	**64.5**	**65.5**	**66.5**	**67.5**	**70.5**	**72.5**	**76**	**80**	**83**	**85**
Reference	**61**	48	48	48	48	48	49	50	51	53	55	55
sub-lethal	**56**	37	39	39	39	39	41	41	44	46	48	49
Lethal	**70**	47	47	47	48	48	49	49	52	54	57	58

APPENDIX 13

a. The times-to-death of amphipods during the 1st IED vs. stochastic NaPCP experiment.

		Time-to-death (hour)											
Treatment	**Total #**	**0**	**1**	**2**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**	**6.5**	**7**
Reference	**58**	0	0	1	1	2	3	4	4	5	6	7	7
Sub-lethal	**59**	0	0	1	1	2	5	6	7	7	7	8	9
Lethal	**65**	0	0	0	1	2	2	2	3	3	3	4	5

		Time-to-death (hour)											
Treatment	**Total #**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**	**12.5**	**13**
Reference	**58**	8	8	10	13	15	16	16	17	17	19	22	23
Sub-lethal	**59**	11	12	14	16	17	18	19	21	21	23	25	26
Lethal	**65**	8	10	10	12	12	13	14	14	15	15	15	16

		Time-to-death (hour)											
Treatment	**Total #**	**13.5**	**14**	**14.5**	**15**	**15.5**	**16**	**16.5**	**17**	**17.5**	**18**	**18.5**	**19**
Reference	**58**	24	25	27	30	31	33	33	33	35	35	36	36
Sub-lethal	**59**	26	28	30	31	33	34	34	34	35	37	38	38
Lethal	**65**	17	17	17	17	20	20	20	22	26	27	28	30

		Time-to-death (hour)											
Treatment	**Total #**	**19.5**	**20**	**20.5**	**21**	**21.5**	**22**	**22.5**	**23**	**23.5**	**24**	**24.5**	**25**
Reference	**58**	37	39	40	40	44	44	44	45	45	45	45	47
Sub-lethal	**59**	39	40	41	43	43	43	45	45	45	45	47	47
Lethal	**65**	32	32	34	36	39	39	40	40	41	41	45	47

		Time-to-death (hour)											
Treatment	**Total #**	**25.5**	**26**	**26.5**	**27**	**27.5**	**28**	**28.5**	**29**	**29.5**	**30**	**30.5**	**31**
Reference	**58**	47	47	47	49	49	50	51	51	51	51	51	52
Sub-lethal	**59**	48	48	48	49	49	50	50	51	52	52	54	55
Lethal	**65**	47	49	49	52	53	53	54	55	56	57	58	59

b. The times-to-death of amphipods during the 2nd IED vs. stochastic NaPCP experiment.

		Time-to-death (hour)										
Treatment	**Total #**	**0**	**1**	**2**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**	**6.5**
Reference	**63**	0	0	0	1	1	1	3	3	3	3	5
Sub-lethal	**59**	0	0	0	0	0	0	0	1	2	3	3
Lethal	**58**	0	0	0	0	0	1	2	2	2	3	4

		Time-to-death (hour)										
Treatment	**Total #**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
Reference	**63**	6	6	7	8	10	10	10	12	15	15	16
Sub-lethal	**59**	4	5	7	8	9	11	14	16	16	19	20
Lethal	**58**	5	5	5	5	6	6	6	7	7	7	8

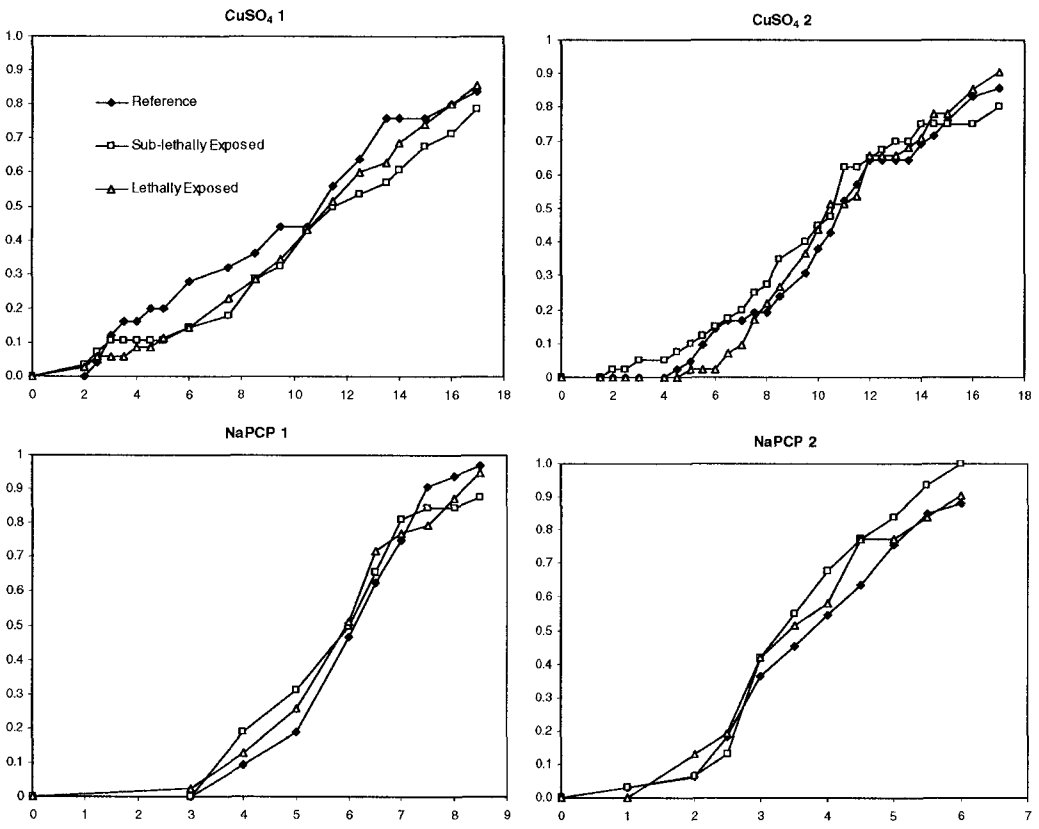
		Time-to-death (hour)										
Treatment	**Total #**	**12.5**	**13**	**13.5**	**14**	**14.5**	**15**	**15.5**	**16**	**16.5**	**17**	**17.5**
Reference	**63**	17	19	20	23	24	27	29	29	31	33	34
Sub-lethal	**59**	20	21	23	23	24	27	28	29	29	31	32
Lethal	**58**	8	9	11	12	13	13	15	19	20	22	22

		Time-to-death (hour)										
Treatment	**Total #**	**18**	**18.5**	**19**	**19.5**	**20**	**20.5**	**21**	**21.5**	**22**	**22.5**	**23**
Reference	**63**	37	39	39	41	43	43	44	44	46	47	49
Sub-lethal	**59**	33	35	38	38	40	40	41	42	45	47	50
Lethal	**58**	25	28	32	34	38	41	42	43	43	45	46

		Time-to-death (hour)										
Treatment	**Total #**	**23.5**	**24**	**24.5**	**25**	**25.5**	**26**	**26.5**	**27**	**27.5**	**28**	**28.5**
Reference	**63**	50	52	53	53	54	55	55	56	57	58	58
Sub-lethal	**59**	50	51	51	52	54	54	54	55	55	55	55
Lethal	**58**	47	47	50	51	52	52	52	52	53	53	53

APPENDIX 14

The cumulative mortality curves for the four IED vs stochastic experiments. They were confounded possibly by the bacteria contamination in RMHRW.



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