Water Research Vol. 15, pp. 107 to 119

0043-1354/81/0101-0107$02.00/0

PARAMETRIC ANALYSES OF MORTALITY RATES IN BIOASSAYS

S. A. L. M. KOOIJMAN

Division of Technology for Society TNO, Postbox 217, 2600 AE Delft, The Netherlands

(*Received June* 1980)

Abstract—Parametric analyses are presented for the mortality rates obtained from bioassays. In this paper we base the large sample theory on the estimation and testing of no, small and ultimate effects of test compounds on the maximum likelihood principle. The relevance of the LC 50 in toxicity tests and hazard evaluation is discussed. The analysis of the accumulation and elimination behaviour of test compounds through mortality rates is described. A method is presented for estimating the disappearance rate of the test compound in a semistatic bioassay from the mortality rates of the test organisms. The application of the theory is illustrated by several examples.

INTRODUCTION

This paper describes some parametric analyses of the results of experiments in which several groups of organisms are exposed to a range of concentrations of test compound, these being chosen such that at least one of the groups of organisms suffers significant but not excessive mortality. This is determined by counting the surviving organisms one or several times after different exposure periods. Other effects of the test compound on the physiology of the organisms, such as growth, feeding and reproductive behaviour, will not be dealt with in this paper.

Methods for evaluating the results of toxicity tests of the kind mentioned are of current interest, see Rosiello *et al.* (1977), Hewlett & Plackett (1979) and Stephan (1977) for parametric approaches, and Wasan (1969), Hammilton *et al.* (1977) and Millar (1973) for more or less non-parametric approaches. All these studies are primarily concerned with estimating what is called the LC 50: the concentration of test compound that causes 50% mortality among the test organisms during a certain exposure time. Litchfield & Wilcoxon's (1949) estimation method is still widely applied because it is relatively simple.

In evaluating hazards caused by compounds discharged into the environment, it is the order of magnitude of LC 50 values which is of interest, rather than the values themselves. The LC 50 values are usually multiplied by a safety factor of 0.01 in order to arrive at what are considered "safe" concentrations. In hazard evaluation, therefore, even the simplest estimation procedure is too complex, because too little is known about the relevant factors and about how much damage at community level is admissible.

In the context of toxicology, existing statistical theories concerning toxicity tests are too neglectful of the dynamic aspects of mortality. Nor do these theories pay due attention to statistical tests on LC 50 values. In this paper we attempt to make up for these deficiencies. The two following sub-sections restate the "standard" analysis and serve as an introduction to the subsequent sections. The more formal analysis of "no effects" and of the time dependence of concentration-response relations is believed to be new.

Although many of the analyses described below might be applied in mammalian toxicity tests, their main area of application lies in aquatic toxicology. The usefulness of the analyses in hazard evaluation will be discussed briefly. The theory presented in this paper is based on the principle of maximum likelihood. For a general background of the latter, see Cox & Hinkley's textbook (1974).

SINGLE CONCENTRATION-RESPONSE RELATIONS

Specification of models

For a fixed exposure time t_1 to the test compound at a concentration c, the classical model for survival of a particular individual, conditional under the parameter α, is

$$F(c|\alpha) \equiv \Pr\{\text{survival at concentration } c|\alpha\} = \begin{cases} q_0 & \text{if } \alpha \geqslant \ln c \\ 0 & \text{if } \alpha < \ln c \end{cases}$$

Here, $p_0 = 1 - q_0$ can be interpreted as the natural mortality probability.

The parameter α is believed to vary from one individual to another. It is conceived of as a random variable having a log-normal (see Finney, 1978) or a log-logistic (see Ashton, 1972) distribution, i.e.

$$F(c) = q_0(2\pi\sigma^2)^{-1/2} \int_{\ln c}^{\infty} \exp\left\{-\frac{1}{2}\left(\frac{x-\alpha}{\sigma}\right)^2\right\} dx \quad (1)$$

for $\underline{\alpha}$ having a log-normal distribution, with mean α and variance σ^2 or

$$F(c) = q_0 \exp\left\{\frac{\alpha - \ln c}{\beta}\right\}\left(1 + \exp\left\{\frac{\alpha - \ln c}{\beta}\right\}\right)^{-1} \quad (2)$$

for $\underline{\alpha}$ having a log-logistic distribution, with mean α and slope parameter β.

In order to relate results based on the two different assumptions, we might equate the two variances of $\underline{\alpha}$, obtaining $\sigma = \pi/\sqrt{3}\,\beta \simeq 1.8\,\beta$. Alternatively, we might equate the two slopes of the plots of survival probability vs the natural logarithm of the concentration, obtaining $\sigma = 4/\sqrt{2\pi}\,\beta \simeq 1.6\,\beta$. As the latter relationship corresponds more closely to the way in which the parameters σ and β are estimated in practice, it is to be preferred. So both σ and β can be conceived of as measuring the variance of $\underline{\alpha}$, the mean of which is widely known as ln(LC 50).

As it is much simpler to estimate the parameters of the logistic distribution than those of the normal one, the procedures described in the sequel all assume the logistic distribution. This assumption is not, however, expected to affect the qualitative results of the analyses.

Maximum likelihood estimation of the parameters

If the organisms die independently with equal probabilities when exposed to a test compound at a concentration c, the number of organisms $\underline{x}(t_1, c)$ surviving at time t_1 has a binomial distribution, i.e.

$$\Pr\{\underline{x}(t_1, c) = x(t_1, c)\} = \begin{bmatrix} x(t_0, c) \\ x(t_1, c) \end{bmatrix}[1 - F(c)]^{x(t_0,c) - x(t_1,c)} F(c)^{x(t_1,c)} \quad (3)$$

where $t_0 = 0$ is the starting time of the experiment.

If the counts $x(t_1, c)$ have been made at concentrations $c_1, \ldots, c_k$, the parameters $\theta' = (p_0, \alpha, \beta)$ of the survival probability function F may be estimated by the maximum likelihood method. This estimation is performed by solving θ in the vector equations:

$$\mathbf{G}(\theta) = \sum_{j=1}^{k} \frac{x(t_1, c_j) - x(t_0, c_j)F(c_j)}{F(c_j)[1 - F(c_j)]} \frac{\partial}{\partial \theta} F(c_j) = \mathbf{0}. \quad (4)$$

The solution $\hat{\theta}$ may be obtained by applying the iteration scheme

$$\hat{\theta}_{i+1} = \hat{\theta}_i + I(\hat{\theta}_i)^{-1}\mathbf{G}(\hat{\theta}_i) \quad (5)$$

where I is the information matrix, i.e.

$$I(\theta) = \sum_{j=1}^{k} \frac{x(t_1, c_j)}{F(c_j)[1 - F(c_j)]}\left[\frac{\partial}{\partial \theta} F(c_j)\right]\left[\frac{\partial}{\partial \theta} F(c_j)\right]' \quad (6)$$

The inverse, I^{-1}, of the information matrix is an estimate of the variance–covariance matrix of the maximum likelihood estimators for $\theta = \hat{\theta}$.

If we wish to have an estimate of the mean and variance of the LC 50, rather than of α, which we took to be ln(LC 50), we may take into account the skewness of the distribution of the LC 50.

Denoting the expectation by E, we obtain:

$$\hat{E}(\text{LC 50}) = \exp\{\hat{\alpha} + \tfrac{1}{2}\,\hat{\text{var}}(\hat{\alpha})\} \quad (7a)$$

$$\hat{\text{var}}(\text{LC 50}) = (\exp\{\hat{\text{var}}(\hat{\alpha})\} - 1)\hat{E}^2(\text{LC 50}). \quad (7b)$$

The confidence limits for the LC 50 can be obtained by taking the exponents of the confidence limits for $\hat{\alpha}$.

In hazard evaluation, one might be interested in effect levels other than the 50% effect level. The estimate for the ln of an arbitrary effect level of $100z\%$, where $0 < z < 1$, has an asymptotic normal distribution, with mean $\hat{\alpha} + a\,\hat{\beta}$, and variance $\text{var}(\hat{\alpha}) + a^2\,\text{var}(\hat{\beta}) + 2a\,\text{cov}(\hat{\alpha}, \hat{\beta})$, where $a = \ln z - \ln(1 - z)$. More specifically, the ln of the 1% effect level has a mean $\hat{\alpha} - 4.6\,\hat{\beta}$, and variance $\text{var}(\hat{\alpha}) + 21\,\text{var}(\beta) - 9.2\,\text{cov}(\hat{\alpha}, \hat{\beta})$. In practice the covariance of $\hat{\alpha}$ and $\hat{\beta}$ appears to be small. The variance of the LC1, and so its confidence limits, are strongly dominated by the variance of the slope parameter β.

Toxicity tests are usually conducted with animals from well-standardized laboratory cultures instead of animals from wild populations. One consequence of this is therefore that β is very small. As a result, its variance is in practice almost impossible to estimate. In addition, the β of wild populations being unknown, tests with laboratory cultures are insufficient for hazard evaluation for wild populations.

Likelihood-ratio tests on parameters

Two types of tests on the parameters of simple concentration–response relations are relevant, viz. the one-sample test and the many-sample test.

The one-sample case arises when a reference compound is tested simultaneously with a test compound, for the purpose of checking the condition of the organisms used. The parameters of the concentration–response relation of the reference compound are in this case known. Under the null hypothesis, one or more of the parameters corresponding to the reference compound tested, are equal to known values. The free parameters under the null hypothesis can be estimated by solving equation (4), conditional under the values of the fixed parameters. Under the null hypothesis, twice the difference between the ln likelihood under the null and that under the alternative hypothesis, is asymptotically chi-square distributed, with a degree of freedom equal to the number of fixed parameters.

The many-sample case arises in, e.g. ring tests, where several concentration–response relations are to be compared. The null hypothesis, which in practice is frequently of interest, is that one or more parameters of the various concentration–response relations are equal. Under the null hypothesis it is simple to estimate the three parameters when they are tested for equality simultaneously. Equation (4) are then solved for the combined counts, which are taken as the result of one experiment. Under the null hypothesis of equality of a single parameter, say, the LC 50, irrespective of the others, estimation of the parameters is more

difficult. The simultaneous likelihood then has to be maximized in $1 + 2n$ parameters, where n is the number of samples. The difficulty of straightforwardly inverting the information matrix rapidly increases with n. Fortunately, the natural mortality probability, $p_0 = 1 - q_0$, can be solved explicitly from (4), i.e.

$$\hat{q}_{0i} = \left[\sum_{j=1}^{k} x(t_1, c_{ij})\right]\left[\sum_{j=1}^{k} x(t_0, c_{ij})\,\frac{e_{ij}}{1 + e_{ij}}\right]^{-1} \tag{8}$$

where

$$e_{ij} = \exp\left\{\frac{\hat{\alpha}_i - \ln c_{ij}}{\hat{\beta}_i}\right\}. \tag{8a}$$

The index i denotes the different samples, i.e. $i = 1, \ldots, n$. The index j corresponds to the concentrations of test compound. The likelihood equations can now be solved by applying the Newton–Raphson method, where the matrix inversion is performed by applying the formulae for the inversion of partitioned matrices; see, e.g. Rao (1965). Under the null hypothesis, twice the difference between the ln likelihoods under the null and the alternative hypothesis is again asymptotically chi-square distributed, with a degree of freedom equal to the difference between the numbers of parameters under either hypothesis.

No-effect levels

It is common practice in toxicology to say that a compound has "no effect" if its concentration is too low to cause "more" than the natural mortality among the test organisms in a given exposure time. The highest concentration tested which has this property is called the "no-effect" level. The "no-effect" level may very well lie above the estimated small-effect level, say the LC1, as defined by (1) or (2). In fact, "no-effect" levels are incompatible with these models. It would seem to be more logical to define a model comprising the "no-effect" level as a parameter. Such a model is:

$$\begin{aligned} F(c) &\equiv \Pr\{\text{survival at concentration } c\} = q_0 \\ &\quad \text{for} \quad c < \gamma \\ &= q_0 \exp\left\{\frac{\alpha - \ln(c - \gamma)}{\beta}\right\} \\ &\quad \times \left[1 + \exp\left\{\frac{\alpha - \ln(c - \gamma)}{\beta}\right\}\right]^{-1} \\ &\quad \text{for} \quad c > \gamma \end{aligned} \tag{9}$$

where γ is the "no-effect" level. A plot of this probability vs the ln concentration is shown in Fig. 1. Maximum likelihood estimates are obtained by solving (4) for $\theta' = (p_0, \alpha, \beta, \gamma)$. Interval estimates can be obtained from the inverse of the information matrix given in (6). Since (9) reduces to (2) for $\gamma = 0$, we have a likelihood ratio test on the presence of a "no-effect" level as defined by (9). Treating "no-effect" levels in this way affords two advantages over the usual one, mentioned above.

(i) It is a valid procedure, in the sense that the estimation of such a "no-effect" level is not the result of a sequence of *mutually dependent* tests on equality of mortality probabilities.

(ii) It utilizes all information available about the observed concentration–response relations, the result being an interval estimate for the "no-effect" level.

It has the disadvantage, like any model parameter, of being dependent on the structure of the model. If (9) is false, the resulting estimate of the "no-effect" level may be misleading. It seems to the point to remark here that the null hypothesis $\gamma = 0$ will only be rejected if (9) fits the data better than does (2). If the slope parameter β is very small, as may be expected when organisms from laboratory cultures have been used, it may be hard to make a suitable choice of test compound concentrations. This disadvantage does not, however, seem to be a serious one, because "no-effect" levels are of primary interest in the hazard evaluation for natural populations.

TIME-DEPENDENT CONCENTRATION–RESPONSE RELATIONS

Specification of models

Mortality is by definition time-dependent. The toxicity tests mentioned in the introduction can therefore by regarded as a number of simultaneous processes in time, and can be analyzed accordingly. If the organisms die independently with equal probabilities at a concentration c, the number of organisms $\underline{x}(t, c)$ surviving at time t has the multinomial distribution:

$$\begin{aligned} &\Pr\{\underline{x}(t_i, c) = x(t_i, c),\ i = 1, \ldots, r\} \\ &\quad = x(t_0, c)! \prod_{i=0}^{r+1} \{[\Delta x(t_i, c)]!\}^{-1} [\Delta F(t_i, c)]^{\Delta x(t_i, c)} \end{aligned} \tag{10}$$

where

$\{t_i\}_{i=1}^{r}$ are the times of counting; $t_0 = 0$ and $t_{r+1} = \infty$

Δ is the difference operator acting on the time parameter, e.g. $\Delta x(t_i, c) = x(t_i, c) - x(t_{i-1}, c)$

F is the survival probability function; $F(\infty, c) = 0$.

Further: $x(\infty, c) = 0$. For $r = 1$, (10) reduces to (3) as expected.

If the counts $\{x(t_i, c_j)\}_{11}^{rk}$ have been made, the parameters θ of the function F may be estimated by the maximum likelihood method. This estimation is performed by solving θ in the vector equations

$$\begin{aligned} \mathbf{G}(\boldsymbol{\theta}) = \sum_{ij} [\Delta x(t_i, c_j)] \left\{\Delta \frac{\partial}{\partial \boldsymbol{\theta}} F(t_i, c_j)\right\} \\ \times \{\Delta F(t_i, c_j)\}^{-1} = \mathbf{0}. \end{aligned} \tag{11}$$

The solution may be obtained by applying the iteration scheme (5) with

$$\begin{aligned} I(\boldsymbol{\theta}) = \sum_j x(t_0, c_j) \sum_i \left[\Delta \frac{\partial}{\partial \boldsymbol{\theta}} F(t_i, c_j)\right] \\ \times \left[\Delta \frac{\partial}{\partial \boldsymbol{\theta}} F(t_i, c_j)\right]' \{\Delta F(t_i, c_j)\}^{-1}. \end{aligned} \tag{12}$$

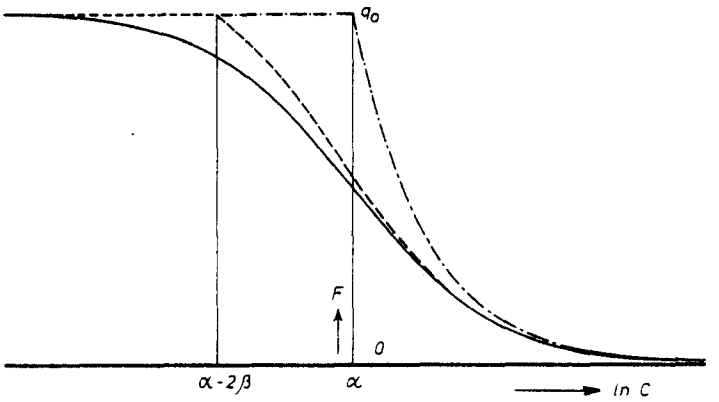

Fig. 1. Survival probability as a function of the ln concentration of test compound. It is shown for "no effect" levels 0 (——), $\alpha - 2\beta$ (– – –), and α (– · –).

Under model (2), $F(t, c)$ has the form

$$F(t, c) = q_0(t)e(t, c)\{1 + e(t, c)\}^{-1} \tag{13}$$

where

$$e(t, c) = \exp\left\{\frac{\alpha(t) - \ln c}{\beta}\right\}. \tag{13a}$$

In order to keep the number of parameters to be estimated as small as possible, we assume the natural mortality to be a simple pure death process, i.e.

$$q_0(t) = \exp\{-\mu t\}. \tag{13b}$$

Age-dependent death processes, however, do not give rise to new estimation problems.

If no further assumptions can be made, we may put

$$\alpha(t_i) = \alpha_i \text{ for } i = 1, \ldots, r. \tag{14}$$

We now have $r + 2$ parameters which, if r is not too large, may be estimated according to (5), (11) and (12). The number of parameters can be reduced by further parametrization of $\alpha(t)$. If the structure of $\alpha(t)$ is tested under the null hypothesis for its goodness of fit, the model specified by (10), (13) and (14) might serve to define the alternative hypothesis.

Ultimate effects

In order to specify the structure of $\alpha(t)$ in (13), we may use the following procedure. First, $\alpha(t)$ is kept fixed, and the concentration c in the water is replaced by the concentration $Q(t)$ in the organism. The interpretation is that an organism dies as soon as it has accumulated a concentration exceeding a threshold value, which may vary from one individual to another. The substitution of $Q(t)$ for $c(t)$ enables one to use the wealth of models that has been developed for describing the accumulation of chemicals in organisms. See, for instance, Jacquez (1972). If, in fact, the outer concentration c does not vary, and the internal concentration $Q(t)$ at equilibrium is proportional to the outer concentration, we have the relation

$$Q(t) = kc\,\frac{\text{LC } 50(\infty)}{\text{LC } 50(t)} \tag{15}$$

where k is the bioaccumulation factor. All compartmental models fall into this broad class. The LC $50(t)$ can be estimated from the mortality rates, and so can $Q(t)/k$.

Although the hyperbolic relation

$$\alpha(t) = \frac{a}{t} + b \tag{16}$$

is sometimes fitted to ln LC 50 values (see Ericksen Jones, 1964), it only has an empirical basis. According to (15), this relation would imply

$$Q(t) = kc \exp\left\{-\frac{a}{t}\right\} \tag{17}$$

which does not correspond to a simple compartment model.

A more attractive model is the one-compartment one

$$\frac{\partial}{\partial t} Q(t) = \frac{k}{\tau} c - \frac{1}{\tau} Q(t) \tag{18a}$$

with the boundary condition $Q(0) = 0$, this gives

$$Q(t) = kc\left(1 - \exp\left\{-\frac{t}{\tau}\right\}\right). \tag{18b}$$

This is the well-known accumulation curve. The bioaccumulation factor is given by k, and the elimination rate by $(1/\tau)$. Substitution of (18b) in (15) leads to

$$\alpha(t) = \alpha - \ln\left(1 - \exp\left\{-\frac{t}{\tau}\right\}\right). \tag{19}$$

Under this model, it is therefore possible to estimate the elimination rate of the test compound from the counts of surviving organisms. The parameter α is interpreted as ln[LC $50(\infty)$].

In toxicity experiments with bivalves, it might be appropriate to shift the time parameter as follows:

$$\alpha(t) = \begin{cases} \alpha - \ln\left(1 - \exp\left\{-\dfrac{t - \delta}{\tau}\right\}\right) & \text{for } t > \delta \\ \infty & \text{for } t \leqslant \delta \end{cases} \tag{20}$$

because these animals tend to keep their valves shut for some time at the beginning of the test.

It should be noted that (18) still applies if the test compound is metabolized to non-toxic compounds by the organism, provided that the rate of the process is proportional to the internal concentration. In this case, however, $1/\tau$ cannot be interpreted as the elimination rate.

If the test compound is absorbed to or desorbed from the outer surface of the test organism, (19) may still apply, but $Q(t)$ in (18) can no longer be regarded as the concentration inside the organisms.

This simple model can readily be developed into multicomparatment ones. The transformation of toxic compounds into even more toxic ones in the organism may be described by what is known as a two-compartment mammilary system with intake (see Jacquez, 1972).

In the model defined by (19) and (13), the threshold concentration is for the sake of consistency with the classical models (1) and (2) assumed to have a log logistic distribution. In this model the bioaccumulation factor and elimination rate are model parameters, i.e. they are identical for every individual. In theory, it is possible to treat them as random variables as well. If they are independent of the threshold concentration, (13) has to be mixed with their (simultaneous) probability density function. This would have the disadvantages that the model would become:

(i) inconsistent with the classical models (1) and (2);

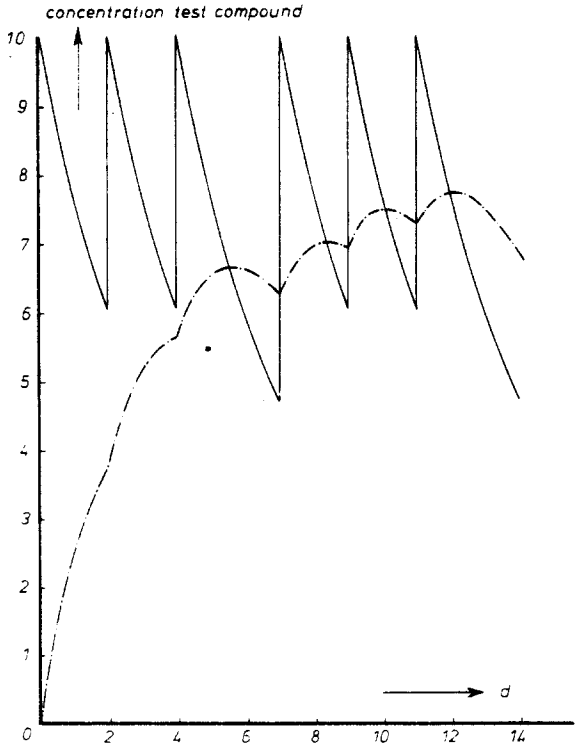

Fig. 2. The water concentration $\hat{c}(t)$ (——) in a 2-week semistatic bioassay, with disappearance rate $\frac{1}{4}$, and the tissue concentration $Q(t)$ (– · –), divided by the bioaccumulation factor, with elimination rate $\frac{1}{3}$.

(ii) impractical, because its parameters would be almost impossible to estimate for well-standardized laboratory cultures, which have a small slope parameter.

Sometimes, instead of counting surviving organisms, we measure the time ET 50 that elapses until 50% of the organisms exposed to a certain concentration of test compound have died. In the absence of natural mortality, we have the relation ET $50 = \alpha^{-1}(c)$, where α^{-1} is the inverse function of α, i.e. $\alpha^{-1}(\alpha(t)) = t$. For (16) we get ET $50 = a/(c - b)$ and for (19): ET $50 = -\tau \ln(1 - \exp\{\alpha - c\})$.

The latter relation may be applied in what are called "range finding" toxicity tests. If ET 50's are determined for two concentrations, the parameters τ and α can be estimated from them, and by application of (19), the LC 50 can be predicted for any exposure time. The parameters τ and α can be estimated from $t_1 = \text{ET } 50(c_1)$ and $t_2 = \text{ET } 50(c_2)$ by solving $t_1 \ln(1 - \exp\{\hat{\alpha} - c_2\}) = t_2 \ln(1 - \exp\{\hat{\alpha} - c_1\})$ in $\hat{\alpha}$. Then, $\hat{\tau}$ is found by $\hat{\tau} = -t_1/\ln(1 - \exp\{\alpha - c_1\})$.

The solution $\hat{\alpha}$ exists if $c_1 t_1 > c_2 t_2$ for $c_1 < c_2$. If the concentrations c_1 and c_2 are relatively high, the variance of the lifetimes of the test organisms will be small. For this reason it is in principle possible to perform such a range finding test with as few as two individuals.

Test on persistence of test compounds

In aquatic toxicity testing, semistatic systems are used beside continuous–flow systems to keep the concentration of test compound as nearly constant as possible. In the former, the solution of test compound is replaced regularly by a freshly prepared one. Nearly all test compounds tend to disappear from the solution as a result of evaporation, adsorption to walls of the vessel(s) or to food particles, formation of inaccessible complexes, or transformation into other compounds, etc. It might be worthwhile to study the process of disappearance more closely. In (18a), c is made time-dependent, i.e.

$$\frac{\partial \hat{c}}{\partial t} Q(t) = \frac{k}{\tau} c(t) - \frac{1}{\tau} Q(t). \tag{21a}$$

We further suppose that the test compound disappears at a rate proportional to its concentration, i.e.

$$\frac{\partial \hat{c}}{\partial t} c(t) = -\frac{1}{\lambda} c(t). \tag{21b}$$

This results in

$$Q(t) = Q(0) \exp\left\{-\frac{t}{\tau}\right\} + kc \frac{\lambda}{\tau - \lambda}\left(\exp\left\{-\frac{t}{\tau}\right\} - \exp\left\{-\frac{t}{\lambda}\right\}\right). \tag{22}$$

Obviously, $Q(t)$ no longer increases monotonously with t, making this model essentially more complex than the one specified by (18). For the new model, equation (15) does not hold.

$Q(t)$ attains a maximum for

$$t = {}^{m}t = \frac{\tau\lambda}{\tau - \lambda}[\ln\tau - \ln\{\lambda + (\tau - \lambda)Q(0)\}]. \quad (23)$$

An organism surviving at ${}^{m}t$, will be subject to its natural mortality rate until $Q(t)$ exceeds $Q({}^{m}t)$ owing to the test solution being replenished (see Fig. 2). Replacement of the test compound at $\{t_i\}_{i=1}^{r}$ results in

$$\alpha(t_i) = \min\{\alpha^*(t_i), \alpha^*(t_{i-1})\}, \quad \text{with } \alpha^*(t_0) = \infty \quad (24a)$$

$$\alpha^*(t_i) = \alpha - \ln Q^*(\min\{t_i, {}^{m}t_i\}) \quad (24b)$$

$$Q^*(t) = Q^*(t_{i-1})\exp\left\{-\frac{t - t_i}{\tau}\right\} + \frac{\lambda}{\tau - \lambda}\left(\exp\left\{-\frac{t - t_i}{\tau}\right\} - \exp\left\{-\frac{t - t_i}{\lambda}\right\}\right) \quad (24c)$$

with

$$Q^*(0) = 0 \quad \text{and} \quad t_i \leqslant t < t_{i+1}.$$

The disappearance rate $1/\lambda$ of the test compound can be estimated from the counts of surviving organisms by (5), (11) and (12) together with (24) and (13). This model can serve as a check on analytical figures, while the estimate of the parameter α in (24b) gives the ultimate ln LC 50 corrected for the disappearance of the test compound. Comparison of the disappearance rate $1/\lambda$ with analytical figures may provide a clue to the chemical form of a test compound that is subject to transformation.

EXAMPLES

One-sample test on LC 50

In fresh water bioassays with young *Poecilia reticulata* (guppy), pentachlorophenol (PCP) dissolved via DSMO has been chosen as a reference compound. Table 1 lists the results after 96 h exposure.

Table 1. Number of surviving young guppies after 96 h exposure to PCP

mg PCP l^{-1}	$t = 0$	$t = 96$ h
0	10	10
0.56	10	5
0.74	10	6
1.0	10	3
1.3	10	0
1.8	10	0

Table 3. Number of surviving young guppies after 96 h exposure to PCP in two laboratories

	Lab 1		Lab 2	
mg PCP l^{-1}	$t = 0$	$t = 96$ h	$t = 0$	$t = 96$ h
0	90	90	100	100
0.32	50	46	100	99
0.41	70	57		
0.56	90	72	100	81
0.74	90	55		
1	90	20	100	14
1.3	40	3		
1.8	20	0	100	0
3.2			40	0

The (unconstrained) parameter estimates of (2) for these results, are given in Table 2. They are obtained by application of (4), (5) and (6). In a ring test, the mean ln(LC 40 (96 h)) was found to be -0.33 for PCP in mg l^{-1}. The parameter estimates of (2), constrained by $\alpha = -0.33$, are also collected in Table 2.

Twice the difference in ln likelihood was 0.31. Under the null hypothesis $\alpha = -0.33$, this difference is asymptotically chi-square distributed, with one degree of freedom. It follows that the result of the bioassay does not contradict the results of the ring test.

Two-sample test on LC 50

The LC 50 (96 h) of PCP, dissolved via DSMO, for young guppies in fresh water has been determined in a ring test conducted by two laboratories. Table 3 gives the results.

The parameters of (2) have been estimated for both laboratories, and are listed in Table 4. They have been obtained by application of (4), (5) and (6); the parameters have also been estimated under the condition $\alpha_1 = \alpha_2$, i.e. equality of the LC 50's for the two laboratories. The estimates have been obtained according to the procedure described after (8). Finally, the parameters have been estimated for the combined results of the bioassays. This gives the parameters under the condition of equality of the concentration–response curves. All parameter estimates are collected in Table 4.

Twice the difference in ln likelihood between the unconstrained model and the model constrained by $\alpha_1 = \alpha_2$ equals 1.33. Under the null hypothesis $\alpha_1 = \alpha_2$, this difference is asymptotically chi-square distributed, with one degree of freedom. It follows that the LC 50 values do not differ significantly. Twice the difference in ln likelihood between the unconstrained model and the model constrained by the condition of equal concentration–response curves equals 14.2. Under the null hypothesis of equal concentration–response curves, this difference is asymptotically chi-square distributed with three degrees of freedom. It follows that the concentration–response curves were not identical. This is due to the difference between the slope parameters.

"No-effect" level

In a semistatic bioassay, the toxicity of an industrial waste coded DSB was determined for young ($\leqslant 24$ h) *Daph-*

Table 2. Unconstrained and constrained parameter estimates for model (2), using the data of Table 1

Parameter	Symbol	Unconstrained	Constrained
Nat. mortality prob.	p_0	0	0
ln LC 50	α	-0.39	-0.33
Slope parameter	β	0.28	0.26

Table 4. Unconstrained and constrained parameter estimates for model (2), using the data of Table 3

Parameter	Symbol	Unconstrained lab 1	Unconstrained lab 2	Constrained lab 1	Constrained lab 2	Combined
Nat. mort. prob.	p_0	0	0	0	0	0
ln LC 50	α	0.75	0.72	0.74	0.74	0.74
Slope parameter	β	0.28	0.17	0.27	0.18	0.23

Table 5. Observed and expected numbers of surviving daphnids after 7 d exposure to DSB

DSB in %	$t = 0$	$t = 7$ d	Expected values with "no-effect level"	Expected values without "no-effect level"
0	40	40	40.0	40.0
0.1	40	40	40.0	40.0
0.18	40	40	40.0	39.8
0.32	40	33	33.3	33.5
0.56	40	5	4.6	4.6
1	40	0	0.2	0.1

Table 6. The parameter estimates for model (2) and (9) using the experimental data of Table 5 and the variance–covariance estimates of the parameters for model (9)

Parameter	Symbol	Model (2)	Model (9)	Var–cov matrix		
Nat. mort. prob.	p_0	0	0	0		
ln LC 50	α	−0.891	−1.241	0	0.353	
Slope parameter	β	0.152	0.212	0	−0.067	0.014
"No-effect" level	γ		0.114	0	−0.093	0.018 0.025

nia magna. The counts of surviving animals and the expected survival values based on the models defined by (9) and (2), together with (3) are given in Table 5.

The parameters of models (2) and (9) with the inverse of the information matrix (6) of the latter are listed in Table 6.

Twice the difference in ln likelihood being 0.013, it can be concluded that the "no-effect" level is not significantly different from 0.

LC 50 for several instants in time

The toxicity of 1,2,3-trichloropropane (TCPP) has been determined for *Chaetogammarus marinus*. In six 3-l. conical flasks, TCPP solutions of concentrations of respectively, 0, 5.6, 10, 18, 32 and 56 mg l^{-1} were refreshed every day. (The test was conducted in duplicate). The animals were fed with *Fucus* the numbers of surviving animals as well as the numbers expected to survive from the model defined by (14), (13) and (10) are given in Fig. 3.

The parameters and their estimated variances are given in Table 7. The conclusion follows that the fit in the lower concentration range is not perfect. The observations indicate age-dependent mortality and non-normality of the threshold values.

Hyperbolic ln LC 50 curve

The toxicity of cupric chloride has been determined for the bivalve *Dreissena polymorpha*. Twelve aquaria each containing 12 l. of hard water were continuously supplied with 1 l. of water per hour. The copper chloride was continuously dosed in mixing chambers to give concentrations of 0, 10, 32, 100, 320 or 1000 μg Cu l^{-1}, respectively in six pairs of aquaria. The number of mussels surviving as well as the numbers expected to survive from the model defined by (16), (13) and (10) are given in Fig. 4. The parameters and the inverse information matrix are given in Table 8. Figure 4 shows that the fit is not perfect, although reasonable. The 95% confidence interval for the LC 50 (∞) is (1.7, 27.7 μg Cu l^{-1}).

Simple accumulation curve

In a static bioassay, the toxicity of para chloro phenol has been determined for 3-week old *Poecilia reticulata* in sea-water. From chemical analyses, it was obvious that the compound did not degrade during the experiment. The counts of surviving animals and the numbers expected to survive by virtue of the model defined by (19), (9) and (10) are given in Fig. 5. The parameters and the inverse information matrix are given in Table 9. The likelihood ratio test on the no effect level being 0, leads to a rejection of this hypothesis (tailprobability was 3 E − 6). From Table 9, the LC 50(∞) was found to be 6.36.

Accumulation curve with time shift

The toxicity of cupric chloride has been determined for the bivalve *Anodonta cygnea*. Twelve aquaria each containing 12 l. of hard water were continuously supplied with 1 l.

Table 7. Parameter estimates and their variances for the model defined by (14), (13) and (10), using the data of Fig. 3

Parameter	Symbol	Estimate	Variance
Nat. Mort. rate	μ	1.1 E −2	4.2 E −6
ln LC 50(1d)	α_1	4.3 E 0	8.3 E−3
ln LC 50(2d)	α_2	4.1 E 0	2.7 E −3
ln LC 50(4d)	α_3	3.8 E 0	2.4 E −3
ln LC 50(7d)	α_4	3.5 E 0	1.9 E −3
ln LC 50(14d)	α_5	3.1 E 0	2.9 E −3
ln LC 50(21d)	α_6	3.0 E 0	3.5 E −3
Slope parameter	β	1.4 E −1	3.4 E −4

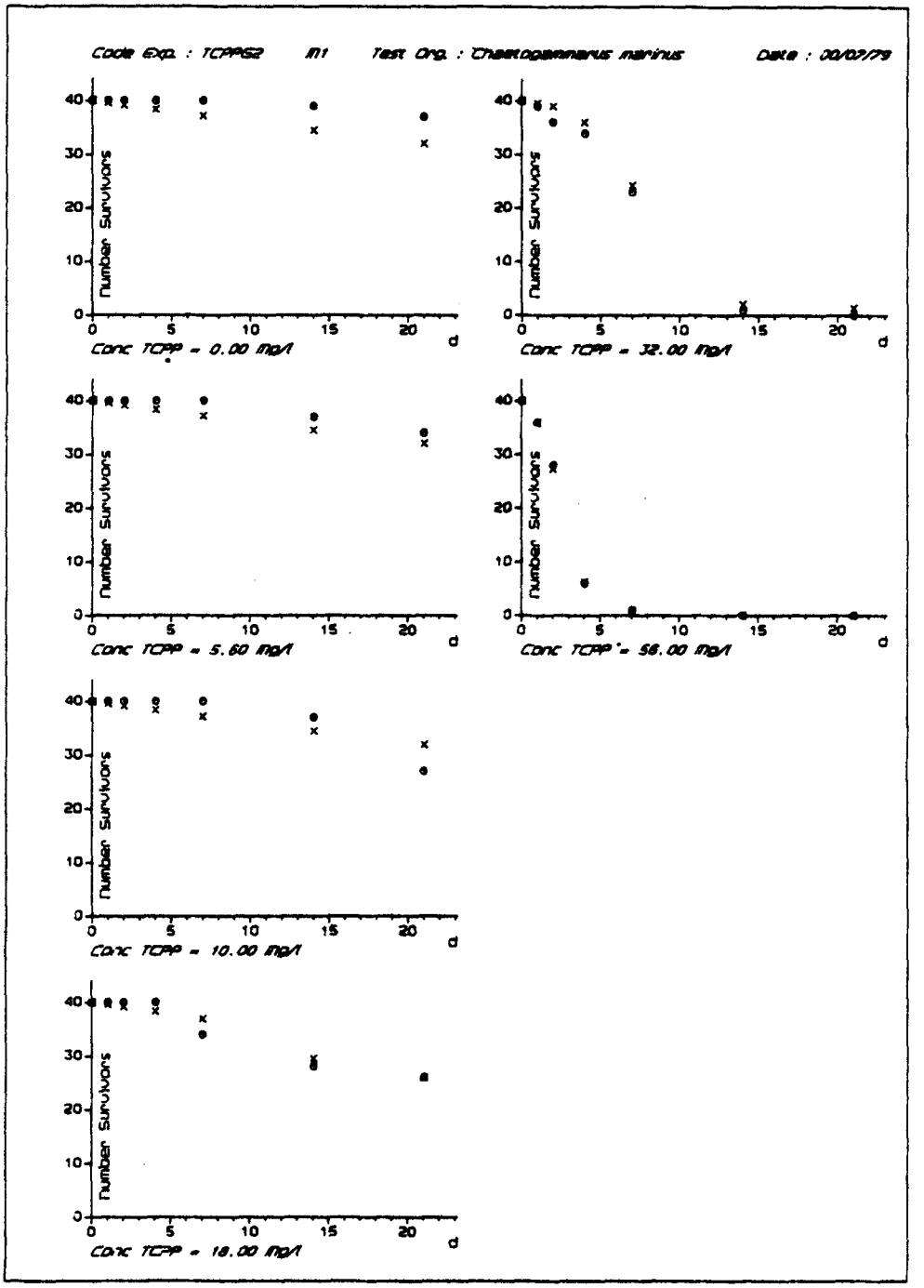

Fig. 3. Observed (dots) and expected (crosses) numbers of surviving amphipods during a three-week's exposure to 1,2,3-trichloropropane. The expected numbers are based on the model defined by (14), (13) and (10).

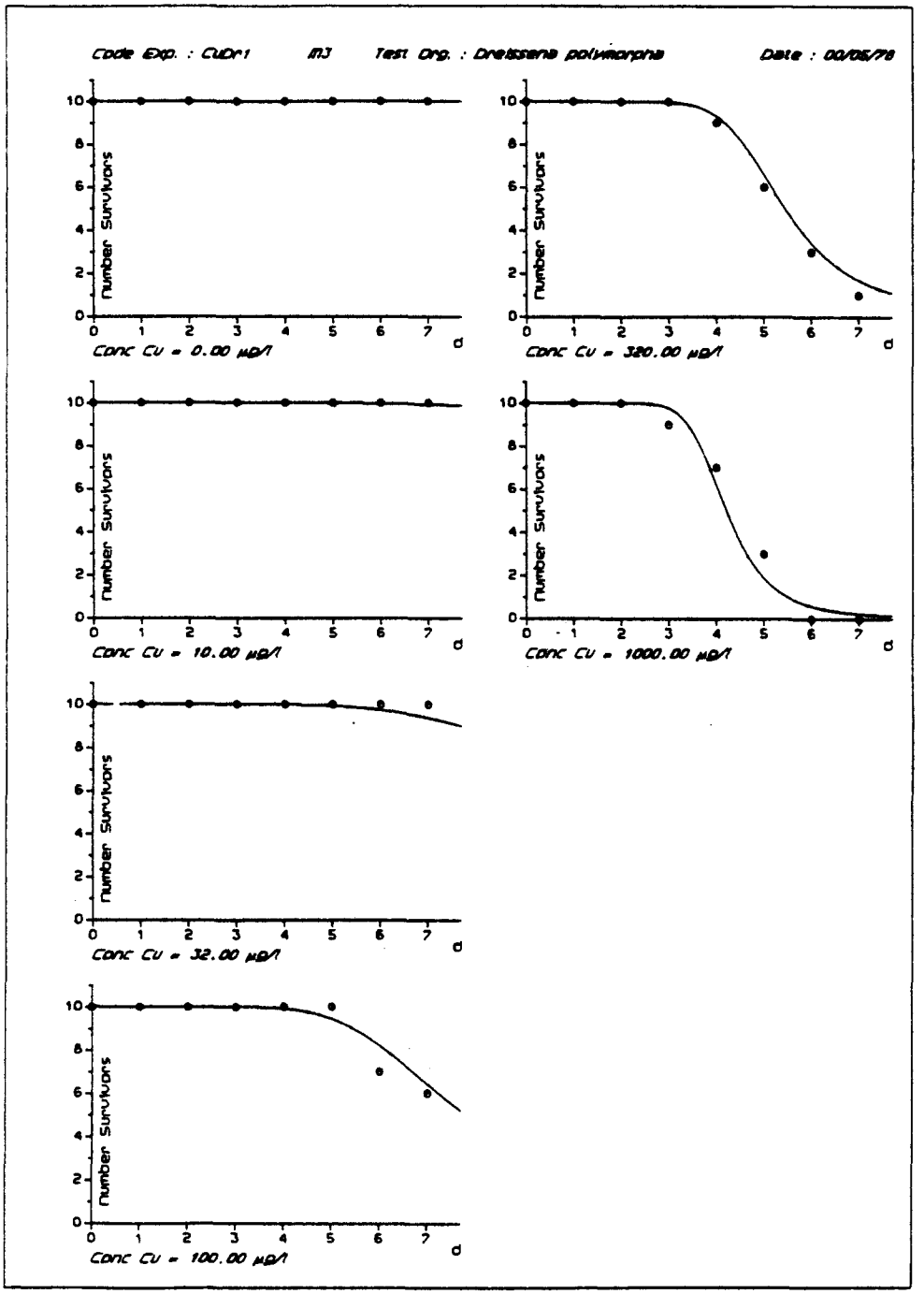

Fig. 4. Observed (dots) and expected (line) numbers of surviving mussels during a week's exposure to copper chloride. The expected numbers are based on the model defined by (16), (13) and (10).

Table 8. Parameter estimates and their variance–covariance matrix for the model defined by (16), (13) and (10), using the data of Fig. 4

Parameter	Symbol	Estimate	Var–cov matrix		
Nat. mort. rate	μ	0	0		
Hyperbolic par.	a	21.00	0	1.4 E 1	
ln LC 50(∞)	b	1.92	0	−2.5 E 0	4.9 E −1
Slope parameter	β	0.54	0	2.0 E −1	−3.3 E −2 1.2 E −2

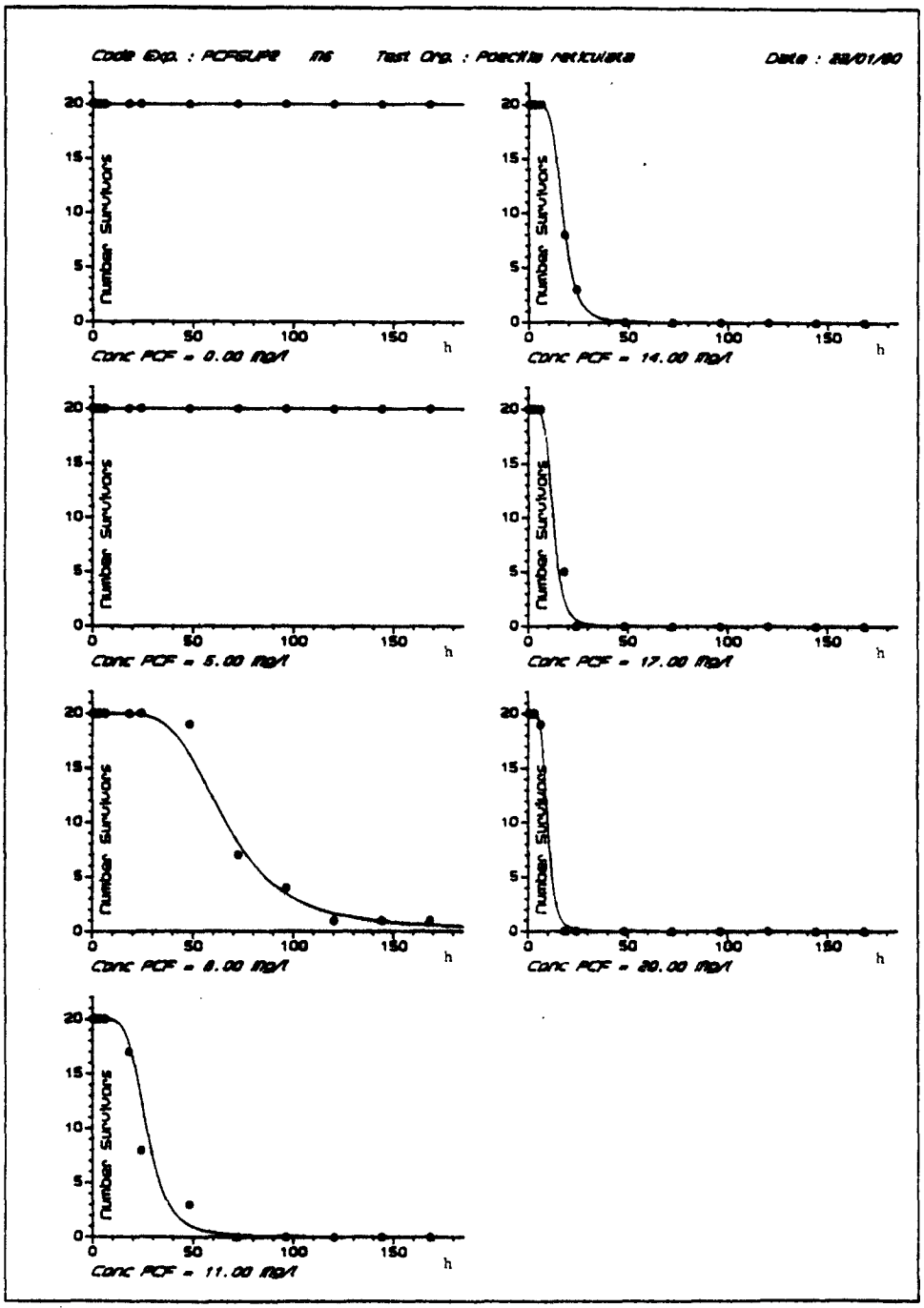

Fig. 5. Observed (dots) and expected (line) number of surviving guppies during a week's exposure to *para*-chlorophenol in sea-water. The expected numbers are based on the model defined by (19), (9) and (10).

Table 9. Parameter estimates and their variance–covariance matrix for the model defined by (19), (9) and (10), using the data of Fig. 5

Parameter	Symbol	Estimate		Var–cov matrix		
Nat. mort. rate	μ	0	0			
(Elim. rate)$^{-1}$	τ	145	0	2.0 E 4		
$\ln(LC\,50(\infty)-\gamma)$	α	−0.055	0	−1.4 E 2	9.7 E −1	
Slope parameter	β	0.18	0	3.0 E 0	−2.1 E −2	7.9 E −4
"No-effect" level	γ	5.4	0	8.3 E 1	−6.0 E −1	1.3 E −2 4.5 E −1

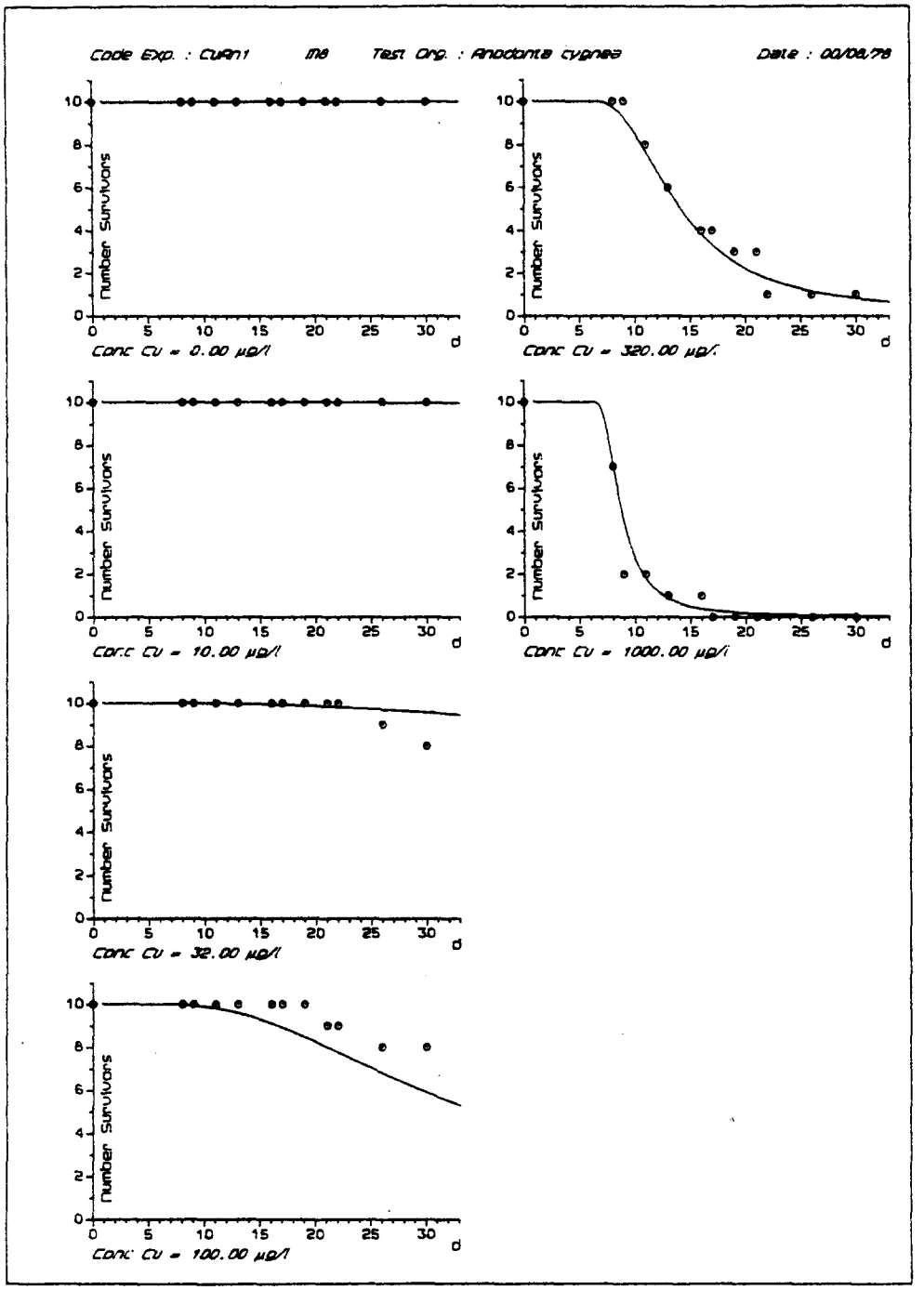

Fig. 6. Observed (points) and expected (line) number of surviving mussels during a month's exposure to copper chloride. The expected numbers are based on the model defined by (20), (13) and (10).

Table 10. Parameter estimates and their variance–covariance matrix for the model defined by (20), (13) and (10), using the data of Fig. 6

Parameter	Symbol	Estimate		Var–cov matrix			
Nat. mort. rate	μ	0	0				
(Elim. rate)$^{-1}$	τ	81	0	5.2 E 4			
ln LC 50(∞)	α	3.38	0	−5.8 E 2	6.5 E 0		
Time shift	δ	6.34	0	−1.1 E 2	1.1 E 0	6.1 E −1	
Slope parameter	β	0.42	0	1.3 E 0	−1.7 E −2	1.9 E −2	6.5 E −3

Table 11. Numbers of surviving daphnids during exposure to $CdCl_2$

conc. Cd in $\mu g\,l^{-1}$ Time in d	0	3.2	5.6	10	18	32	56
0	50	50	49	50	53	50	59
2	50	50	49	50	53	50	28
4	50	50	49	50	53	35	6
7	50	50	49	50	40	14	0
9	50	50	49	47	28	4	0
11	50	49	47	45	26	0	0
14	50	49	46	42	17	0	0
16	50	49	46	40	17	0	0
18	50	49	44	40	15	0	0
21	49	48	42	38	11	0	0

Table 12. Parameter estimates and their variance–covariance matrix for the model defined by (24), (13) and (10), using the data of Table 11

Parameter	Symbol	Estimate	Var–cov matrix			
Nat. mort. rate	μ	1.9 E −3	6.8 E −7			
(Elim. rate)$^{-1}$	τ	1.1 E 1	−2.5 E −4	2.9 E 0		
ln LC 50(∞)	α	1.9 E 0	2.1 E −5	−2.5 E −1	7.5 E −2	
(Disapp. rate)$^{-1}$	λ	2.3 E 0	2.3 E −5	−5.6 E −1	3.5 E −1	1.9 E 0
Slope parameter	β	2.2 E −1	2.2 E −1	1.1 E −2	−5.9 E −4	−2.3 E −4	3.3 E −4

of water per hour. The copper salt was continuously dosed in mixing chambers to give concentrations of 0, 10, 32, 100, 320 or 1000 $\mu g\ Cu\ l^{-1}$, in respectively six pairs of aquaria. The number of mussels surviving and the numbers expected to survive from the model defined by (20), (13) and (10) are given in Fig. 6.

The parameters and the inverse information matrix are given in Table 10. From the information matrix it is obvious that, in view of the variance of the elimination rate, this bioassay is not very conclusive as regards this parameter. The 95% confidence interval of the LC 50(∞) is (0.18, 4832 $\mu g\ Cu\ l^{-1}$), so the bioassay is not conclusive as regards this parameter either.

Disappearing test compounds

In a 3-week semistatic bioassay, the toxicity of cadmium chloride has been determined for young (< 24 h) *Daphnia magna* in hard water at 20°C. The daphnids were fed daily with *Chlorella* sp. The media were refreshed on days 2, 4, 7, 9, 11, 14, 16, 18 and 21. The counts of the surviving daphnids in the various concentrations of cadmium are given in Table 11.

The parameters of the model defined by (24), (13) and (10) and the inverse information matrix are given in Table 12. The 95% confidence interval for the elimination rate is (0.07, 0.14 μg Cd $(ld)^{-1}$) and for the LC 50(∞) it is (4.05, 12.13 $\mu g\ Cd\ l^{-1}$).

In addition to the counts of the daphnids, the concentration of cadmium in the water has been measured during the bioassay by means of atomic absorbtion spectroscopy. The measured concentrations are shown in Fig. 7 together with the values predicted by a disappearence rate of 2.33^{-1} (see Table 12). The figure shows that the concentrations of cadmium are in remarkable agreement with the concentrations predicted by the model defined by (24), (13) and (10) for mortality rates in test animals.

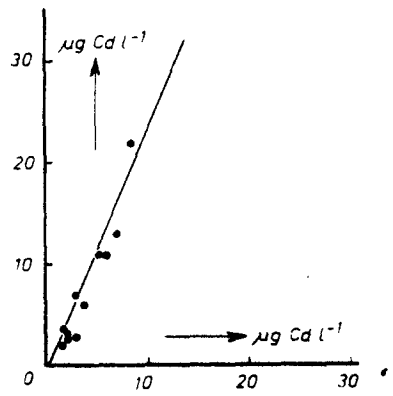

Fig. 7. Cadmium concentration in the water before (abscissa) and after (ordinate) refreshment of the test media. The line is not based on the points shown, but on the counts of the daphnids of Table 11.

DISCUSSION

From the above it will be clear that parametric analyses of the type presented are much more profound than are non-parametric analyses. Although parametric analyses are therefore advantageous in many respects, their validity is inextricably linked with the validity of the models used. The proposed tests on goodness of fit will save the user of models from wild assumptions.

The distribution theory here presented is based on large samples and may be misleading when applied to small ones. Great care has to be exercised in extrapolating effects beyond the ones actually observed. Using confidence intervals instead of point estimates will stimulate such care.

A simple accumulation curve, which has an asymptote, will fit tissue analyses of cadmium in fish quite well for a short time (up to several weeks), but Pentreath (1977) found a correlation of the cadmium con-

tent of plaice and ray with their age. So the model may not describe long-term effects equally well. In case such correlation exists, a more suitable method of hazard evaluation of long-term effects may be found within the theory of competing risks developed by David & Moeshberger (1978).

It should be emphasized that in (3) as well as in (10), it is assumed that each individual has the same mortality probability for a certain time, when exposed to a certain concentration of test compound. This assumption may be false if one uses animals from wild populations, or animals from different cultures, of different ages, etc. In that case (3) and (10) have to be mixed with an appropriate distribution. This is likely, however, to widen confidence intervals by an order of magnitude.

The analyses unifying tests on mortality rates on the one hand, and measurements of accumulation and elimination of test compounds on the other, are hoped to strengthen each other.

APL software for the analyses described in this paper can be obtained from the author at moderate cost.

REFERENCES

Ashton W. D. (1972) *The Logit Transformation.* Griffin, London.

Cox D. R. & Hinkley D. V. (1974) *Theoretical Statistics.* Chapman & Hall, London.

David H. A. & Moeshberger M. L. (1978) *The Theory of Competing Risks.* Griffin, London.

Ericksen Jones J. R. (1964) *Fish and River Pollution.* Butterworths, London.

Finney D. J. (1978) *Statistical Method in Biological Assay,* 5th edition. Griffin, London.

Hammilton M. A., Russo R. C. & Thurston R. V. (1977) Trimmed Spearmann-Karber method for estimating median lethal concentrations in toxicity bioassays. *Envir. Sci. Technol.* **11**, 714–719.

Hewlett P. S. & Plackett R. L. (1979) *The Interpretation of Quantal Responses in Biology.* Arnold, London.

Jacquez J. A. (1972) *Compartmental Analyses in Biology and Medicine.* Elsevier, Amsterdam.

Litchfield J. T. & Wilcoxon F. (1949) A simplified method of evaluating dose effect experiments. *J. Pharmac. exp. Ther.* **96**, 99–113.

Millar R. G. (1973) Non parametric estimators of the mean tolerance in bioassay. *Biometrika* **60**, 535–542.

Pentreath R. J. (1977) The accumulation of cadmium by the plaice *Pleuronectes platessa* L. and the thornback ray, *Raja clavata* L. *J. exp. Kar. Biol. Ecol.* **30**, 223–232.

Rao C. R. (1965) *Linear Statistical Inference and its Applications.* Wiley, New York.

Rosiello-A. P., Essigmann J. M. & Wogan G. N. (1977) Rapid and accurate determination of median lethal dose (LD 50) and its error with a small computer. *J. Toxic. envir. Hlth* **3**, 797–809.

Stephan C. E. (1977) Methods for calculating an LC 50. In *Aquatic Toxicology and Hazard Evaluation* (Edited by Mayer F. L. & Hamelink J. L.). American Society for Testing and Materials, Philadelphia.

Wasan M. T. (1969) *Stochastic Approximation.* Cambridge University Press.