CHAPTER 8

Ecologically Meaningful Estimates of Lethal Effect in Individuals

Michael C. Newman and Philip M. Dixon

I. OVERVIEW

An individual organism's fitness is diminished in the presence of sufficiently high concentrations of a toxicant. As survival is the most easily measured fitness component, most of the early efforts to protect aquatic biota focused on lethality. Methods, borrowed from mammalian toxicology, were first applied to acute or intense toxicant exposures and later extended to chronic exposures. For decades, the foundation of methods and assessment procedures (e.g., Sprague, 1969, 1971; EPA, 1975; Stephanz, 1977; Buikema et al., 1982; ASTM, 1989) was classic work in mammalian toxicology (e.g., Bliss, 1935; Litchfield and Wilcoxon, 1949; Armitage and Allen, 1950; Gaddum, 1953; Finney, 1964).

The predominant approach adopted in the U.S. (dose- or concentration-response approach) quantified mortality at a predetermined time endpoint to estimate the toxicant concentration producing a certain level of mortality, e.g., 96-h LC50 (Sprague, 1969). The predicted concentration at which 50% of exposed individuals survived exposure of a specified duration (LC50) was selected as the primary measure of toxic effect as it was the most statistically reliable estimate, i.e., the estimate with the narrowest 95% confidence interval (Trevan, 1927). It was not selected because it was the most ecologically meaningful estimate. An extensive regulatory structure was built with this approach as the cornerstone.

Despite their early utility, direct application of dose-response or time endpoint techniques became increasingly awkward as attention shifted from acute exposure toward chronic exposure scenarios. Temporal dynamics of toxicant effects, less pertinent in early mammalian toxicity testing, grew in importance during assessment of long-term toxicant impacts on aquatic

1-56670-1127-9/95/$0.00+$.50

populations. Modification of time endpoint methods became necessary to incorporate variation of exposure duration into effects models. The incipient LC50 (the predicted concentration below which at least 50% of exposed individuals would live indefinitely, relative to the lethal effects of the toxicant) was formulated from plots of LC50 versus exposure time. This estimate of toxic incipiency generated from LC50 values also has ambiguous ecological significance (Newman, 1995). The long-term consequences of 50% mortality on population viability is impossible to assess from such studies. Empirical models continue to be developed in attempts to encompass temporal dynamics with the time endpoint approach, e.g., Wang and Hanson (1985) and Mayer et al. (1994).

Sprague (1969) argued unsuccessfully for equal consideration of the alternate, time-response approach. Instead of noting deaths only at one specific time, the times-to-death for individuals exposed to various treatments were to be noted. He reemphasized Finney's (1964) point that ignoring mortality information prior to a time endpoint seriously reduces statistical power. He quoted Gaddum (1953), "... theoretically it may be expected that about half the information is lost, so that twice as many observations will be needed for any given degree of accuracy." Sprague followed Gaddum's estimate with Burdick's (1960) more extreme estimate that ten times more replicates would be needed if time endpoint methods were used instead of time-to-death methods. Despite these sound arguments, use of the endpoint methods was deemed "good enough" and expanded to dominate ecotoxicology while the survival time approach did not progress much beyond the Litchfield (1949) method for estimating median time of survival (LT50). Today, endpoint methods are used without critical comparison to alternative approaches, such as the survival time approach, and without much understanding of underlying assumptions.

Consequently, it is the purpose of this chapter to provide a brief treatment of the often-neglected basis for time endpoint methods and then to detail the implementation of the heretofore neglected survival time methods. Discussion of survival time methods will pull together material scattered throughout several of our past publications, including Diamond et al. (1989, 1991), Dixon and Newman (1991), Heagler et al. (1993), Keklak et al. (1994), Newman et al. (1989, 1994), Newman and Aplin (1992), and Newman (1995). Data sets from Diamond et al. (1989) and Newman and Aplin (1992) will be used throughout to illustrate application of survival time methods. Time endpoint methods will not be described in as much detail because specifics are provided in many other sources (e.g., Hamilton et al., 1977; Stephan, 1977; Buikema et al., 1982; Newman, 1995).

II. THE DOSE-RESPONSE (TIME ENDPOINT) APPROACH

A wide range of time endpoint methods are applied in ecotoxicology (Figure 1). Most are used to estimate the LC50 and its 95% confidence

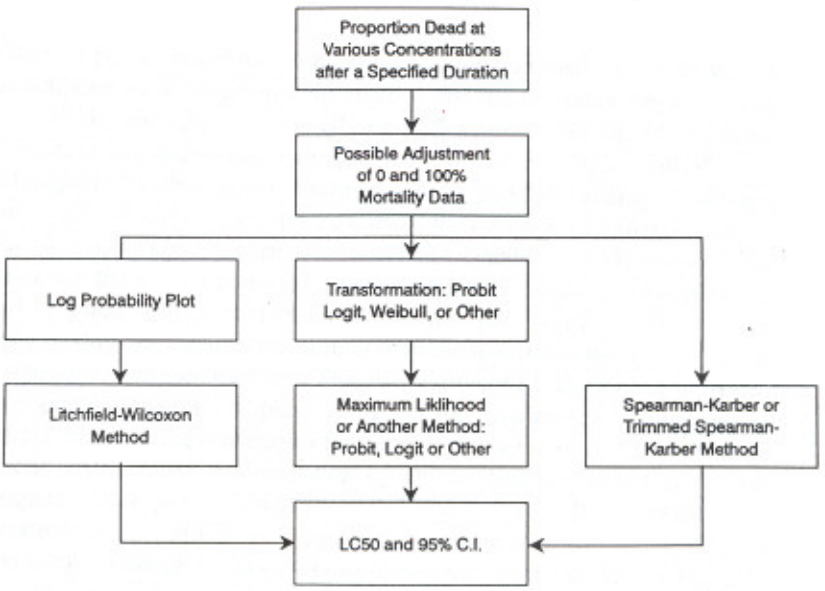

Figure 1 Dose-response (time endpoint) methods for analyzing mortality data.

interval.* One such method, the semigraphical Litchfield-Wilcoxon approach (Litchfield and Wilcoxon, 1949), is subject to error because a line is fit by eye in one step. The Litchfield-Wilcoxon method can be improved by using statistically fit data, but methods such as maximum likelihood estimation fit data assuming a specific underlying distribution and provide more reliable estimates than the Litchfield-Wilcoxon approach. Maximum likelihood methods have good precision yet generate slightly biased estimators.** But this bias is small relative to those of alternative methods except when there are very few observations (Finney, 1971). A range of underlying distributions can be assumed, including a log normal (probit transformations), log logistic (logit transformations), or Weibull. Finney (1964) also describes the less common Wilson-Worcester, Cauchy-Urban, and "linear" or rectangular sigmoid models. The arcsine square root transformation may also be used, although its use is based less on conformity to any specific underlying model than on the desire to produce transformed data with constant variance over the range of exposure concentrations. These techniques are implemented conveniently with a wide range of software including CT-TOX (CT Dept. of Environmental Protection, 1990), PROBIT (EPA, 1988), SAS (SAS Institute, 1988), and TOXSTAT (WEST, Inc. and Gulley, 1994).

* Although concentrations killing other proportions of exposed individuals, e.g., LC10, can also be estimated, the precision of the estimate is generally poorer than that of the LC50.

** Alternatively, the Spearman-Karber method is applicable if a symmetrical but unspecified distribution is assumed.

Fitting a probit model with a maximum likelihood method carries with it the assumption of a log normal distribution for the mortality response. The probit* of the proportion dead (P) plotted against the log of exposure concentration should generate a straight line. Bliss (1935) explains the process underlying this distribution of mortalities within a population using the concept of individual lethal dose, i.e., there exists a characteristic minimal amount of toxicant needed to kill any particular individual. Gaddum (1953) also explains the basis for the log normal model in an identical manner but refers to the individual lethal dose as the individual effective dose or I.E.D. He gives the example of slow intravenous infusion of digitalis into individual cats until just enough is present to stop the heart. The distribution of I.E.D.s among individuals in a population often displays a log normal distribution because "... in biological material the variation often shows a geometrical rather than an arithmetic distribution, an observation which has been confirmed by several investigators in respect to toxicological characteristics" (Bliss, 1935). This explanation is forwarded to support the present-day use of probits in ecotoxicology although I.E.D.s are never measured and the validity of extrapolating from poisoned "biological material" to whole organism exposure from an aqueous media is questionable.

The use of logits implies a log logistic model. A plot of the logit (logit = $\ln[P/(1-P)]$) against the log of exposure concentration may produce a straight line.** Use of the logit has been defended by linkage to either the Hill equation for enzyme kinetics or Langmuir adsorption models (Gaddum, 1953). Also, the differential form of the logistic model describes many diverse and pertinent processes including autocatalysis, bimolecular reactions, and enzyme-mediated hydrolysis (Berkson, 1951). Regardless of similarities of curves and underlying mechanisms, the rate of change in the logistic model is proportional to an "amount" to be acted upon (e.g., number of individuals alive at any moment) and is also proportional to some additional factor that increases as this "amount" decreases (Berkson, 1951). In support of the log logistic model, Berkson (1951) points out that the log normal model assumes a static distribution of tolerances but the log logistic model is based on dynamic mechanics. He discusses a study of human tolerance (I.E.D.) to high altitude conditions that, upon repetition, did not support the assumption that the I.E.D. was an inherent quality of an individual. Because the I.E.D. was the usual explanation of the log normal (probit) model, Berkson argued that this study illustrated the inadequacy of the log normal model. Gaddum (1953) countered this criticism by suggesting that the log normal model reflects a process in which several "hits" are needed at a target site to result in death. Finney (1964) provides more

* The probit is the normal equivalent deviate + 5. The normal equivalent deviate is the distance from the mean of a normal distribution expressed in units of standard deviations. The normal equivalent deviate is also called the z, standard normal deviate, normal equivalent deviation, and standard score in various statistical textbooks.

** Often a transformed logit (transformed logit = logit/2 + 5) is used because the resulting transformed values are very similar to probit values.

detail but no definitive resolution regarding the theoretical foundations for the probit or logit transformations.

More recently the Weibull model, a flexible generalization of the exponential model, was advocated for fitting survival data (Pinder et al., 1978), including time endpoint data (Christensen, 1984; Christensen and Nyholm, 1984; Newman, 1995). A straight line is produced by plotting the Weibull transform ($\ln(-\ln(1-P))$) against the log of exposure concentration. This model has been applied successfully to algal (Christensen, 1984; Christensen and Nyholm, 1984) and fish (Christensen, 1984; Newman, 1995) toxicity data. Speculation about the underlying mechanism for a Weibull model has included its conformity to one hit or multiple hit models of carcinogenesis (Christensen, 1984). Christensen and Nyholm (1984) link the Weibull model to Teisser's equation also. They assume that the slope of the Weibull transform curve is a measure of the number of toxicant molecules interacting with each receptor site. No evidence has been presented to date to support or refute this insightful yet speculative assertion.

There are two important points to be made regarding the application of these methods to time endpoint data. First, these transforms produce very similar estimates near the LC50 but estimates become increasingly divergent toward the extremes, e.g., LC10. Also, confidence intervals at the extremes become larger, regardless of the model. Consequently, effective model selection* becomes more important as ecotoxicology shifts its focus downward away from the LC50. Second, these well-established methods have no theoretical superiority to alternative approaches and, as applied today, are simply empirical or redescription models (see Chapter 1).

III. THE TIME-RESPONSE (SURVIVAL TIME) APPROACH

A. INTRODUCTION

The time-response (survival time, failure time, time-to-death, resistance time, or waiting time) approach is a powerful alternative to dose-response methods. These methods make more effective use of mortality data than do dose-response methods (see Figure 2). In Figure 2, mortality is noted through time at five concentrations. Only four data points (marked by ▲'s) would be used in applying a dose-response method to estimate an LC50. Complete mortality before 96 h at the highest concentration would render this treatment useless or marginally useful in calculations. In contrast, almost an order of magnitude more data would be available from the same experiment if mortality were noted every 12 h. This larger data set would include data from the highest concentration. This approach entails some extra effort; however, it is customary with toxicity testing to periodically remove dead individuals and to note

* Newman (1995) provides a detailed discussion and example of such comparisons of probit, logit, and Weibull transformations.

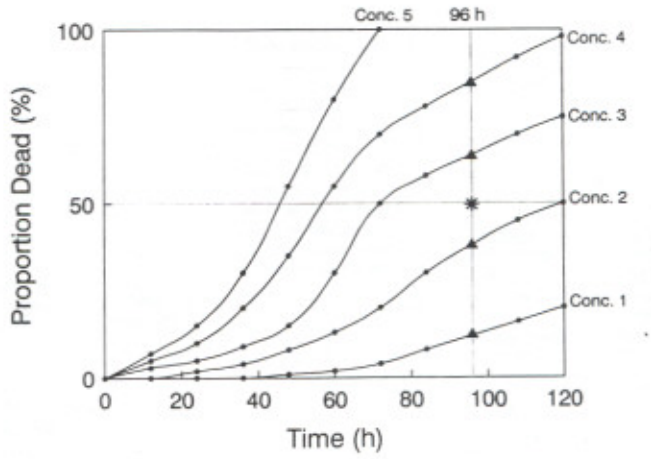

Figure 2 More data are collected from a toxicity test if times-to-death are noted. Only four data points are available if proportion dead by 96 h is the measurement noted.

mortality, e.g., the sample data sheet for toxicity tests provided in Parrish (1985).

Litchfield's method (Litchfield, 1949) for estimating median lethal time (LT50) and its 95% confidence interval is the only time-response method routinely presented to aquatic toxiciologists by instructors, textbooks, and methods manuals. Although this graphical method is useful, the time-response approach embraces a much richer suite of methods routinely used in clinical sciences, economics, engineering, and epidemiology (Figure 3). Nonparametric methods, including product-limit (Kaplan-Meier) and life table methods, are available. Neither requires that a specific distribution be assigned to the survival curve. Product-limit methods have the advantage of allowing observation intervals to vary in length and carry fewer assumptions than life table methods (Newman, 1995). Life (actuarial) tables have the advantage of linkage to many ecological concepts and parameters. (The reader is referred to Chapter 9 in this volume and Miller (1981) for details regarding life table methods.) Survival curves for different groups or classes of exposed individuals can be tested for equality using log-rank, Wilcoxon, or other methods.

The remaining methods in Figure 3 compare covariate effects to a reference survival curve, e.g., survival of smokers relative to that of nonsmokers. A Cox proportional hazard model can be used if no underlying model can or need be assumed for the reference survival curve. The hazards (proneness to die) of the various classes are assumed to be proportional. If a specific underlying distribution is assumed for the survival curves, data can be fit using one of two parametric formulations, proportional hazard or accelerated failure time. The hazards of various classes are assumed proportional in the parametric proportional hazard model as with the semiparametric Cox proportional hazard model. With the accelerated failure time model, covariates act to modify the ln of time-to-death (TTD). A covariate may shorten the ln TTD of an individual

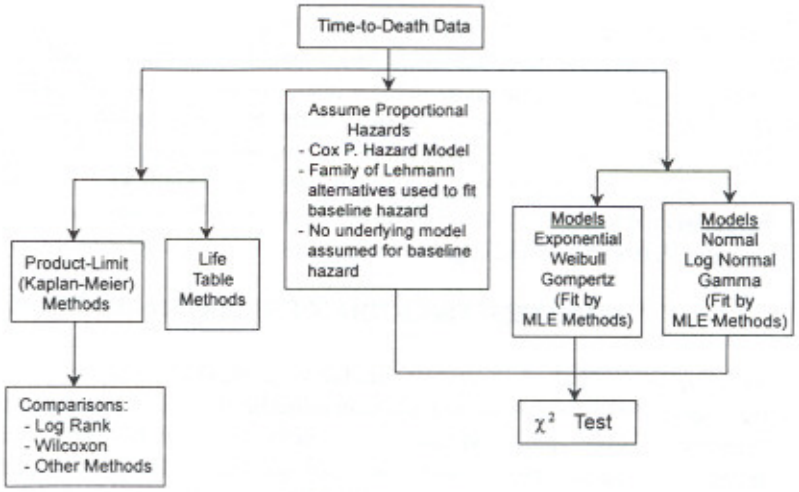

Figure 3 Time-response (survival time) methods for analyzing mortality data.

or group of similar individuals, that is "accelerate" the process resulting in death. Hypothesis tests are used to test for differences among classes or effects of continuous variables with these semiparametric and parametric models.

Nonparametric, semiparametric, and fully parametric methods are easily implemented with a wide range of popular software packages, including BMDP (Dixon, 1985), GLIM 4 (Francis et al., 1993), SAS (SAS Institute, 1988), S-Plus (Statistical Sciences, 1993), Survcalc (Lachenbruch, 1986), and SYSTAT (Steinberg and Colla, 1988). Code for the widely used SAS package (SAS Institute, 1988) will be used to illustrate implementation here.

Before detailing each method, it is necessary to define several unfamiliar terms. F(t) is the cumulative mortality distribution function and f(t) is the associated probability density function. The F(t) is 0 at $t = 0$ and slowly increases toward 1 as the cumulative number of deaths increases through time. The survival function (S(t)) begins at 1 and slowly decreases toward 0 through time. S(t) is equal to $1 - F(t)$. The hazard function, h(t), describes the probability of dying in a time interval. H(t) is the cumulative hazard function. Dixon and Newman (1991) provide further details and examples of hazard and survival curves.

Another conspicuous feature of time-to-death data is the common occurrence of censoring. Often, the exact time-to-death is not known for some individuals. For example, the only information available from a censored individual may be that it survived at least a certain number of hours of exposure. Censoring often involves termination of the exposure before all individuals are dead. The time-to-death for a survivor is some undetermined time greater than the duration of the experiment. This is referred to as right censoring because the missing times-to-death are from the right-hand side of the survival curve. Alternatively, individuals may have been removed at set times during the course of the exposure to assay some physiological or

biochemical change during exposure. A series of censoring times would be generated for sets of fish removed during the trial. This is referred to as multiple, right censoring. Finally, time-to-death may be measured within a sufficiently long interval that time-to-death can only be noted to have occurred between the beginning and end of the interval, e.g., between 24 and 30 h of exposure. This interval censoring can bias results if intervals are large relative to the total duration of the exposure. Newman et al. (1994) and Newman (1995) provide a detailed example of interval censoring in survival model development.

B. NONPARAMETRIC (PRODUCT-LIMIT) METHODS

S(t) or the cumulative survival density function can be described by a nonparametric product-limit estimator (Kaplan and Meier, 1958) as detailed in Miller (1981), Blackstone (1986), Dixon and Newman (1991), and Newman (1995). It is initially 1 and declines over time as individuals die. It remains undefined beyond the end of the exposure period (T) if right censoring is present, i.e., there are survivors.

$$\hat{S}(t_i) = \prod_{j=1}^{i} \left(1 - \frac{d_j}{n_j}\right) \tag{1}$$

where n_j = the number of individuals alive just before t_j, and d_j = the number of individuals dying at t_j. Greenwood's formula (Equation 2) estimates the variance for the product-limit $\hat{S}(t_i)$ which becomes Equation 3 for all times prior to the end of the exposure if no individuals were censored before the end of the experiment.

$$\hat{\sigma}^2 = \sum_{j=1}^{i} \frac{d_j}{n_j s_j} \tag{2}$$

where $s_j = n_j - d_j$.

$$\hat{\sigma}^2(t_i) = \frac{\hat{S}(t_i)[1 - \hat{S}(t_i)]}{N} \tag{3}$$

where N = the total number of individuals exposed.

The standard error for $\hat{S}(t_i)$ is the square root of the variance calculated above. This standard error and $z_{1-\alpha/2}$ can be used to produce confidence intervals as described in SAS Institute (1988), Dixon and Newman (1991), and Newman (1995).

Dixon and Newman (1991) provide a detailed application of these methods to survival data, including SAS code to produce survival curves with confidence

intervals. They also illustrate the use of log-rank and Wilcoxon tests for equivalence of survival curves for treatment classes of tuberculosis-infected mice, trout exposed to different dissolved oxygen concentrations, and mosquitofish of different sexes and genotypes exposed to arsenate. With these methods, Newman (1995) tested and accepted the null hypothesis that survival time data for duplicate tanks in a salt toxicity test (Newman and Aplin, 1992) came from the same distribution. Appendix 1 provides SAS program code to implement such a comparison. This code also generates product-limit estimates and associated standard errors. More detailed description of these results is given in Newman (1995). Rejection of the null hypothesis using the calculated χ^2 statistic implies that the duplicates were not acceptably similar, much as Fisher's exact test might be used to test whether duplicate treatments in a dose-response test were homogeneous. The Wilcoxon test tends to be more sensitive than the log-rank test to deviations at the onset of exposure (Dixon and Newman, 1991).

C. SEMIPARAMETRIC COX PROPORTIONAL HAZARD METHODS

This approach is based on the assumption that the effect of a covariate such as toxicant concentration is to shift the baseline hazard (probability of dying during an interval). The amount by which the baseline hazard is shifted remains constant with time: the effect of each covariate does not change over the duration of exposure (Equation 4).

$$h(t, x_i) = e^{f(x_i)} h_0(t) \tag{4}$$

where $h(t,x_i)$ = the hazard at t given the covariate value x_i, $h_0(t)$ = the baseline hazard, and $e^{f(xi)}$ = the function of the covariate x acting on the baseline hazard.

More precisely, the Cox proportional hazard model assumes "linearity and additivity of the predictors with respect to log hazard" (Harrell, 1988). No specific distribution is assumed for the baseline hazard to which hazards of other types are scaled. Instead an empirical function (from a family of Lehmann alternatives) is fit for the baseline hazard ($h_0(t)$) using the rank order of the TTDs. (See Miller, 1981, or Cox and Oakes, 1984 for further discussion of Lehmann alternatives.) The neglect of the specific form of the baseline hazard may be by design or necessity. In many clinical trials, the exact form of the underlying hazard is much less important than the effect of various treatments on diminishing the likelihood of dying. Alternatively, Heagler et al. (1993) exposed mosquitofish to inorganic mercury and found no acceptable model for the hazard function: a Cox proportional hazard model was necessary. Steadman et al. (1991) also used such a model to describe the effect of trout pre-exposure to No. 2 fuel oil on TTD during a second exposure.

The term, $e^{f(x_i)}$ scales hazards to the baseline hazard. The significance of a covariate is tested with a χ^2 statistic. The mercury-mosquitofish data from

Table 1 Summary of Cox Proportional Hazard Model for Diamond et al. (1989) data

Variable	df	Estimate (S.E.[a])	χ^2	*P* of obtaining this χ^2 value by chance alone
NTANK	1	0.1207 (0.0861)	1.965	0.1610
NSEX	1	−0.8033 (0.0894)	80.830	0.0001
LWT	1	−2.7551 (0.2470)	124.474	0.0001

[a] Standard error of the estimate.

Diamond et al. (1989) are used here to illustrate this approach (Table 1). The SAS code to perform the associated analyses is provided in Appendix 2. The cumulative mortality curves for the two exposure tanks and one control tank are provided in Figure 4. The effects of exposure tank (NTANK),. fish sex (NSEX), and fish weight (ln of wet weight, LWT) are tested with the SAS PHGLM procedure. Note that two of the covariates (NTANK and NSEX) are class variables and one (LWT) is a continuous variable. The tank effect is not significant at $\alpha = 0.05$; however, mosquitofish sex and wet weight are highly significant. The maximum likelihood estimate of $f(x_i)$ for ln of wet weight was negative, indicating that the hazard increased as size decreased. Smaller fish were more sensitive than larger fish. Sex also has a negative estimate. Note from the code in Appendix 2 that females are arbitrarily designated as 1 and males are designated as 0. Therefore, a decrease in NSEX from 1 to 0 (risk associated with being female is changed to that of a male) increased the hazard. Males were more sensitive than females.

These differences can be expressed as relative risks. (See Dixon and Newman, 1991 for more details.) For a class variable such as sex, the relative risk is $e^{\hat{T}_i}$ where $\hat{T}_i$ is the estimated effect of the ith type within the class. The relative risk of females and males in this Cox proportional hazard model are $e^{-0.8033\times1}$ (=0.447) and $e^{-0.8033\times0}$ (=1), respectively. Males are more than twice (1/0.447 = 2.23) as likely to die as females. For continuous variables such as ln of wet weight, relative risk is $e^{\hat{\beta}_i\Delta x}$ where $\hat{\beta}_i$ is the estimated effect of the continuous variable and Δ_x is the change in the variable x. For example, the relative risk associated with the difference between a 0.1 g (ln 0.1 = −2.30258) and 0.5 g (ln 0.5 = −0.69315) fish is $e^{-2.7751*(-2.30258-(-0.69315))}$ or approximately 87. A 0.1-g fish is 87 times more likely than a 0.5-g fish to die at any time during exposure. Under the assumption of additivity of covariate effects on the ln hazard, the risk of a 0.1-g male fish relative to a 0.5-g female is approximately 87*2 or 174 times higher.

D. PARAMETRIC METHODS

i. General

In other cases, it is possible and advantageous to define an underlying distribution for the baseline mortality. There are two general forms of such parametric models. A proportional hazard model with the same general form as Equation 4 can be used; however, $h_0(t)$ is now fit to a specific distribution. The exponential and Weibull distributions are those that result in a proportional

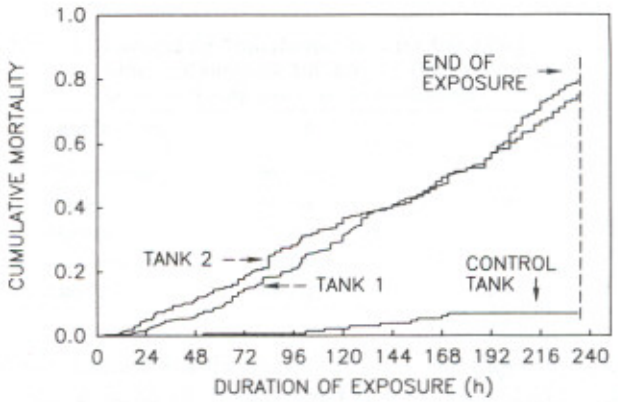

Figure 4 Cumulative time-to-death of mercury-exposed (tanks 1 and 2) and control fish. (Fom Diamond, S.A. et al., 1989. Environ. Toxicol. Chem. 8, 613–622. Reproduced by permission of Pergamon Press.)

hazard model. The second form is the accelerated failure time model, which has the form given in Equation 5.

$$\ln t_i = f(x_i) + \varepsilon_i \tag{5}$$

where t_i = time-to-death, $f(x_i)$ = a function of the covariate effecting ln t_i, and ε_i = an error term. The distributions most often applied to an accelerated failure model are the log normal, log logistic, and gamma. Cox and Oakes (1984) describe less commonly used distributions in their Table 2.1.

Why do the different distributions result in proportional hazard or accelerated failure time models? Cox and Oakes (1984), and Dixon and Newman (1991) point out that the accelerated failure time model (Equation 5) can be expressed also as a model of hazard (Equation 6). Under the assumption of an exponential or Weibull distribution, this general hazard model is a proportional hazard model. Hazard is either constant (exponential distribution) or changing monotonically (Weibull distribution) with time for these two distributions. With the other distributions mentioned above, hazards do not remain proportional through time and an accelerated failure model (Equation 5) is appropriate.

$$h(t, x_i) = e^{f(x_i)} h_0(t, e^{f(x_i)}) \tag{6}$$

The $f(x_i)$ can take on a wide range of forms in both the proportional hazard and accelerated failure time models. Dixon and Newman (1991), and Newman et al. (1994) provide several examples. The function may simply describe the mean response of each class if the variable in question is a class variable, e.g., the mean response for males and females. Alternatively, it may be some function of a continuous variable, e.g., $f(\text{Weight}_i) = \alpha + \beta \ln(\text{Weight}_i)$.

Inclusion of several covariates in the model is easily accomplished. For example, Newman et al. (1994) describe size-dependent mortality during

Table 2 Linearizing Transformations for Selecting Among Candidate Underlying Distributions in Formulating Survival Time Models

Distribution	X	Y
Exponential	$\ln \hat{S}(t)$	t
Weibull	$\ln(-\ln \hat{S}(t))$	ln t
Normal	Probit $(\hat{F}(t))$	t
Log normal	Probit$(\hat{F}(t))$	ln t
Log logistic	$\ln(\hat{S}(t)/\hat{F}(t))$	ln t

sodium chloride exposure with the model, $f([NaCl_i], Weight_j) = \alpha + \beta_s \log[NaCl_i] + \beta_w \log Weight_j$. A vector of $\hat{\beta}$ values and $\hat{\alpha}$ are estimated and multiplied by the data matrix to predict TTD. Maximum quasi-likelihood estimates ($\hat{\beta}$s and $\hat{\alpha}$) are generated assuming a distribution for ε.

ii. Application of Parametric Models

Several tools are available for selecting the best model for survival data. A series of linearizing transformations can be done on the data and their effectiveness examined visually. The most common linearizing transformations are provided in Table 2. No linearizing transformation is available for the gamma distribution. (Appendix 3 contains SAS program code for plotting the various linearizing transformations for data from Diamond et al., 1989 shown in Figure 4.) For example, Figure 5 is a plot of the data from Newman and Aplin (1992) assessing the Weibull model. $\hat{S}(t)$ is estimated up to the end of the exposure as one minus the cumulative mortality at each time, i.e., one minus the total number of fish dying by time t divided by the total number of exposed fish. (Similarly, $\hat{F}(t)$ used in other transformations in Table 2 is estimated as the total number of fish dying by time t divided by the total number of exposed fish.) The relatively straight and parallel lines for the five salt treatments suggest that the Weibull model is appropriate for these data.

The log likelihood statistic may also be used to compare candidate models much as sums of squares are used to compare model fit with least-squares methods. The model with the largest (least negative) log likelihood statistic has the best fit to the data. The SAS code provided in Appendix 4 generates the log likelihood statistics required to assess the relative goodness of fit for various underlying distributions (exponential, Weibull, log normal, log logistic, and gamma) in combination with various covariate (salt concentration and fish wet weight) transformations. The log likelihood statistics cannot be used directly in this situation because the number of parameters varies among the candidate models. Instead Akaike's information criterion (AIC), which adjusts the log likelihood value to account for the varying number of parameters, can be used for model comparison (Atkinson, 1980; Harrell, 1988).

$$AIC = -2(\log \text{ likelihood}) + 2P \tag{7}$$

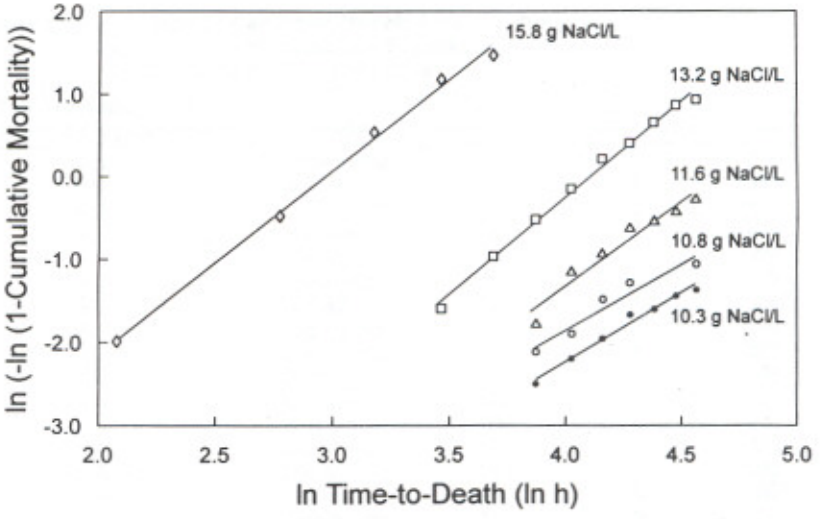

Figure 5 Transformed sodium chloride TTD data. (From Newman, M.C. and M.S. Aplin, 1992. Aquat. Toxicol. (Amst.) 23, 85–96. Reproduced by permission of Elsevier Science Ltd.)

where P = the number of parameters fit with the model. In the exponential model, three parameters are estimated, the two $\hat{\beta}$s for the effects of size and salt concentration plus the exponential parameter ($\hat{\mu}$). In the Weibull model, the two $\hat{\beta}$s for the effects of size and salt concentration plus the two Weibull parameters (the scale and shape parameters) are estimated. Similarly, the log normal, log logistic, and gamma models involve estimation of the two $\hat{\beta}$s plus the parameters required to define the log normal (two parameters), log logistic (two parameters), or gamma (three parameters) distributions. As the log logistic model using ln transformed wet weight and ln transformed salt concentration has the smallest AIC, it fits these data best (Table 3). The parameter estimates generated for these data using the log logistic model with log transformed covariates are provided in Table 4. Notice that two additional parameters are provided for the parametric, log logistic model (Table 4) relative to the Cox proportional hazard model (e.g., Table 1). The values of $\hat{\mu}$ and $\hat{\sigma}$ are estimates of the central tendency and logistic scale parameter. The log logistic model fit to these data is the following.

$$\text{Ln TTD} = \hat{\mu} + \hat{\beta}_s \ln[\text{NaCl}] + \hat{\beta}_w \ln \quad \text{Weight} + \hat{\varepsilon} \tag{8}$$

or

$$\text{TTD} = e^{\hat{\mu}} e^{\hat{\beta}_s \ln[\text{NaCl}]} e^{\hat{\beta}_w \ln \text{Weight}} e^{\hat{\varepsilon}} \tag{9}$$

The scale parameter and the value for 50% from a standardized error distribution assuming a logistic distribution are used for estimation of the

Table 3 Comparison of Candidate Models for Describing Survival Time of Mosquitofish Exposed to a Series of Sodium Chloride Concentrations

Transformations[a] of weight/salt concentration	Distribution	Log likelihood	No. of parameters	AIC
A/A	Exponential	−385	3	776
A/A	Weibull	−194	4	396
A/A	Log normal	−202	4	412
A/A	Log logistic	−198	4	404
A/A	Gamma	−193	5	396
A/L	Exponential	−383	3	772
A/L	Weibull	−193	4	394
A/L	Log normal	−193	4	394
A/L	Log logistic	−190	4	388
A/L	Gamma	−189	5	388
L/A	Exponential	−377	3	760
L/A	Weibull	−196	4	400
L/A	Log normal	−195	4	398
L/A	Log logistic	−190	4	388
L/A	Gamma	−191	5	392
L/L	Exponential	−376	3	758
L/L	Weibull	−194	4	396
L/L	Log normal	−186	4	380
L/L	Log logistic	−182	4	372
L/L	Gamma	−185	5	380

[a] A, arithmetic; L, ln of covariate. A/L would indicate that wet weight was used in the arithmetic and salt concentration was transformed to its natural logarithm in the model.

From Newman, M.C. and M.S. Aplin, 1992. Aquat. Toxicol. (Amst.) 23, 85–96. With permission of Elsevier Science Ltd.

Table 4 Summary of the Log Logistic Model for the Mosquitofish-Sodium Chloride Toxicity Data of Newman and Aplin (1992)

Variable	df	Estimate (S.E.[a])	χ^2	Probability of obtaining this χ^2 value by chance alone
Intercept (μ)	1	15.2860 (0.2563)	3555.98	<0.0001
Ln [NaCl] (β_s)	1	−4.2129 (0.0830)	2575.80	<0.0001
Ln Weight (β_w)	1	0.2545 (0.0386)	43.50	0.0001
Scale (σ)[b]	1	0.2081 (0.0104)		

[a] Standard error of the estimate.

[b] The scale parameter for the logistic distribution.

median TTD. The value ($L_{0.5}$) corresponding to 50% for a logistic distribution (0) is taken from a table such as Appendix 7 in Newman (1995). The median TTD (MTTD) for this log logistic model is predicted with Equation 10. Equation 10 reduces to Equation 12. (Appendix 5 also provides SAS program code to calculate MTTD and its associated confidence interval.)

$$\text{MTTD} = e^{\hat{\mu}} e^{\hat{\beta}_s \ln[\text{NaCl}]} e^{\hat{\beta}_w \ln \text{Weight}} e^{\hat{\sigma} L_{0.5}} \tag{10}$$

$$\text{MTTD} = e^{\hat{\mu}} e^{\hat{\beta}_s \ln[\text{NaCl}]} e^{\hat{\beta}_w \ln \text{Weight}} e^{\hat{\sigma} * 0} \tag{11}$$

$$\text{MTTD} = e^{\hat{\mu}} e^{\hat{\beta}_s \ln[\text{NaCl}]} e^{\hat{\beta}_w \ln \text{Weight}} \tag{12}$$

These MTTD can be estimated for a wide range of salt concentrations (Figure 6). If the sodium chloride data had been used to estimate 96-h LC50 with a conventional dose-response method such as the trimmed Spearman-Karber method, only one point and its confidence interval would have been generated in Figure 6. However, a series of estimates of toxic effect, including confidence intervals, can be generated using the methods just described for these same data. Even more useful predictions can be generated from these models. For example, Figure 7 displays predicted MTTD at various sodium chloride concentrations for various sized fish.

Other TTD for other percentages of mortality can be calculated using the corresponding $L_{0.X}$ values from the standardized error distribution for the logistic distribution. In the SAS program code in Appendix 5, the requested quantile (Q = 0.5 for median) can be easily changed to facilitate these calculations. Further, the parameter estimates for other models, such as the Weibull model discussed immediately below, can be used to estimate TTD for any percentile using the appropriate standardized error distribution. Respectively, $W_{0.5} = -0.36651$ and $N_{0.5} = 0.00000$ would be used instead of $L_{0.5}$ in equations such as those above for estimation of the MTTD for the Weibull and log normal models.

The Weibull model for these data (Table 5) can be used to illustrate the estimation of relative risk with a parametric proportional hazard model. The Weibull model employing arithmetic covariates (salt concentration and fish wet weight) suffices as it has an AIC value only slightly larger than the log logistic (transformed covariates) model and it generates similar predictions in the pertinent toxicant concentration range (Figure 6). The approach is similar to that described previously for the Cox proportional hazard model, except the scale parameter estimate is incorporated. The relative risk is $e^{-T_i/s}$ for a class variable such as fish sex or $e^{-\beta_i \Delta_i / s}$ for a continuous variable such as salt concentration or fish wet weight. Using this Weibull model with untransformed covariates as an example, the risk of a 0.1-g fish relative to a 1.0-g fish is $e^{(-1.0602*-0.9)/0.3046}$ or approximately 23 times higher during salt exposure. Combined risks associated with differences in two or more covariates may be estimated as done with the Cox proportional hazard model results.

Conventional estimates of toxicity such as the 96-h LC50 can be generated with these parametric models (Newman and Aplin, 1992). Equation 13 predicts LC50 for the Weibull model with untransformed covariates. LC50 could be estimated for any time by changing ln 96 to the ln of the particular time of interest. The LC50 for fish of various weights could be calculated by specifying the particular weight of interest (Weight).

$$96\text{-h LC50} = \frac{\ln 96 - \hat{\mu} - \hat{\beta}_w \text{Weight} - \hat{\sigma} W_{0.5}}{\hat{\beta}_s} \tag{13}$$

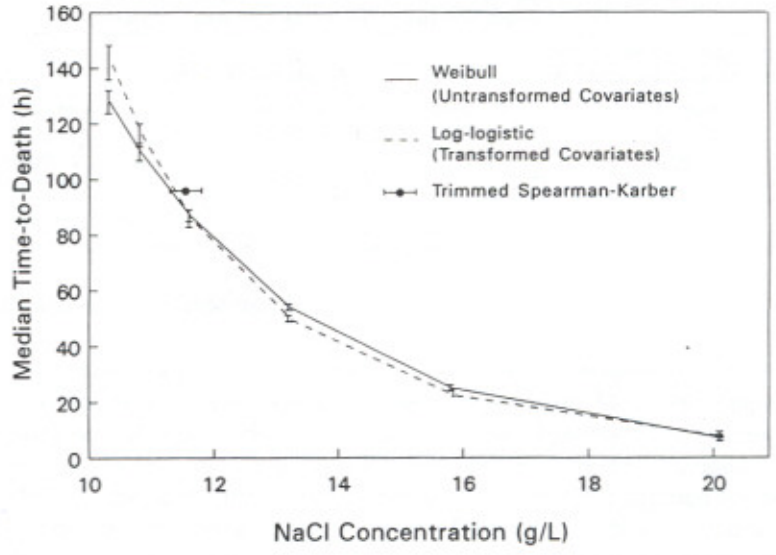

Figure 6 Prediction of median times-to-death during sodium chloride exposure using a Weibull model with untransformed covariates and a log logistic model with transformed covariates. (From Newman M. C. and M.S. Aplin, 1992. Aquat. Toxicol. (Amst.) 23, 85–96. Reproduced by permission of Elsevier Science Ltd.)

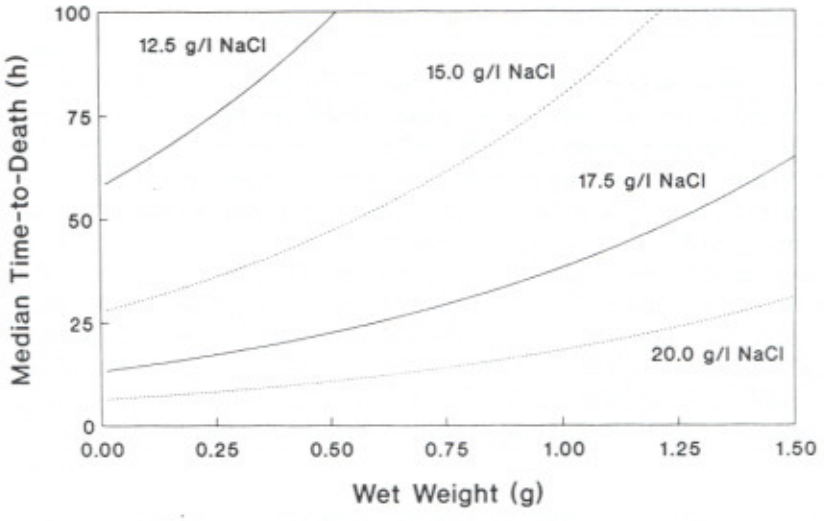

Figure 7 Prediction of median times-to-death for different concentrations of sodium chloride and mosquitofish sizes. (From Newman M.C. and M.S. Aplin, 1992. Aquat. Toxicol. (Amst.) 23, 85–96. Reproduced by permission of Elsevier Science Ltd.)

Table 5 Summary of the Weibull Model for the Mosquitofish-Sodium Chloride Toxicity Data of Newman and Aplin (1992)

Variable	df	Estimate (S.E.[a])	χ^2	Probability of obtaining this χ^2 value by chance alone
Intercept (μ)	1	7.8579 (0.0853)	8487.27	<0.0001
[NaCl] (β_s)	1	–0.2953 (0.0052)	3257.53	<0.0001
Weight (β_w)	1	1.0602 (0.2566)	17.07	0.0001
Scale (σ)[b]	1	0.3046 (0.0137)		

[a] Standard error of the estimate.

[b] The scale parameter for the Weibull distribution.

iii. Multiple Comparisons

The class variables considered to this point have been those involving only two types: tank 1 versus tank 2, or male versus female. Means of comparing among several types within a class, such as several genotypes at a locus, are presented in this section using data generated initially by Diamond et al. (1989). In this study, mosquitofish in duplicate tanks were exposed for 240 h to 0.964 µg/l of inorganic mercury. The TTD for each fish was noted within 3-h intervals. The TTD, fish weight, fish sex, and genotype at eight loci were noted. Data for fish from the duplicate tanks were pooled, as preliminary analyses indicated no significant tank effect on TTD (Table 1). The effect of genotype (six genotypes) at the glucosephosphate isomerase-2 locus (PGI-2) on TTD is analyzed here in addition to the effects of fish sex and size noted as significant in Table 1. The $\log_{10}$ of wet weight is used in contrast to the original analysis of Diamond et al. (1989) to improve goodness-of-fit. The code detailed in Appendix 6 applied to these data produced the model estimates in Table 6.

Note that both sex and GPI-2 genotype are treated as class variables. For sex classes, males were arbitrarily selected as the reference sex and the effect of being female estimated based on the differences between the means of the two sexes. In contrast with continuous variables for which the value of the variable is used, an indicator variable is used for class variables. One is used for all types within a class, except the reference type, which is given an indicator value of zero. The effects of all GPI-2 genotypes are estimated relative to the 38/38 genotype: the 38/38 genotype has an indicator variable of zero and the remaining genotypes have indicator variables of one. For example, the MTTD for a male fish of 0.15 g weight ($\log_{10}$ of 0.15 = –0.8239) and genotype of 100/100 would be 124 h as calculated with Equation 14. That for a female fish with the same weight and genotype would be 182 h as calculated with Equation 15.

$$\text{MTTD} = e^{5.7191} e^{(0*0)} e^{(1.2971*-0.8239)} e^{(0.3571*1)} e^{(0.5082*-0.3665)} \quad (14)$$

$$\text{MTTD} = e^{5.7191} e^{(0.3806*1)} e^{(1.2971*-0.8239)} e^{(0.3571*1)} e^{(0.5082*-0.3665)} \quad (15)$$

Table 6 Summary of the Weibull Model for the Mosquitofish-Mercury Toxicity Data of Diamond et al. (1989)

Variable	df	Estimate (S.E.[a])	χ^2	Probability of obtaining this χ^2 value by chance alone
Intercept (μ)	1	5.7191 (0.1478)	1496.93	<0.0001
Sex (β_s)	1		70.00	0.0001
Female	1	0.3806 (0.0455)	70.00	0.0001
Male	0	0(0)	—	—
Log_{10} Weight (β_w)	1	1.2971 (0.1254)	107.03	0.0001
PGI-2 genotype	5		16.86	0.0048
100/100	1	0.3571 (0.1162)	9.44	0.0021
66/100	1	0.4544 (0.1152)	15.55	0.0001
38/100	1	0.3551 (0.1216)	8.53	0.0035
66/66	1	0.3737 (0.1236)	9.14	0.0025
38/66	1	0.3235 (0.1393)	5.39	0.0202
38/38	1	0(0)		
Scale (σ)[b]	1	0.5082 (0.0189)		

[a] Standard error of the estimate.

[b] The scale parameter for the Weibull distribution.

The effect of each class variable is indicated by an overall χ^2 e.g., 16.86 with df = 5 for an overall effect of GPI-2 genotype on TTD (Table 6). There was clearly a significant effect of GPI-2 genotype on TTD with the 38/38 being the most sensitive genotype. Additional χ^2 values are provided for pairwise comparisons of the reference genotype (38/38) to each of the other five genotypes. Adjusting the experimentwise α level to account for five comparisons, differences between the reference genotype and each of the other genotypes can be tested. However, additional information is required to compare all $k(k-1)/2$ possible genotype pairs. (k is the number of types in the class, e.g., six GPI-2 genotypes will require 15 pairwise comparisons.) The convenient SAS code provided by Fox (1993) is modified for this purpose in Appendix 7. Values from the parameter covariance matrix output from the SAS program are used in Equation 16 (Fox, 1993) to estimate a z score.

$$Z = \frac{\left|\hat{\beta}_i - \hat{\beta}_j\right|}{\sqrt{V_{ii} + V_{jj} - 2(V_{ij})}} \tag{16}$$

where $\hat{\beta}_i$ and $\hat{\beta}_j$ = the estimates for the ith and jth type, and V_{ii}, V_{jj}, V_{ij} = the iith, jjth, and ijth element of the covariance matrix.

The associated probabilities (P) are calculated or taken from a table using these z scores. These probabilities are then compared to an experimentwise α. Fox (1993) uses the following adjustment (Dunn-Šidák adjustment with a one-sided interval) to ensure that the experimentwise α remains constant (e.g., 0.05).

$$\alpha' = 1 - \left(\frac{1}{2}\right)^{1/p} (2-\alpha)^{1/p} \tag{17}$$

or

$$\alpha' = 1 - \left(1 - \frac{\alpha}{2}\right)^{1/p} \tag{18}$$

where p = the number of comparisons. Fox's use of the one-sided interval is not appropriate and, in our modified SAS program code in Appendix 7, Dunn-Šidák adjustment for a two-sided interval is made according to Equation 19 instead. Other adjustments such as the Bonferroni adjustment may also be used, but they are generally more conservative than the Dunn-Šidák adjustment.

$$\alpha' = 1 - (1 - \alpha)^{1/p} \tag{19}$$

The results suggest that the 38/38 genotype is significantly different (experimentwise $\alpha = 0.05$) from all genotypes except the 38/66 genotype (Table 7). There are no other significant differences between genotypes.

iv. Summary of Parametric Methods

The mechanisms underpinning the most common models remain as speculative as those discussed earlier for the dose-response models. For example, the exponential model suggests a simple process with a constant hazard over time. The constant hazard implies no system memory, i.e., the hazard at any time interval is unrelated to what occurred in previous intervals (Cox and Oakes, 1984). The best example of this type of process is radioactive decay, in which the probability of a radionuclide atom decaying is independent of any processes that took place in past time intervals. Obviously, this type of model would be inappropriate if a slow accumulation of damage or "wear" over time was suspected to result in death (Nelson, 1969). Cox and Oakes (1984) give an example of a distribution of "loads" described by a Poisson process: there are many "loads" or toxicant damages that the organism could experience during exposure. Failure occurs the first time that an extreme "load" is experienced. This explanation is similar to that given earlier for a dose-response model based on the I.E.D. Use of the more general Weibull model suggests a "weak link" failure mode (Dixon and Newman, 1991). The Weibull shape parameter is speculated to reflect the number of "components," "parts," or "links in a chain" available to fail and cause death. "... if a unit can be regarded as a system of many parts each with a failure time from the same distribution and if the unit fails with the first part failure, then a Weibull distribution may be appropriate" (Nelson, 1969). It follows mathematically and conceptually that the Weibull model with a shape parameter of one (one component available to fail) reduces to an exponential model.

Regardless of the predominantly nonmechanistic motivations behind their application, a wide spectrum of powerful parametric models for survival time can be used to predict survival as a function of exposure time and covariates. In addition to the methods described here, means of incorporating covariates

Table 7 Summary of Comparisons Among GPI-2 Genotypes

Genotype	100/100	66/100	38/100	66/66	38/66	38/38
100/100	—	N	N	N	N	Y
66/100		—	N	N	N	Y
38/100			—	N	N	Y
66/66				—	N	Y
38/66					—	N
38/38						–

An experimentwise $\alpha = 0.05$ was maintained by adjusting the pairwise αs using the Dunn-Sidàk method ($\alpha' = 0.003$).

Note: Comparisons were based on the modified code of Fox (1993) as provided in Appendix 7. See Table 6 for estimates of effect for each genotype.

that change over the duration of the exposure, e.g., toxicant accumulating in a target organ, are available. Chapter 8 in Cox and Oakes (1984) and Chapter 6 in Miller (1981) provide general discussions of time-dependent covariates. Several statistical computer programs, e.g., SAS (SAS Institute Inc., 1988) allow inclusion of time-dependent covariates. Such models have promise in linking bioaccumulation to toxicant effect. Concentration present in a critical tissue or cumulative amount of exposure in the tissue during exposure, i.e., the area under the time-tissue concentration curve, could be explored as the time-dependent covariate depending on the assumptions made regarding the mode of failure. These methods can also be extended to estimation of other ecologically relevant qualities, such as genetic fitness (Manly, 1985; Newman, 1995), seedling emergence and flowering (Fox, 1990a, b, 1993), and foraging behavior (Muenchow, 1986).

IV. CONCLUSION

Relative to dose-response methods, those based on time-response permit more meaningful inclusion of time, increased precision of estimates, and, because of their enhanced power, more effective incorporation of covariates. Enhancing the precision of estimates becomes increasingly important as our focus moves downward from 50% mortality to lower percentages. Resulting models may be linked to demographic and population genetics models. For these reasons, use of the time-response methods described herein will enhance our ability to predict (or test the significance of) covariate effects on toxicity. They provide more ecologically meaningful estimates of lethal effect than dose-response methods.

ACKNOWLEDGMENTS

This work was supported by contract DE-AC09-76SROO-819 between the U.S. Department of Energy and the University of Georgia. We appreciated the thoughtful reviews by Drs. G. Fox, T. La Point, P. Landrum, F. Mayer, M. Mulvey, and C. Strojan of an earlier version of the manuscript.

Appendix 1 Statistical Analysis System (SAS) Code for Nonparametric Tests of Equality Between Classes (Duplicate Tanks of Fish Exposed to Various Salt Concentrations)

```
DATA TOXICITY;
 INFILE "B:TOXICITY.DAT";
 INPUT TTD 1-2 TANK $ 4-5 PPT 6-10 WETWGT 12-16 STDLGTH 18-20;
 IF TTD>96 THEN FLAG=1;
  ELSE FLAG=2;
RUN;
PROC SORT;
 BY PPT TANK TTD;
RUN;
PROC LIFETEST;
 TIME TTD*FLAG(1);
 STRATA TANK;
 BY PPT;
RUN;
```

The data in file "TOXICITY.DAT" are identified and their format defined. The time-to-death (TTD), replicate tank (TANK), sodium chloride concentration in g/l (PPT), fish wet weight (WETWGT), and fish standard length (STDLGTH) are defined. If the TTD is greater than 96 h (longer than the duration of the experiment), the fish survived to the end of the exposure and it is identified as censored (FLAG = 1). After these data are sorted, the LIFETEST procedure is used to test for significant differences between duplicates at each salt concentration. Product-limit survival estimates (including standard errors) are tabulated in the resulting output. A χ^2 statistic and an associated P is calculated to facilitate testing of differences based on the Log-rank, Wilcoxon, and –2 log (likelihood ratio) methods.

Note: These data come from Newman and Aplin (1992). A detailed description of this example is contained in Newman (1995).

Appendix 2 Statistical Analysis System (SAS) Code for Using a Cox Proportional Hazard to Analyze the Mercury-Exposed Mosquitofish Data (Diamond et al., 1989)

Fish sex (NSEX as a numerical variable), fish weight (LWT as the log of wet weight), and duplicate tank (NTANK as a numerical variable) are tested for significant effect.

```
DATA FISH;
 INFILE "B:FISH.DAT";
 INPUT TTD 5-9 TANK $ 11 SEX $ 13 SIZE 15-18;
 IF TANK NE "C";
 IF SEX = "F" THEN NSEX=1;
  ELSE NSEX=0;
 IF TANK="1" THEN NTANK=1;
  ELSE NTANK=0;
 LWT=LOG10(SIZE);
 IF TTD>234 THEN FLAG=1;
  ELSE FLAG=2;
RUN;
PROC SORT;
 BY NTANK NSEX;
RUN;
PROC PHREG;
 CLASS NTANK NSEX;
 MODEL TTD*FLAG(1)=NTANK NSEX LWT;
RUN;
```

No underlying distribution is assumed for the baseline hazard in this model. However, hazards are assumed proportional among classes (duplicate tanks and sexes).

Appendix 3 Statistical Analysis System (SAS) Code for Plotting Various Linear Transformations of Mercury Survival Data from Diamond et al. (1989)

Cumulative mortality (CDF_TTD) in two, replicate tanks containing 367 (TANK 1) and 375 (TANK 2) mosquitofish are plotted to visually assess the appropriateness of the exponential, Weibull, log normal, and log logistic distributions.

```
DATA FISH;
 INFILE "B:FISH.DAT";
 INPUT ID 1-3 TTD 5-9 TANK $ 11 SEX $ 13 SIZE 15-18;
 IF TREAT NE "C";
 IF TTD>234 THEN FLAG=1;
   ELSE FLAG=2;
RUN;
PROC SORT;
 BY TANK TTD;
RUN;
DATA FIGURE;
 KEEP TANK TTD CUM_TTD CDF_TTD ST FT PRIT E H ODD LODD LOG_H
 LOG_T;
 SET FISH;
 BY TANK TTD;
 RETAIN CUM_TTD 0;
  IF FIRST.TANK THEN CUM_TTD=0;
  CUM_TTD=CUM_TTD+1;
  IF LAST.TTD THEN DO;
   IF TANK="1" THEN CDF_TTD=CUM_TTD/367;
     ELSE CDF_TTD=CUM_TTD/375;
  ST=1-CDF_TTD;
  FT=1-ST;
  PRIT=PROBIT(FT);    /* Five may be added to Prit (normal equivalent deviate or */
  E=LOG(ST);          /* inverse normal distribution function) to get the probit  */
  H=-LOG(ST);
  ODD=ST/FT;
  LODD=LOG(ODD);
  LOG_H=LOG(H);
  LOG_T=LOG(TTD);
  OUTPUT;
 END;
RUN;
PROC PLOT;
 PLOT E*TTD=TANK;            /* EXPONENTIAL   */
 PLOT LOG_H*LOG_T=TANK;      /* WEIBULL       */
 PLOT PRIT*LOG_T=TANK;       /* LOG NORMAL    */
 PLOT LODD*LOG_T=TANK;       /* LOG LOGISTIC  */
RUN;
```

A series of potentially linearizing plots are generated with this code. Gross assessment of goodness-of-fit of the data to the underlying model can be made with these plots.

Appendix 4 Statistical Analysis System (SAS) Code for Selection Among Various Model Formulations by Comparing Underlying Distributions and Variable Transformations of the Newman and Aplin (1992) data

```
DATA TOXICITY;
 INFILE "B:TOXICITY.DAT";
 INPUT TTD 1-2 TANK $ 4-5 PPT 6-10 WETWGT 12-16 STDLGTH 18-20;
 IF PPT>0;
 IF TTD>96 THEN FLAG=1;
  ELSE FLAG=2;
 LWETWGT=LOG(WETWGT);
```

```
 LPPT=LOG(PPT);
RUN;
PROC SORT;
 BY PPT WETWGT TTD;
RUN;
PROC LIFEREG;
  MODEL TTD*FLAG(1)=PPT WETWGT/DISTRIBUTION=EXPONENTIAL;
  MODEL TTD*FLAG(1)=PPT WETWGT/DISTRIBUTION=WEIBULL;
  MODEL TTD*FLAG(1)=PPT WETWGT/DISTRIBUTION=LNORMAL;
  MODEL TTD*FLAG(1)=PPT WETWGT/DISTRIBUTION=LLOGISTIC;
  MODEL TTD*FLAG(1)=PPT WETWGT/DISTRIBUTION=GAMMA;
  MODEL TTD*FLAG(1)=PPT LWETWGT/DISTRIBUTION=EXPONENTIAL;
  MODEL TTD*FLAG(1)=PPT LWETWGT/DISTRIBUTION=WEIBULL;
  MODEL TTD*FLAG(1)=PPT LWETWGT/DISTRIBUTION=LNORMAL;
  MODEL TTD*FLAG(1)=PPT LWETWGT/DISTRIBUTION=LLOGISTIC;
  MODEL TTD*FLAG(1)=PPT LWETWGT/DISTRIBUTION=GAMMA;
  MODEL TTD*FLAG(1)=LPPT WETWGT/DISTRIBUTION=EXPONENTIAL;
  MODEL TTD*FLAG(1)=LPPT WETWGT/DISTRIBUTION=WEIBULL;
  MODEL TTD*FLAG(1)=LPPT WETWGT/DISTRIBUTION=LNORMAL;
  MODEL TTD*FLAG(1)=LPPT WETWGT/DISTRIBUTION=LLOGISTIC;
  MODEL TTD*FLAG(1)=LPPT WETWGT/DISTRIBUTION=GAMMA;
  MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=EXPONENTIAL;
  MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=WEIBULL;
  MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=LNORMAL;
  MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=LLOGISTIC;
  MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=GAMMA;
RUN;
```

The data in file "TOXICITY.DAT" are identified and their format defined as detailed in Appendix 1. Two additional variables are created (LPPT = natural log of salt concentration and LWETWGT = natural log of wet weight). After sorting, the LIFEREG procedure is used to generate models using the various distributions and variable transformations. The log likelihood statistic is used indirectly to compare relative goodness of fit for the candidate models.

Appendix 5 Statistical Analysis System (SAS) Code for Generating Predicted Times-to-Death and Associated Standard Errors for the Newman and Aplin (1992) Data Set

```
DATA TOXICITY;
 INFILE "B:TOXICITY.DAT";
 INPUT TTD 1-2 TANK $ 4-5 PPT 6-10 WETWGT 12-16 STDLGTH 18-20;
 IF PPT>0;
 IF TTD>96 THEN FLAG=1;
  ELSE FLAG=2;
 LWETWGT=LOG(WETWGT);
 LPPT=LOG(PPT);
RUN;
PROC SORT;
 BY LPPT LWETWGT TTD;
RUN;
PROC LIFEREG;
 MODEL TTD*FLAG(1)=LPPT LWETWGT/DISTRIBUTION=LLOGISTIC;
 OUTPUT OUT=TIMEL Q=0.5 P=LLPRED CDF=LLALIVE STD=LLSTD;
RUN;
PROC PRINT;
 VAR PPT WETWGT TTD LLPRED LLSTD.
```

The data in file "TOXICITY.DAT" are identified and their format defined as detailed in Appendix 1. Two additional variables are created (LPPT = natural log of salt concentration and LWETWGT = natural log of wet weight). After sorting, the LIFEREG procedure is used

to generate a log logistic model. The predicted TTD for each record (fish) and its associated standard deviation is calculated, output to a file (TIMEL), and printed. Predictions can also be generated with SAS for fish with other qualities, e.g., predicted median TTD and associated standard deviations for 0.1-g fish held at various salt concentrations. Lines of data with a negative ID, an average standard weight, missing TTD, and different exposure concentrations of interest (e.g., PPT of 10, 12.5, 15.0, and 15.5) are added to the original data set. Associated predicted TTD will be those of "average fish" identical except for the concentration to which they were exposed. Because the TTDs are missing for these fabricated records, these records will not be used for the calculations although predicted values will be generated for each. To conveniently output the predictions from these records only, the code is modified so that only those records with negative IDs are printed.

Appendix 6 Statistical Analysis System (SAS) Code for Generating a Proportional Hazard Model (Weibull Distribution) to Incorporate the Effects of Mosquitofish Sex (SEX), Wet Weight (log of wet weight in g or SIZE), and Genotype at the Glucosephosphate Isomerase-2 Locus (PGI-2) on Time-to-Death During Exposure to Inorganic Mercury

The covariance matrix is requested at the end of the model statement for use later in estimating the significance of differences between all genotypes.

```
DATA HG;
 INFILE "B:HG.DAT";
 INPUT ID 1-3 PGI2 $ 38-39;
RUN;
PROC SORT;
 BY ID;
DATA FISH;
 INFILE "B:FISH.DAT";
 INPUT ID 1-3 TTD 5-9 TANK $ 11 SEX $ 13 SIZE 15-18;
 LSIZE=LOG10(SIZE);
 IF TREAT NE "CONTROL";
 IF TTD>234 THEN FLAG=1;
   ELSE FLAG=2;
RUN;
PROC SORT;
 BY ID;
RUN;
DATA ALL;
 MERGE HG FISH;
 BY ID;
RUN;
PROC SORT DATA=ALL;
 BY SEX PGI2;
RUN;
PROC LIFEREG;
 CLASS SEX PGI2;
 MODEL TTD*FLAG(1)=SEX LSIZE PGI2/COVB;
RUN;
```

A model is generated that estimates β's for the effects of fish sex, log of wet weight, and GPI-2 genotype on TTD. Tests of significant differences (χ^2) are performed. The addition of /COVB to the model statement results in an output containing the covariance matrix. This matrix can then be used for making multiple comparisons, e.g., pairwise comparisons among all genotypes (see Appendix 7).

Appendix 7 Statistical Analysis System (SAS) Code for Doing Multiple Comparisons Between GPI-2 Genotypes in the Mercury-Exposed Mosquitofish Data

The code detailed in Appendix 6 is used to generate estimates of β for each genotype and a covariance matrix. Three files are then made with the output from the code: names of the genotypes (NAMES.DAT), the covariance matrix with 0s in the table for the reference genotype (COVAR.DAT), and the β estimates for each genotype (STATS.DAT). This code is modified from that of Fox (1993). The results are placed into a file (MERCURY.OUT).

```
DATA TOT;
 ARRAY NAMES[6] $;
 ARRAY STATS[6];
 ARRAY COVAR[6,6];
 ARRAY ZSCORE[6,6];
 ARRAY PROB[6,6];
 INFILE "B:NAMES.DAT";
  INPUT NAMES [*] $;
 INFILE "B:STATS.DAT";
  INPUT STATS[*];
 INFILE "B:COVAR.DAT";
  INPUT COVAR[*];
FILE "B:MERCURY.OUT";
PUT 'MULTIPLE COMPARISONS OF PGI-2 GENOTYPES';
PUT 'BASED ON LIFEREG BETA STATISTICS';
PUT;
DO I=1 TO 6;
 DO J=(I+1) TO 6;
      ZSCORE[I,J]=ABS(STATS[I]-STATS[J])/
            SQRT(COVAR[I,I]+COVAR[J,J]-2*COVAR[I,J]);
     PROB[I,J]=1-PROBNORM(ZSCORE[I,J]);
    PUT 'COMPARISON:
     'NAMES[I] '&' NAMES [J]':'
        'Z = ' ZSCORE[I,J]
        'PR{FROM SAME SAMPLE} ='
        PROB[I,J];
      PUT;
    END;
  END;
  PUT;
  PUT 'THESE GIVE PROBABILITIES FOR SINGLE COMPARISONS ONLY.';
  PUT 'FOR MULTIPLE COMPARISONS, USE THE FOLLOWING:';
ALPHA=0.05;
NPOP=6;
NCOMPARE=NPOP*(NPOP-1)/2;
INVCOMP=1/NCOMPARE;
ADJUST=1-(1-ALPHA)**INVCOMP; /* DUNN-SIDAK ADJUSTMENT */
ZADJUST=PROBIT(ADJUST);
PUT 'TO ACCEPT A DIFFERENCE AS SIGNIFICANT AT THE ' ALPHA
    'LEVEL,';
PUT 'USE ONLY Z-VALUES WITH P < '
    ADJUST;
RUN;
```

The SAS data set NAMES.DAT is the following: 100100 10066 10038 6666 6638 3838.

These values are the six genotypes for this three-allele locus, e.g., the 100/100 genotype is designated 100100.

The SAS data set STATS.DAT containing the estimates of β is the following: 0.35713182 0.45437342 0.35505211 0.37369819 0.3235346 0.00000.

These estimates could also have been statistics generated with the nonparametric product-moment method. Indeed, this code was modified slightly from that of Fox (1993), which used the Wilcoxon scores as generated by the SAS procedure LIFETEST. In Fox's analysis, the covariance matrix in COVAR below would contain the covariance matrix for the Wilcoxon scores.

The SAS data set COVAR.DAT is the following:

```
0.013513 0.011806 0.011805 0.011779 0.011768 0.000000
0.011806 0.013278 0.011823 0.011793 0.011779 0.000000
0.011805 0.011823 0.014787 0.011776 0.011799 0.000000
0.011779 0.011793 0.011776 0.015270 0.011762 0.000000
0.011768 0.011779 0.011799 0.011762 0.019413 0.000000
0.000000 0.000000 0.000000 0.000000 0.000000 0.000000
```

The columns containing 0.000000 are those associated with the reference genotype (3838). The remaining values were extracted from the appropriate columns in the covariance matrix generated by SAS. For example, 0.013513 is that associated value with the 100100, 100100 element in the matrix. Below it in the matrix is the value 0.011806 which is associated with the 100100, 10066 element of the matrix.

REFERENCES

Armitage, P. and I. Allen, 1950. Methods of estimating the LD50 in quantal response data. J. Hyg. 48, 298–322.

ASTM, 1989. Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. ASTM E 729–88. American Society for Testing and Materials, Philadelphia, PA, pp. 480–499.

Atkinson, A.C., 1980. A note on the generalized information criterion for choice of a model. Biometrika 67, 413–418.

Berkson, J., 1951. Why I prefer logits to probits. Biometrics 7, 327–339.

Blackstone, E.H., 1986. Analysis of death (survival analysis) and other time-related events. In: Current Status of Clinical Cardiology, edited by F.J. Macartney, MTP Press, Boston, MA, pp. 55–101.

Bliss, C.I., 1935. The calculation of the dosage-mortality curve. Ann. Appl. Biol. 22, 134–167.

Buikema, Jr., A.L., B.R. Niederlehner and J. Cairns, 1982. Biological monitoring. IV. Toxicity testing. Water Res. 16, 239–262.

Burdick, G.E., 1960. The use of bioassays by the water pollution control agency. Biol. Problems Water Pollution Transactions of Second Seminar, 1959, U.S. Public Health Service, R.A. Taft Sanit. Eng. Center Tech. Report W 60–3, U.S. Department of Health, Education and Welfare, Atlanta, 145–148.

Christensen, E.R., 1984. Dose-response functions in aquatic toxicity testing and the Weibull model. Water Res. 18, 213–221.

Christensen, E.R. and N. Nyholm, 1984. Ecotoxicological assays with algae: Weibull dose-response curves. Environ. Sci. Technol. 18, 713–718.

Cox, D.R. and D. Oakes, 1984. Analysis of Survival Data. Chapman and Hall, New York.

CT Department of Environmental Protection, 1990. CT-TOX MULTI-METHOD PROGRAM. Bureau of Water Management, CT Dept. Environ. Protect., Water Toxics Lab., 122 Washington St., Hartford, CT 06106.

Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon and D. Martinson, 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard), during acute exposure to inorganic mercury. Environ. Toxicol. Chem. 8, 613–622.

Diamond, S.A., M.C. Newman, M. Mulvey and S.I. Guttman, 1991. Allozyme genotype and time-to-death of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure: a comparison of populations. Aquat. Toxicol. (Amst.) 21, 119–134.

Dixon, W.J., 1985. BMDP statistical software 1985. University of California Press, Berkley, CA.

Dixon, P.M. and M.C. Newman, 1991. Analyzing toxicity data using statistical models for time-to-death: an introduction. In: Metal Ecotoxicology. Concepts and Applications, edited by M.C. Newman and A.W. McIntosh, Lewis Publishers, Chelsea, MI, pp. 207–242.

EPA, 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Ecological Research Series No. EPA-660/3-75-013. U.S. Environmental Protection Agency, Washington, D.C.

EPA, 1988. Users guide for a computer program for probit's analysis of data from acute and short-term chronic toxicity tests with aquatic organisms. Biological Methods Branch, Environ. Mon. and Support Lab., Office of Res. and Devel., U.S. Environmental Protection Agency, Cincinnati, OH 45268.

Finney, D.J., 1964. Statistical Method in Biological Assay. Hafner, New York.

Finney, D.J., 1971. Probit Analysis, Cambridge University Press, London.

Fox, G.A., 1990a. Components of flowering time variation in a desert annual. Evolution 44, 1404–1423.

Fox, G.A., 1990b. Perennation and the persistence of annual life histories. Am. Nat. 135, 829–840.

Fox, G.A., 1993. Failure-time analysis: emergence, flowering, survivorship, and other waiting times. In: Design and Analysis of Ecological Experiments, edited by S.M. Scheiner and J. Gurevitch, Chapman and Hall, New York, pp. 253–425.

Francis, B., M. Green and C. Payne, 1993. The GLIM system, release 4 manual. Clarendon Press, Oxford.

Gaddum, J.H., 1953. Bioassays and mathematics. Pharmacol. Rev. 5, 87–134.

Hamilton, M.A., R.C. Russo and R.V. Thurston, 1977. Trimmed Spearmen-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714–719.

Harrell, F.E., Jr., 1988. Survival and Risk Analysis. Duke University Medical Center, Durham, NC.

Heagler, M.G., M.C. Newman, M. Mulvey and P.M. Dixon, 1993. Allozyme genotype in mosquitofish, *Gambusia holbrooki*, during mercury exposure: temporal stability, concentration effects and field verification. Environ. Toxicol. Chem. 12, 385–395.

Kaplan, E.L. and P. Meier, 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. 53, 457–481.

Keklak, M.M., M.C. Newman and M. Mulvey, 1994. Enhanced uranium tolerance of an exposed population of the Eastern mosquitofish (*Gambusia holbrooki* Girard 1859). Arch. Environ. Contam. Toxicol. 27, 20–24.

Lachenbruch, P.A., 1986. SurvCalc. Survival Analysis on the IBM PC. John Wiley & Sons, New York.

Litchfield, Jr., J.T., 1949. A method for rapid graphic solution of time-per cent effects curves. J. Pharmacol. Exp. Ther. 97, 399–408.

Litchfield, Jr., J.T. and F. Wilcoxon, 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.

Manly, B.F.J., 1985. The statistics of natural selection on animal populations. Chapman and Hall, London.

Mayer, F.L., G.F. Krause, D.R. Buckler, M.R. Ellersieck and G. Lee, 1994. Predicting chronic lethality of chemicals from acute toxicity test data: concepts and linear regression analysis. Environ. Toxicol. Chem. 13, 671–678.

Miller, R.G., Jr., 1981. Survival Analysis. John Wiley & Sons, New York.

Muenchow, G., 1986. Ecological use of failure time analysis. Ecology 67, 246–250.

Nelson, W., 1969. Hazard plotting for incomplete failure data. J. Qual. Technol. İ, 27–52.

Newman, M.C. 1995. Quantitative Methods in Aquatic Ecotoxicology. Lewis Publishers, Chelsea, MI.

Newman, M.C. and M.S. Aplin, 1992. Enhancing toxicity data interpretation and prediction of ecological risk with survival time modeling: an illustration using sodium chloride toxicity to mosquitofish (Gambusia *holbrooki*). Aquat. Toxicol. (Amst.) 23, 85–96.

Newman, M.C., S.A. Diamond, M. Mulvey and P. Dixon, 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute toxicant exposure: a comparison of arsenate and inorganic mercury. Aquat. Toxicol. (Amst.) 15, 141–156.

Newman, M.C., M.M. Keklak and M.S. Doggett, 1994. Quantifying animal size effects on toxicity: a general approach. Aquat. Toxicol. (Amst.) 28, 1–12.

Parrish, P.R., 1985. Acute toxicity tests. In: Fundamentals of Aquatic Toxicology, edited by G.M. Rand and S.R. Petrocelli, Hemisphere Publishing, Washington, D.C., pp. 31–57.

Pinder, J.E., III, J.G. Wiener and M.H. Smith, 1978. The Weibull distribution: a new method of summarizing survivorship data. Ecology 59, 175–179.

SAS Institute Inc., 1988. SAS/STAT User's Guide, Release 6.03 edition. SAS Institute Inc., Cary, NC.

Sprague, J.B., 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res. 3, 793–821.

Sprague, J.B., 1971. Measurement of pollutant toxicity to fish. III. Sublethal effects and "safe" concentrations. Water Res. 5, 245–266.

Statistical Sciences, 1993. S-PLUS Guide to Statistical and Mathematical Analysis, Version 3.2. Mathsoft, Inc., Seattle, WA, pp. 17-1–17-36.

Steadman, B.L., W.A. Stubblefield, T.W. LaPoint and H.L. Bergman, 1991. Decreased survival of rainbow trout exposed to No. 2 fuel oil caused by sublethal preexposure. Environ. Toxicol. Chem. 10, 355–363.

Steinberg, D. and P. Colla, 1988. SURVIVAL: A supplementary module for SYSTAT. SYSTAT, Inc., Evanston, IL.

Stephan, C.E., 1977. Methods for calculating an LC50. In: Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, edited by F.L. Mayer and J.L. Hamelink, American Society for Testing and Materials, Philadelphia, PA, pp. 65–84.

Trevan, J.W., 1927. The error of determination of toxicity. Proc. R. Soc. London Ser. B Biol. Sci. 101, 483–514.

Wang, M.P. and S.A. Hanson, 1985. The acute toxicity of chlorine on freshwater organisms: time-concentration relationships of constant and intermittent exposures. In: Aquatic Toxicology and Hazard Evaluation, ASTM STP 891, edited by R.C. Bahner and D.J. Hansen, American Society for Testing and Materials, Philadelphia, PA, pp. 213–232.

WEST, Inc. and D.D. Gulley, 1994. TOXSTAT 3.4. Western Ecosystems Technology, Inc., Cheyenne, WY.

ECOTOXICOLOGY
A Hierarchical Treatment

Edited by
Michael C. Newman
Charles H. Jagoe

LEWIS PUBLISHERS
Boca Raton New York London Tokyo

Library of Congress Cataloging-in-Publication Data

Ecotoxicology : a hierarchical treatment / editors, Michael C. Newman and Charles H. Jagoe.
p. cm. -- (Savannah River series on environmental sciences)
Includes bibliographical references (p.) and index.
ISBN 0-56670-127-9 (alk. paper)
1. Pollution--Environmental aspects--Congresses. 2. Pollutants--Toxicology--Congresses. I. Newman, Michael C. II. Jagoe, Charles H. III. Series.
QH545.A1E285 1996
574.5'222--dc20 93-31128
CIP

Lewis Publishers is an imprint of CRC Press

No claim to original U.S. Government works
International Standard Book Number 0-56670-127-9
Library of Congress Card Number 95-31128
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper