THE UNIVERSITY OF MANITOBA

MODELS FOR THE JOINT EFFECTS OF TOXICANTS IN AQUEOUS MIXTURES - A STUDY OF THE ACUTE LETHAL RESPONSE OF THE FRESHWATER AMPHIPOD GAMMARUS LACUSTRIS TO TWO-TOXICANT MIXTURES.

by

B. G. E. DE MARCH

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BRIGITTE G.E. DE MARCH

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ABSTRACT

Methods of predicting the joint effects of several toxicants in biological systems are of interest to scientists and to persons involved in regulatory decisions.

In this study it is shown that the three modes of action usually presented as alternatives, namely simple similar action (SSA), independent joint action with no correlation of susceptibilities (IJA, r=0), and independent joint action with complete correlation of susceptibilities (IJA, r=1), are unrelated descriptions about dependent variables, independent variables or about mathematical relationships. Indices intended to describe modes of action intermediate between two of the above, such as Bliss' (1939) correlation between susceptibilities ("r"), and Koenemann's (1981) Mixture Toxicity Index, add to the spectrum of models capable of describing the same results. Because of these problems, a classificatory framework, essentially an expansion of joint effects models which eliminates inconsistencies, was proposed.

Eighteen experiments, each testing the acute lethal toxicity of a two-toxicant mixture with two of the five potentially lethal ions Cu^{++}, Cd^{++}, Zn^{++}, Mg^{++}, and K^{+} to the freshwater amphipod Gammarus lacustris, were performed. The ability of response surfaces representing major modes of joint action to describe the results of experiments was then assessed. All response surfaces describing the response to two toxicants were of a form such that the effects of a single toxicant were described as follows:

$$Q = a*C + b*C^2 + c, \quad \text{and}$$

$$\text{logit}(1-Q) = \underline{\ln}((1-Q)/Q) = d*C + e*C^2 + f,$$

in which Q = the probability of non-response (survivorship), $\underline{\ln}$ is the natural logarithm, a to f are fitted numerical constants, and C is the concentration of the toxicant.

Results in 15 of 18 experiments were described by the two most commonly scrutinized modes of action, IJA (r=0) and SSA, significantly and equally well. Joint action with completely correlated susceptibilities (IJA, r=1) and with partly correlated susceptibilities (IJA, -1<=r<1) were not not strongly supported as modes of joint action. The experimental results support the theoretical expectations that the existing major reference points serve little purpose.

Experimental results suggest that "additive effects" of several toxicants on the probability of response is worth considering as a mode of joint action that is a reference point. The joint effects of copper, cadmium, and zinc on the probability of response were usually additive. The effects of potassium and magnesium in combination with other ions were often less-than- or more-than-additive.

Modifications of the toxic units model relating to the response surfaces were described and applied to the experimental data.

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INTRODUCTION

1.1. General Introduction

The effects of mixtures of toxicants in man's environment is a major concern among scientists and persons involved in policy decisions. Hazard is usually assessed on the basis of tests of individual chemicals, not tests of of combinations that are likely in fact to occur. situations. Scientists have avoided studies with variable mixtures because of their complexity of design and interpretation and because of their cost. This situation has resulted in important gaps in the knowledge of chemical interactions.

The need for productive philosophical and experimental approaches to the effects of mixtures has been expressed in review papers (Sprague 1970) and at major conferences and symposia on the effects of mixtures of toxicants. Among these have been the "Workshop on the combined effects of xenobiotics" (Stich et al. 1981); "Introduction" and "Research needs" by P.D. Anderson in "Symposium on structure-activity correlations in studies of toxicity and bioconcentration with aquatic organisms, (International Joint Commission 1975); International Joint Commission (1976, 1981); "Workshop on methods for assessing the effects of mixtures of chemicals", Scientific Group of Methodologies for the Safety Evaluation of Chemicals (SGOMSEC 1986), and "Report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water", European Inland Fisheries Advisory Committe (EIFAC 1980). It is generally expressed that desirable models should have a

reasonable theoretical basis, be amenable to testing, and should be capable of predicting effects with knowledge of component toxicants.

The problem of toxicant mixtures in the aquatic environment has been especially conspicuous in Canada because of industrial pollution of the Great Lakes. The International Joint Commission (1977, 1978, 1981) has set objective concentrations for individual contaminants. The following equation has been proposed for estimating the potential toxicity of mixtures of metals in Great Lakes waters:

> "The sum of the ratios of each metal concentration (M_i) and its respective objective concentration (O_i) should not exceed 1.0, that is,
>
> $$\text{SUM OF } (M_i/O_i) <= 1.0." \qquad [1].$$

The model is described as "a pragmatic approach to assessing mixtures effects. While it is scientifically indefensible, its application to surveillance data analysis may provide an early indication of areas that are potentially harmful and to which special attention should be paid in a search for impacts and sources" (International Joint Commission 1981).

In spite of extensive theoretical treatment of the topic of the joint effects of toxicants in mixtures in the 1950's and 1960's, the above equation represents the state of the art in applied work. The temporary acceptance of this model for setting standards has also been suggested by national agencies of other countries and in summary and

methods papers (Ministry of Technology 1966, 1968, National Technical Advisory Committee 1968, Committee on Methods for Toxicity Tests with Aquatic Organisms 1975, Stephan 1975, National Research Council 1981, Alabaster and Lloyd 1982, Lloyd in SGOMSEC 1986, Calamari and Alabaster 1980, EIFAC 1980).

The "objective concentration" is often determined from acute mortality studies, although it could conceivably be based on any test. The most common statistic expressing acute lethality, the concentration producing 50% mortality in 48 or 96 hours (the 48- or 96-hour LC50), is routinely determined for new chemicals, and is readily available in the literature and in data bases. The LC50 is generally believed to be directly proportional to one that allows an indefinite survival time (Henderson 1957, Sprague 1971, Tarzwell 1962, 1966, Kenaga 1982). In this study it is assumed that defining models for the joint effects of toxicants in acute tests is relevant to long-term application problems.

1.2. A Summary of Acute Mortality Studies with Toxicant Mixtures

Methods for describing and predicting the joint effects of toxicants applied as mixtures to whole organisms come from several areas of the biological sciences. Following is a review of the literature with terms as used by the authors.

1.21. Probit Analysis

The earliest acute toxicity data and results of true bioassays were analyzed and described using one branch of pure statistical theory, namely probit analysis. Probit analysis is a collection of statistical techniques concerned with the relation of the frequency of occurrence of a specific response, usually the probability of survival or non-survival, and the numerical amount of a dose or other stimulus that may cause the response (Bliss 1934, 1935a, 1937, Finney 1947, 1971, Hubert 1980). The underlying assumption in applying the techniques of probit analysis is that the population has a normal distribution of tolerances, with tolerance defined as the smallest doses that will cause mortality in an individual. Through the use of the probit transformation of percent response, (in this case % mortality) data, an S-shaped curve of % response vs the independent variable becomes a straight line which is more amenable to statistical analysis. A simple logarithmic transformation of the dose of toxicant is usually also applied, this transformation believed to be appropriate for biological reasons. Thus

the underlying model in all probit analysis is:

$$\text{Probit}(p) = a * \underline{\ln}(\text{dose}) + b \ , \qquad [2],$$

in which p = the probability of mortality or fractional mortality observed (Bliss 1934), a and b are constants, and ln is a logarithmic transformation, usually the logarithm to the base 10. The two constants a and b are directly related to the mean and standard deviation of the tolerance concentrations (Finney 1947).

Probit analyses was once used extensively to compare individual drugs and sera to each other, but not to assess their effects in mixtures. Responses were compared by examining the parameters of the probit vs ln(dose) response curves (a and b in Equation [2]). Generally, if two drugs had a similar mode of action, the two response curves were parallel, and only the intercept, indicative of the relative potencies of the compounds, had to be compared (Parallel line assays, Bliss 1935b, Finney 1947, 1971).

The technique of probit analysis, as it is known today, includes methods of weighting the fit of the equation with the inverse of the expected variance to obtain homogeneous variances over the response range, maximum likelihood methods of fitting the response curve, analyses of variance for more complex experiments, and methods for estimating critical doses and fiducial limits (Finney 1947, 1971, Hubert 1980). Probit analysis is still used extensively to produce summary statistics for tests with individual toxicants. The statistics of

interest are the time to the 50% response or the median survival time (MST), the LC50 (lethal concentration which kills 50%), and the LD50 (lethal dose which kills 50%), and their confidence limits.

The logit transformation, $\ln(p/(1-p)) = \ln(p)-\ln(1-p)$, was also described as an appropriate transformation for bioassay use (Finney 1947, Berkson 1944, 1949, 1951, 1953, 1955). It is highly correlated with the probit transformation, but has an underlying model based on autocatalytic processes. The transformation is attractive since it is algebraically more tractable than the probit. Other transformations of the sigmoid curves exist (summary in Ashton 1972), but none have been commonly used for bioassay data.

1.22. Joint Action Models

Bliss (1939) defined the following possible modes of joint action.

> (1) Independent joint action. "The poisons or drugs act independently and have different modes of toxic action .... The toxicity of the mixture can be predicted from the dosage mortality curve for each constituent applied alone and the correlation in susceptibilities of the two poisons. The observed toxicity can be computed on this basis whatever the relative proportions of the components."

By definition, "r", the correlation between susceptibilities, is a constant that can take on values between 1 and -1. It describes the changed probability of responding to the more toxic compound due to the presence of the other, that is, p is changed to p + r*p. If r=0, then the outcome of the exposure to the mixture is known from component probabilities associated with responses to individual toxicants, specifically the probability of non-response (survival) is obtained by multiplying the probabilities of non-response associated with each compound. The equation is:

$$q_c = q_a * q_b = (1-p_a) * (1-p_b),$$

in which q_c is the probability of non-response in the mixture of toxicants A and B, q_a and q_b are the probabilities of non-response in toxicants A and B respectively, and p_a and p_b are the probabilities of response.

A general equation for the probability of non-response, given an "r" for the stronger toxicant A in this case is:

$$q_c = (1 - p_a) * (1 - p_b + r*p_b) = q_a * (q_b + r*(1-q_b)).$$

If we have the case of "complete correlation of susceptibilities" (r=1), then the response is described in terms of only one toxicant, that is $q_c=q_a$. Bliss (1939) wrote "the combined mortality would be attributable to the more toxic ingredient applied alone".

Two other types of joint action are defined as follows:

(2) Similar joint action. "The poisons or drugs produce similar but independent effects, so that one component can be substituted at a constant proportion to the other; .... The toxicity of a mixture is predictable directly from that of the constituents if their relative proportions are known." This mode of action applies if C1, the concentration of one toxicant, can be replaced by a linear combination of toxicant concentration values, that is, C1 + b*C2 + c*C3 .... , in any response curve. The probit response curve describing the joint response to a mixture of two compounds with simple similar action would be:

$$\text{Probit}(p) = a * \ln(C1 + b*C2) + k \ , \qquad [3],$$

(3) Synergistic action. "The effectiveness of the mixture cannot be assessed from that of the individual ingredients but depends on a knowledge of their combined toxicity when used in different proportions. One component synergizes or antagonizes the other." The case of zero synergism is not defined as either simple similar action or independent joint action.

The authors P.S. Hewlett and(or) R.L. Plackett expanded these models with a series of publications on joint action models (Hewlett and

Plackett 1950, 1952, 1959 1964; Plackett and Hewlett 1948, 1952, 1963, 1967). Their described physiological categories of joint action and the definitions are as follows: (Hewlett and Plackett 1959):

	Similar	Dissimilar
Non-interactive	simple similar	independent
Interactive	complex similar	dependent

Similar action. "If two poisons, whether administered separately or jointly, elicit a certain quantal response by causing the same physiological system to react or fail, then joint action is said to be similar with respect to that response."

Dissimilar action. "If two poisons, whether administered separately or jointly, elicit a certain quantal response by causing respectively different systems to react or fail, then joint action is said to be dissimilar with respect to that response.".

Interaction. (Compounds) "A and B are said to interact if the presence of A influences the amount of B reaching B's site of action, or the changes produced by B at B's site of action; and/or reversely with A and B interchanged."

The combined categories are defined as follows:

Simple similar action. This mode of joint action is defined identically to Bliss' (1939) definition of similar joint action, in which one compound can be substituted at a constant proportion to the other. Complex similar action occurs when the effects of one toxicant can be described in terms of the other in any manner.

Independent action. This mode of joint action is identical to Bliss' (1939) independent joint action with complete correlation of tolerances. Any deviation is dependent action.

Bliss' (1939) definition of independent joint action with no correlation of tolerances is equivalent to dependent action as defined by Hewlett and Plackett (1959, 1967). They wrote "dependent action .... is more likely to occur in reality than independent action, because an organism is at least largely a coordinated whole. Nevertheless, independent action, if approximated only occasionally in reality, is important theoretically, for it leads to a limiting mathematical model that appears to be an essential step toward to construction of others more commonly realistic" (Hewlett and Plackett 1967).

The concept of simple similar action is generally considered to be the most useful one arising out of this early work (Finney 1971). It is appealing because it expresses a relationship between the action of the compounds in any model, not necessarily something about a chosen

response such as a probability. There is the underlying assumption that if simple simliar action applies in describing one response, then it will also apply to any other.

The two categories of complex similar action and dependent action as defined by Hewlett and Plackett (1959) encompass so many possibilities that no mathematical generalizations are made about them. In a later paper Hewlett and Plackett (1964) describe another intermediate category, "partial similar action", intermediate between simple similar and independent.

Hewlett and Plackett (1964,1967), Finney (1972) and Ashford and Smith (1965) extended the probit response curve (Equations [2] and [3]) to describe a variety of responses, including independent joint action. These more complex models are highly parameterized ones which require knowledge of variables such as upper and lower threshold levels, number of available sites of action, component probabilities and correlations between susceptibilities.

Ashford and Smith (1964, 1965) argued that Hewlett and Plackett's (1959) physiological definitions did not necessarily relate to specific mathematical equations, and also that the presented models were not necessarily alternatives nor inclusive of all reasonable options. They presented a very general system of classifying joint effects based on ratios of partial derivatives obtained from an overall model or response surface. According to the authors only two types of joint effects are possible - interactive and non-interactive. Models or modes of action are non-interactive for two toxicants if they can be rewritten as $\underline{f1}(R)$

= f2(C1) + f3(C2), in which f1, f2 and f3 are any functions that satisfy the relationship. Such transformations exist if $(d^2R/dC1dC2)/(dR/dC1)*(dR*dC2)$ = k for any R, with the d's representing partial derivatives and k a constant. One special sub-category of interaction is termed "independent action", and is identical to Bliss' (1939) independent joint action with r=1.

The function f1(R) does not necessarily need to be known. In practice it might be a transformation or function which can only by obtained after obtaining model parameters. From this point on, I will refer to a defined response variable as one which is chosen to express the results or one which has a known form before the response surface is fitted, and an undefined response variable as one that can is defined only after the response equation has been rearranged to obtain a desired form of the independent variables. The two types of response variables will be referred to as "R" and "f(R)" respectively.

1.23. Joint Effects Models

Joint effects classifications are based entirely on numerical relationships among linear combinations of independent variables describing a defined response variable. These are the same types of linear models used in analysis of variance and regression analyses. Simple joint effects models have been used in the agricultural sciences for describing and predicting the effects of mixtures of herbicides, pesticides or mixtures of these with solvents (Gowing 1960, Colby 1967,

Burrell and Corke 1980).

Loewe and Muischnek (1926) and Gaddum (1933) independently proposed graphical methods of representing joint effects. Both suggested the use of "isoboles", isobols, isolines, or lines of constant response to classify the type of joint effect. Types of effects, taken from Tammes (1964) are represented in Figure 1. Only the isolines for 100% response are shown, this assumed to be the only response of interest in these models. The axes in Figure 1 range from 0 to 100% effective concentration, which might be any transformation of the concentration. Joint effects are defined as follows:

I. Independent effects. "The total effect is equal to the effect of the most active compound alone". (This mode now has several names: dependent, independent, correlated, "no interaction" (Brown 1968, Sprague 1970), and "no addition" (Koenemann 1981)).

II. Addition. "Cooperative action, such that the total effect is equal to the sum of the effects of the components taken independently. The components may be substituted for each other in amounts inversely proportional to their activity."

III. Synergism. "Cooperative action of two components of a mixture, such that the total effect is greater or more prolonged than the sum of the effects of the two taken independently."

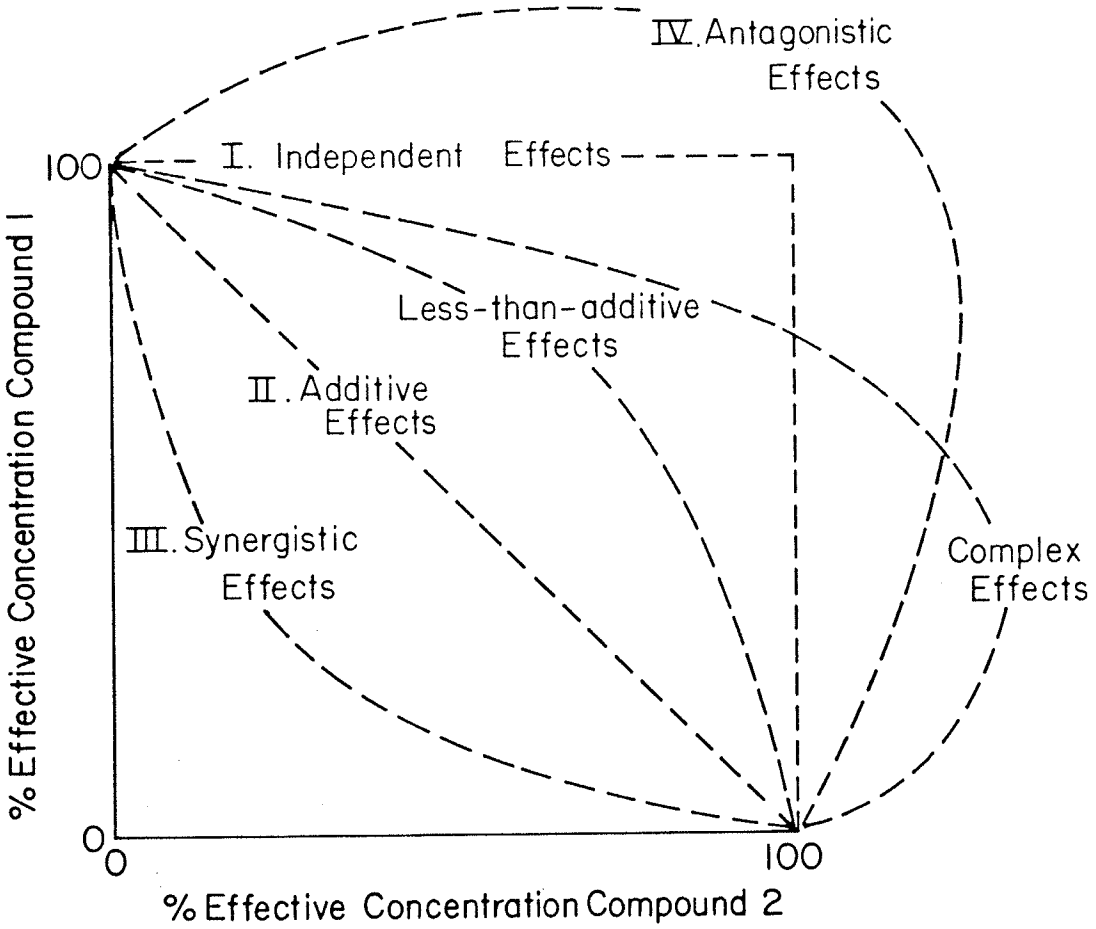

Figure 1. 100% Response Isoboles adapted from Loewe and Muischnek (1926), Gaddum (1933), Tammes (1964) and Sprague (1970).

IV. Antagonism. "The total effect is smaller than the effect of the most active component alone."

Ashford and Smith's (1964) classification of all models as interactive or non-interactive also relate to the isoboles. Ashford and Smith (1964) write of non-interactive models "The system of isobols may be changed into a system of parallel lines by suitable choices of dose metamers". That is, with the right choice of axes (not necessarily linear, increasing, or continuous) and of response variable (not necessarily defined before the response surface is estimated), additive effects are defined.

1.24. Tolerance Addition

In the earlier part of this century and in the 1960's numerous European scientists (Southgate 1932, Cherkinsky 1957, Corner and Sparrow 1956, Devlaminck 1960, Marchetti 1962, LLoyd and Jordan 1963, 1964) used an experimental and interpretational approach which greatly influenced future work with toxicant mixtures. The tolerance model they claimed to be testing was:

1 = SUM OF (Actual concentration/Tolerance concentration). [4].

The model states that the effect of each toxicant is inversely proportional to its tolerance value, and that the proportional

contributions of the fractions of the tolerances of each toxicant can be summed. Any mixture of toxicants satisfying the above relationship should be 100% effective, that is give the tolerance response. The model has also been called the "toxic units concept" (Sprague 1970), a toxic unit being the reciprocal of a tolerance value, "tolerance addition" (Brown 1968, Koenemann 1981), and "concentration addition" (Anderson and Weber 1975, 1976).

Experiments testing the model were usually performed in the following manner. The acute toxic levels or tolerance concentrations of all individual toxicants in question, usually expressed as 48-hour LC50's, were first determined. Then several dilution levels of a mixture, in which all compounds had concentrations proportional to their LC50's, were used in acute toxicity tests. If, with a mixture of n compounds, the mixture in which all concentrations were LC50/n, was the one that caused 50% mortality (the 100% effective response or tolerance response) in 48 hours, then the model and concept of "tolerance addition" was assumed to be confirmed. One point was often the basis of accepting or rejecting the model.

The model was confirmed with the above experimental design for mixtures of zinc and copper (Lloyd 1961), of ammonia and zinc (Herbert and Shurben 1964), of copper and ammonia, and zinc and phenol (Herbert and van Dyke 1964), worked reasonably well for a mixture of ammonia, phenol, zinc, copper, and cyanide (Brown 1968), and underestimated toxicity in more complex mixtures of sewage components (Lloyd and Jordan 1963, 1964). More recent studies testing the concept with various

compounds, usually heavy metals, were done by Cairns and Scheier (1957), Brown et al. (1969), Brown and Dalton (1970), Eaton (1973), Heisinger et al. (1979), Spehar et al. (1978), Sprague (1964), Sprague et al. (1965), Sprague and Ramsay (1965), and Woodward (1982). Sprague (1970) wrote that the method is "not quite perfect" but quite acceptable and the best available. As mentioned in the introduction, this model has become the main one used for setting standards for mixtures of toxicants (International Joint Committee 1981, National Technical Advisory Committee 1968, EIFAC 1980).

1.25. Experiments Testing the Applicability of Modes of Joint Action

Anderson and Weber (1975) tested the applicability of two modes of joint action, simple similar action and independent joint action, for toxicant mixtures expected to act jointly in either mode.

Anderson and Weber (1975) equated the terms "similar joint action" as defined by Bliss (1939) and "concentration addition" as defined by Brown (1968) and Sprague (1970). They argued that because the probit vs ln(dose) response curves of individual metals were parallel in the case of metals with known similar modes of action, and because the effects of the two compounds could be described as constant multiples of each other, that "the toxic units approach could be considered a practical .... simplification of the similar joint action mechanism proposed by Bliss (1939) in his theory of multiple toxicity."

Starting with the assumption that copper and nickel would conform to the concentration addition model because of 1) parallel response curves and 2) knowledge of their modes of action, Anderson and Weber (1975) confirmed "concentration addition" in an experiment testing 6 levels of a mixture of two metals, with 10 fish at each level, and with mixtures with equitoxic concentrations and constant concentration ratios. "Concentration addition" or "simple similar action" as a mode of action was not rejected when the observed and predicted number of animals determined from the probit vs ln(concentration) response curves were compared with a Chi-square test with 4 degrees of freedom. It was possible to calculate predicted values because both metals were used at a constant ratio of concentrations and their relative potency was known from tests with individual toxicants. Thus the combination of component concentrations could be expressed as the concentration of one toxicant.

Anderson and Weber (1975) also equated the terms "independent joint action" as defined by Bliss (1939) and Plackett and Hewlett (1948, 1952) and "response addition". The following experimental method was used in experiments with compounds not having parallel dose-response curves, in this case, one experiment with potassium pentachlorophenate (PCP) and dieldrin, and another with PCP and cyanide. To test for response addition, response curves for individual compounds were examined, and concentrations of each associated with several chosen probabilities of dying were calculated. Mixtures of equitoxic concentrations, in this case concentrations associated with the same probabilities of dying were tested. The observed number and the predicted numbers of fish that died,

based on the assumption of independent joint action, were compared. In experiments with 6 experimental tanks, and 10 fish in each tank, the hypothesis of response addition could not be rejected on the basis of a Chi-square test with 4 degrees of freedom.

In a similar experiment, mixtures of copper and zinc were shown to be "supra-additive", that is, the effect was stronger than expected on the basis of the concentration addition model. Anderson and Weber (1975) also defined a category, "infra-additive", not demonstrated with any of their mixtures, in which the effects were weaker than expected on the basis of their calculations.

These methods of classifying joint effects were then used in further studies (Anderson 1975, Anderson and Weber 1976, Anderson et al. 1979, Koenemann 1981).

Koenemann (1981) developed an index, the Mixture Toxicity Index (MTI), which he claimed described the toxicity of a mixture compared to its components. MTI = 1 for concentration addition, MTI = 0 for completely correlated action, MTI < 0 for antagonism, and MTI > 1 for supra-addition or synergism. Like the index "r", it ranges from -1 to +1 and places a response intermediate between two modes of action, simple similar action and independent joint action with r=1. Other modes of action such as complex similar action and independent joint action with $r \neq 1$ do not occupy constant positions in this scale.

Koenemann (1981) examined the toxicity of six mixtures of 3 to 50 compounds, specifically low molecular weight organochlorides and benzene derivatives, expected to have similar modes of action on the basis of

their physical characteristics. Simple similar action was generally confirmed with the Mixture Toxicity Index. Hermens and Leenwaugh (1982) similarly confirmed this mode of action with mixtures of 8 to 24 lipophilic compounds.

1.26. Experiments using Linear Models

Linear modelling techniques such as analysis of variance and regression analysis have been used to describe the results of experiments with mixtures. Various forms and transformations of the dependent and independent variables have been used, thus the results are difficult to compare to other studies.

Gray (1974) performed balanced experiments measuring the growth reduction of a marine ciliate protozoan Cristigera sp. in mixtures of mercury, zinc and lead. Results expressed as the angular transformation of percent growth reduction were analysed with a complete analysis of variance including all polynomial and interactive orthogonal contrasts. Non-interactive factors accounted for 86% of the model variance, and interactions with zinc accounted for 13% of of the model variance.

Parker (1979) performed balanced experiments examining the growth rate of a marine protozoan Uronema marinum exposed to mixtures of lead, mercury, and zinc. He concluded that adding the percent reductions in growth rate gave good predictions of the the total reduction. Analysis of variance of results transformed with the angular transformation suggested that the non-interactive factors accounted for most of the

variance.

Voyer and Heltsche (1984) reanalyzed two published toxicological data sets in order to compare several predictive models and to "assess the potential practical significance of factor interactions". The term factor interactions is used in the statistical sense, that is, the presence of a significant interaction term in a chosen model describing the data. Their defined alternative models were:

1) Simple additive model, $R = R1 + R2$, where R is the overall response, and R1 and R2 are expected responses when the other toxicant is absent. This model represents the addition of response curves.

2) Linear additive model, $R = k + a*C1 + b*C2$, multiple regression equation in which C1 and C2 represent concentrations of toxicants.

3) Quadratic response model, $R = k + a*C1 + b*C2 + c*C1*C2 + d*C1^2 + e*C2^2$, also a multiple linear regression equation which provides for more complex responses.

Voyer and Heltsche (1984) assumed that the simple-additive model was the most likely to apply, and thus more complex models were not accepted unless there was a substantial increase in their predictive capacity. The reanalysis of Parker's (1979) data suggested that the linear additive model was the most desirable. For this analysis, Voyer and

Heltsche (1984) used either the rate of cell division or percent normal growth as the independent variable - the exact form of the response variable was not given. The reanalysis of the other data set, from Voyer et al. (1982) testing the viability, expressed as percent viable hatch, of eggs of winter flounder Pseudodopleuronectes americanus, exposed to mixtures of cadmium and silver, suggested that the quadratic model was best. The simple additive model overestimated responses in all cases.

1.3. Conceptual Problems in Previous Work

The described literature on the joint effects of toxicants, largely motivated by the work of Bliss (1939), is still believed to define the major reference points for studies on the joint effects of toxicants. It will be shown here that this existing framework is not a satisfactory basis for the testing of alternative hypotheses nor for the consistent quantification of possible modes of joint action.

Simple similar action (SSA) was originally defined as a mode of action in which a function of linear combinations of toxicant concentrations described the response. The definition can be abstracted as follows:

$$\underline{f}(R) = \underline{g}(C1 + b*C2 + c*C3 \ldots. + k),$$

in which R is any response, $\underline{f}$ and $\underline{g}$ are any functions, C1, C2, ... Cn are the concentrations of the toxicants, and lower case letters are numerical constants.

The definition of independent joint action with no correlation of susceptibilities (IJA, r=0), can be abstracted as follows:

$$\underline{h}(q_c) = \underline{h}(q_1*q_2) = \underline{h}(\underline{m}(C1)*\underline{n}(C2)),$$

in which $\underline{h}$, $\underline{m}$, and $\underline{n}$ are any functions and the q's are the probabilities of non-response as previously defined. The definition of SSA is in terms

of any response, most likely a function of the probability of survival. The definition of IJA (r=0) is in terms of the factorability of the expression describing the joint probability of non-response.

Joint effects models are defined in terms of relationships between functions of independent variables describing a defined response variable. Types of joint effects can be identified by examination of the following general model:

$$R = \underline{f}(C1) + \underline{g}(C2) + \underline{h}(C1,C2).$$

in which $\underline{f}$, $\underline{g}$, and $\underline{h}$ are functions of concentrations, and R is a defined response variable which incorporates the control response. Additive effects are described if $\underline{h}(C1,C2)$ is equal to 0 while other categories are described by different forms of $\underline{h}(C1,C2)$. Ashford and Smith's (1964) categories of interactive and non-interactive can be similarly defined, but for an undefined response variable. The two classifications systems coincide only in that additive effects are always non-interactive. Other joint effects classifications can be either interactive or non-interactive.

Independent joint action with correlated tolerances (IJA, r=1) is defined in terms of the toxicant with the "stronger" effect. However, any effect could be described as being due to only one toxicant if that toxicant is defined to be the stronger under a given condition, specifically the presence of both toxicants. The definition has an inherent circularity.

Any of the described modes of action can be identical if certain functions are chosen. For example, if the following curve describes a response:

$$R = 6*C1*C2 + 2*C1 + 3*C2 + 1 = (1+2*C1) * (1+3*C2), \text{ thus}$$

$$\underline{\ln}(R) = \underline{\ln}(1+2*C1) + \underline{\ln}(1+3*C2),$$

the type of joint effects can be described in several ways. If regarded as a joint effects model, C1 and C2 have a more-than-additive or synergistic effects on the response R. If we were interested only in combinations of factors which yield R=0, we might conclude that IJA (r=1) was the appropriate mode of action since the equation describes the square "independent effects" isolines on Figure 1. If R is a probability of survival or non-response, that is R = q, then the above might be an equation expressing IJA (r=0) since it can be factored into component probabilities. The equation could also be considered to be one describing additive effects for the response $\underline{\ln}(R)$. It is also a non-interactive model. In any given situation, more than one definition would likely apply.

Similar problems exist with the response curve:

$$R = 4*C1^2 + 9*C2^2 + 12*C1*C2 = (2*C1 + 3*C2)^2.$$

Examined as a joint effects model, this equation describes more-than-additive effects for the response R. It also describes simple

similar action since a linear combination of concentrations describes the response. If $\ln(q_1 * q_2)$ = SQRT(R), then IJA (r=0) is also described since $q_1 * q_2 = (e^{2*C1}) * (e^{3*C2})$. It is also a non-interactive model.

The transformations of R in the above examples are similar to alternative responses that can be chosen to describe results of experiments. Particularly in experiments in which cumulative temporal responses are observed, possible responses with the same information content might be instantaneous rates, rates at a given point, percent or actual changes, integrated values such as total response times, the difference between or the ratio of two responses of interest, asymptotes and inflection points, or a convenient transformation of any one of these. The fact that interpretational problems are caused by working with alternative forms of the response variable still is not generally recognized by persons working with mixtures. Borgmann (1980) showed that different models describing results led to different conclusions about the joint effects of toxicants. The effects of mixtures on growth rates of algae were less-than-additive, and effects on growth times were more-than-additive. On the basis of this observation, he urges very careful use of models such as the toxic units concept (Section 5 in International Joint Commission 1981). Also, Hermens et al. (1984) reported that in experiments with Daphnia magna, additive effects were demonstrated in acute toxicity tests with mixtures of 14 organic pollutants (used at equitoxic concentrations), but less-than-additive effects were demonstrated in reproduction impairment tests. Acute toxicity was measured as a fraction of the LC50 and reproduction was

measured as a percent reduction of the normal rate. The fact that their conclusions might be due to the choice of response variables is not considered. The work of Voyer and Heltsche (1984) also does not acknowledge that the choice of response variable affects the probability of significant factor interactions in the model.

It is reasonable that not only the identity of modes of action, as demonstrated above, but also the similarity between modes of action, is dependent on chosen functions in the described general models. The extent to which modes of action defined within the same general model differ has not been considered by previously cited authors. The degree of similarity is examined for the probit vs ln(dose) model by the author (de March, in prep.), and for the linear models chosen in this study (explained in Section 2.2) in Appendix A. In both cases it is shown that predictions for simple similar action and independent joint action, given component probabilities of response, are either similar or highly correlated. Moreover, joint effects models also give similar predictions. It is concluded that few experiments are ever likely to be precise, voluminous, and complete enough to be able to distinguish the major modes of action. Also, the two indices, "r" and Koenemann's (1981) Mixture Toxicity Index, intended to modify the above two modes of action, modify the response very similarly.

Therefore, any one of the presented models could be taken as a baseline model to be extended to a diversity of situations. The major described modes of action cannot be considered to be alternatives.

1.4. A Proposed Framework for Model Consistency

The proposed model framework is an expansion of the joint effects models previously described. For the sake of simplicity, the model will be described in terms of levels of only two toxicants. In this model, the response to the mixture of the two toxicants is described by:

$$R = f1(C1) + f2(C2) + f3(C1,C2) + f0$$
$$= f1 + f2 + fc + f0, \text{ by definition,} \quad [5],$$

and thus,

$$R - f0 = f1 + f2 + fc, \text{ and ,} \quad [6],$$
$$R/f0 - 1 = f1/f0 + f2/f0 + fc/f0.$$

R is a defined response variable and C1 and C2 are concentrations of the two toxicants. The expressions f1(C1), f2(C2), and f3(C1,C2), abbreviated as f1, f2, and fc, denote any mathematical functions of the concentrations which take on the value "0" when one or either of the enclosed concentration values are "0". This is an important feature of the model since it ensures that the toxic units model is derived from the response curve equation (to follow). The lower case letters a,b, ..., z represent any numerical constants. The expression f1(C1) or f1 could have forms such as a*C1, $C1^2$, ln(C1+1), $a*C1^{3.2}$, or a*IF(C1), in which IF(C1) has the value 0 or 1 depending on whether or not Toxicant 1 is present. Expressions such as ln(C1) and 1/C1 could not be used since they are not 0 when C1 = 0. The expression f3(C1,C2) or fc could have

forms such as a*C1*C2, minimum of(C1, C2), IF(C1)*C2, ln(a*C1*C2+1), or any other expression that cannot be factored into a linear combination of functions of C1 and C2. The function f0, the response if all toxicants are absent, is a constant or a function related to factors other than toxicant concentrations. The actual or relative amount of change in the response, shown in the last two equations [6], is usually the actual response of interest.

R' represents a fixed R, usually a response of particular interest, for example, 0, 50, or 95% mortality, or the exact time to this mortality. C1' and C2' represent specific concentrations of toxicant associated with specific R''s.

If we are interested in the specific response R', we can calculate concentrations of individual toxicant expected to give this response in the following manner.

$$R' - f0 = f1(C1') \quad \text{and} \quad R' - f0 = f2(C2'), \qquad [7],$$

and therefore,

$$C1' = f1^{-1}(R' - f0) \text{ and } C2' = f2^{-1}(R' - f0), \qquad [8],$$

in which $f1^{-1}$ and $f2^{-1}$ are inverse functions, that is, the series of manipulations used to calculate C1' or C2'.

There is an infinite number of two-toxicant combinations which would yield a specific R'. We can define a function:

$$f4(C1',C2') = f4 = R' - f0 , \qquad [9],$$

since R'-f0 can be expressed in terms of either C1' or C2' or both.

If we divide Equation [6] by expressions equivalent to R' - f0 , specifically Equations [7] or [9], we obtain:

$$1 = \frac{f1(C1)}{f1(C1')} + \frac{f2(C2)}{f2(C2')} + \frac{f3(C1,C2)}{f4(C1',C2')}. \qquad [10].$$

This equation will be referred to as the "tolerance model" since it expresses conditions under which the tolerance level of response is reached. Equation [10] states that if we are interested in a specific response R', know which concentrations of individual toxicants (C1' and C2') that predict R', and have knowledge of f4, then we can determine combinations of levels of the component toxicants which will satisfy the standard of "1" or yield the tolerance response R'.

For example, let us assume that the probability of mortality in relation to concentrations of two toxicants can be described by

$$P = 0.2*C1 + 0.4*C2 + 0.5*C1*C2 + 0.01.$$

Let us assume that we are interested only in toxicant combinations which cause 5 % mortality, that is P' = 0.05. The concentrations of toxicants individually which would cause P' are C1' = 0.20 and C2' = 0.10.

A tolerance equation for the response P' = 0.05 and given the above model would be:

$$1 = \frac{0.2*C1}{0.2*C1'} + \frac{0.4*C2}{0.4*C2'} + \frac{0.5*C1*C2}{P'-0.01} = \frac{C1}{0.20} + \frac{C2}{0.10} + \frac{C1*C2}{0.08}.$$

in which (P'-0.01) = (R'-f0) in Equation [9]. Combinations of concentrations of toxicants which would yield a prediction greater than "1" would therefore cause mortality larger than P' or 0.05.

1.41. Relationship to Previously Described Modes of Joint Action

The described model defines joints effects more precisely than the previous graphical methods. Effects are additive for the response variable R if fc = 0, synergistic or more-than-additive if fc/(f1*f2) > 0, and antagonistic or less-than-additive if fc/(f1*f2) < 0. These ratios may be numerical constants, in which case the classification of type of joint effect is applicable to all levels of the toxicants, or may be functions of concentrations, in which case they apply only a certain concentration levels. In the latter case, lopsided isolines, such as the one labelled "complex effects" on Figure 1, or isolines with discontinuities, may be described.

The square isolines on Figure 1, labelled "independent effects", also previously defined as IJA (r=1) describe a special case of antagonism. This mode of action can be described only by functions containing class variables such as "r" or containing maxima and minima. It is also described when R = f0-((f1*f2)/fc).

The categories of interactive, non-interactive, simple similar action and independent joint action are additional and potentially informative, but not major classifications. They do not overlap consistently with any of the above.

The toxic units concept relates to the Equation [10]. If a response surface is described by the simple equation:

$$R = a*C1 + b*C2 + k,$$

then the associated tolerance equation for the responses R, obtained by reference to Equation [10] is:

$$1 = C1/C1' + C2/C2',$$

identical to the toxic units model (Brown 1968, Sprague 1970). Thus, the toxic units model is scientifically defensible if the response surface is a plane or hyperplane.

The toxic units concept can be derived from any model containing a linear function of concentrations, that is,

$$R = \underline{f}(a*C1 + b*C2 + k), \quad \text{thus} \quad \underline{f}^{-1}(R) = a*C1 + b*C2 + k.$$

$\underline{f}^{-1}(R)$ is not necessarily a defined response variable, that is, its form may not be not known until the response surface has been fitted. This variable could differ greatly between experiments with different types of toxicants. This would make the results of more than one experiments difficult to combine into one tolerance model.

If the response surface is:

$$R = a*C1^b + c*C2^d + k, \quad \text{and}$$
$$R - k = a*C1^b + c*C2^d,$$

then the associated tolerance equation is:

$$1 = (C1/C1')^b + (C2/C2')^d. \qquad [11].$$

A curvilinear equation might describe the response as well as the above. For example, a response might be described by:

$$R = e*C1 + f*C1*C1 + g*C2 + h*C2*C2 + k. \qquad [12].$$

The associated tolerance equation is:

$$1 = \frac{C1 + (f/e)*C1*C1}{C1' + (f/e)*C1'*C1'} + \frac{C2 + (h/g)*C2*C2}{C2' + (h/g)*C2'*C2'}$$

$$= \frac{C1}{C1'} * \frac{1 - C1/(T1*2)}{1 - C1'/(T1*2)} + \frac{C2}{C2'} * \frac{1 - C2/(T2*2)}{1 - C2'/(T2*2)}, \qquad [13],$$

in which T1 =-2*e/f and T2=-2*g/h, and represent the concentrations at which an inflection point occurs in the response curve, that is, high or low threshhold concentrations.

If we have a simple model for more-than- or less-than-additive effects such as the following:

$$R = a*C1 + b*C2 + c*C1*C2,$$

then the corresponding tolerance equation, obtained by reference to Equation [10], and by definition of a constant J = (f4(C1',C2'))/c (with f4 as defined in Equation [9]), is:

$$1 = C1/C1' + C2/C2' + C1*C2/J, \qquad [14],$$

$$1 = C1/C1' + C2/C2' + (C1*C2)/SQRT(C1'*C2') * c/SQRT(a*b). \qquad [15].$$

J could be SQRT(C1'*C2')*fc/(f1*f2) or SQRT(C1'*C2')*(SQRT(a*b)/c since R'-fo = a*C1' and = b*C2' (Equation [7]). Similarly, J could be chosen as f(C1')*fc/f1 or f(C1')/c = a*C1')/c. The tolerance equation would be:

$$1 = C1/C1' + C2/C2' + (C1*C2)/C1') * c/a. \qquad [16].$$

In the above cases, one requires knowledge about the constants (SQRT(a*b))/c or a/c which could be obtained from response surfaces. Such ratios of constants would have real physical meaning and should be as amenable to tabulation as LC50's.

A modified toxic units concept of the following form is described in the agricultural sciences (Gowing 1960, Colby 1967):

$$1 = (C1 + p1)/(C1' + p1) + C2/C2',$$

in which p1 is the fractional changes expected in the tolerance of the first compound due to the presence of the second. The response surface equation related to the above would be:

$$R = a*C1 + b*C2 + a*(p1)*IF(C2) + c.$$

where the coefficient p1 applies only if toxicant 2 is present. In other words, IF(C2) takes on the values 0 or 1.

Expressions similar to IF(C2) would have to be used in tolerance models if the original responses contained variables such as correlations of susceptibilities ("r") (Bliss 1939) or Koenemann's (1981) Mixture Toxicity Index. Also models describing the addition of total responses or response curves with individual intercepts, such as Voyer and Heltsche's (1984) "Simple additive model", would require class variables in the modified toxic units model.

1.5. Expected Toxicity of Tested Chemicals Individually and in Mixtures

The five potentially toxic ions Cu^{++}, Cd^{++}, Zn^{++}, Mg^{++}, and K^{+}, were chosen as toxicants in the experiments because they were believed to show a variety of joint effects when combined.

Death by heavy metals is mainly due to the coagulation or precipitation of mucus secreted by the gills, and damage to the gill tissue. Insoluble metal-protein compounds are believed to interfere with the respiratory function of the gills (Doudoroff and Katz 1953). Danielli and Davis (1951) suggested that metal ions exert their toxic influence by covalent binding at cell surfaces, and that the differences in electronegativity of the various ions is the toxicity determining factor. Metals are expected to act similarly, competing at active physiological sites, due to their common divalence. Thus the applicability of simple similar action applied to metal mixtures has often been tested.

Zinc is an essential component of the enzyme carbonic anhydrase which catalyses the hydration of CO_2 in the gills and red blood cells (Prosser and Brown 1961). Zinc could thus upset the blood buffer system. It is not necessarily expected to have the same effects as copper and cadmium.

Potassium near freshwater LC50 values is lethal primarily due to sodium-potassium imbalances in the nervous system.

Review papers by Gregory (1973) and Clarke (1974) summarize heavy metal LC50's available to that time. Values for 96-hour LC50's of copper

range from 0.0098 to 10.2 mg/L. Cu^{++} for both fish and invertebrates. 48- and 96-hour cadmium LC50's for fish range between 0.029 to 73.5 mg/L Cd^{++}. 96-hour LC50's for zinc range between 0.42 and 41 mg/L Zn^{++} (Cairns and Scheier 1968, Skidmore 1964, Gregory 1973). Reported lethal values of potassium to Daphnia magna and Lymneae sp., expressed either as 48-hour, 96-hour, or three-week LC50's, range between 15 and 572 mg/L K^{+} (Biesinger and Christensen 1972, Dowden and Bennett 1965). Magnesium is toxic to fish and invertebrates at concentrations in the hundreds of mg/L Mg^{++} range (Gregory 1973).

There are reported differences in sensitivity between taxa. For example, Arthur and Leonard (1970) reported 48-hour LC50's of 0.020 mg/L Cu^{++} for the amphipod Gammarus pseudolimneaus and LC50's of 1 to 3 mg/L Cu^{++} for the crayfish Orconectes rusticus. In general, invertebrate or fish taxa which are consistently associated with low or high LC50 values cannot be identified.

Reported differences are believed to be primarily due to modifying chemical factors. Water hardness is known to increase resistance to copper (Sprague 1964, Sprague and Ramsay 1965, Pickering and Henderson 1966, Mount 1968, Mount and Stephan 1969). Variations in LC50's have been shown to be due to differences in pH, since the metal ions are more likely to be dissociated and physiologically available at low pH values. For example, Howarth and Sprague (1978) report 96-hour copper LC50's for rainbow trout from 0.020 to 0.520 mg/L Cu^{++}, the values dependent on both water hardness and pH. The effects of pH and hardness have been demonstrated primarily within experiments done at the same time or in

the same laboratory - they do not explain results reported by different authors. Variation within laboratories is often high, for example, the Ministry of Technology (1968) reported that 48-hour LC50's varied by a factor of 2.5 in successive tests for no apparent reasons.

Nearly all of the available literature on metal mixtures suggests that the toxicity of mixtures is greater than any of the metals tested singly (review in International Joint Commission 1981, EIFAC 1980). Nearly all of the reported results of experiments with metal mixtures were obtained with mixtures of equitoxic concentrations or with mixtures of toxicants at constant ratios. Usually the applicability of only simple similar action at concentrations yielding 50% mortality in 96 hours was tested. Brown and Dalton (1970) concluded that the joint effects of copper, zinc, and nickel were approximately additive on the basis of an experiment using mixtures of equitoxic concentrations and using 50% mortality at 48 hours as the tolerance response. Hewitt and Anderson (1979) and Eisler and Gardiner (1973) reported more-than-additive effects for cadmium and copper. The results of experiments with mixtures of copper and zinc have generally been reported as synergistic or as more-than-additive effects (Eisler and Gardiner 1973). Sprague and Ramsay (1965) reported that at concentrations near the incipient lethal levels (32 ug/L Cu^{++} and 420 ug/L Zn^{++}), effects were additive, while in mixtures totalling two to five toxic units, effects were more than additive. Eaton (1973) reported that copper and zinc had additive effect on 90% egg production, and less-than-additive effects on 50% egg production of fathead minnow

Pimephales promelas. Eisler and Gardiner (1973) and Spehar et al. (1978) reported that effects were considerably more than additive. Anderson et al. (1979) and Gallimore and Anderson (1979) reported additivity, but a changed vulnerability at different life stages of zebrafish. Wong et al. (1978) reported that mixtures of 10 heavy metals were more toxic than expected if zinc was present.

It is believed that the divalent cations Ca^{++} and Mg^{++} compete with metals for active sites at cell surfaces, and thus lessen the toxic effects of heavy metals. Thus Mg^{++} might lessen or enhance toxicity.

Doudoroff and Katz (1953) describe interactive effects of calcium, magnesium, and potassium as being due to chemical equilibrium processes, primarily precipitation, in test water. This conclusion is based on toxicity tests of very short duration (minutes) at concentrations orders of magnitudes higher than reported LC50's and than concentrations used in my experiments. Equilibrium processes were not believed to be important in the work presented here.

MATERIALS AND METHODS

2.1. Acute Toxicity Tests

All experiments were performed at the Freshwater Institute, Department of Fisheries and Oceans, Winnipeg, Manitoba, with dechlorinated water available in controlled environment rooms. Average chemical measurements were: pH, 7.8; hardness, 80 mg/L $CaCO_3$; conductivity, 200 uS/cm; total dissolved inorganic carbon, 720 umole/L; dissolved organic carbon, 900 umole/L; K^+, 1.16 mg/L; Mg^{++}, 5.84 mg/L; Cl^-, 6 mg/L; and SO_4^{--}, 7.7 mg/L.

All experiments were performed in the dosing apparatus shown in Plates 1 and 2, and outlined diagramatically in Figure 2. In this apparatus, a series of water-metering cells was filled approximately once every two hours. After the last water-metering cell was filled, a siphon was activated, causing a partial vacuum in the vacuum manifold. This activated the stand-up siphons in the water-metering cells, which then emptied into mixing cells, where the water was mixed with stock solution already dispensed into the mixing cells. When the water reached a certain level in the mixing cells, another series of siphons was activated, and the mixing cells emptied into the test cells. The volume of new solution introduced every two hours was equivalent to the volume of the test cells, approximately 500 ml.

The peristaltic pump dispensing water was controlled by a single-pole, single-throw microswitch which was "on" when the float in the overflow chamber was down. When the overflow chamber filled, the

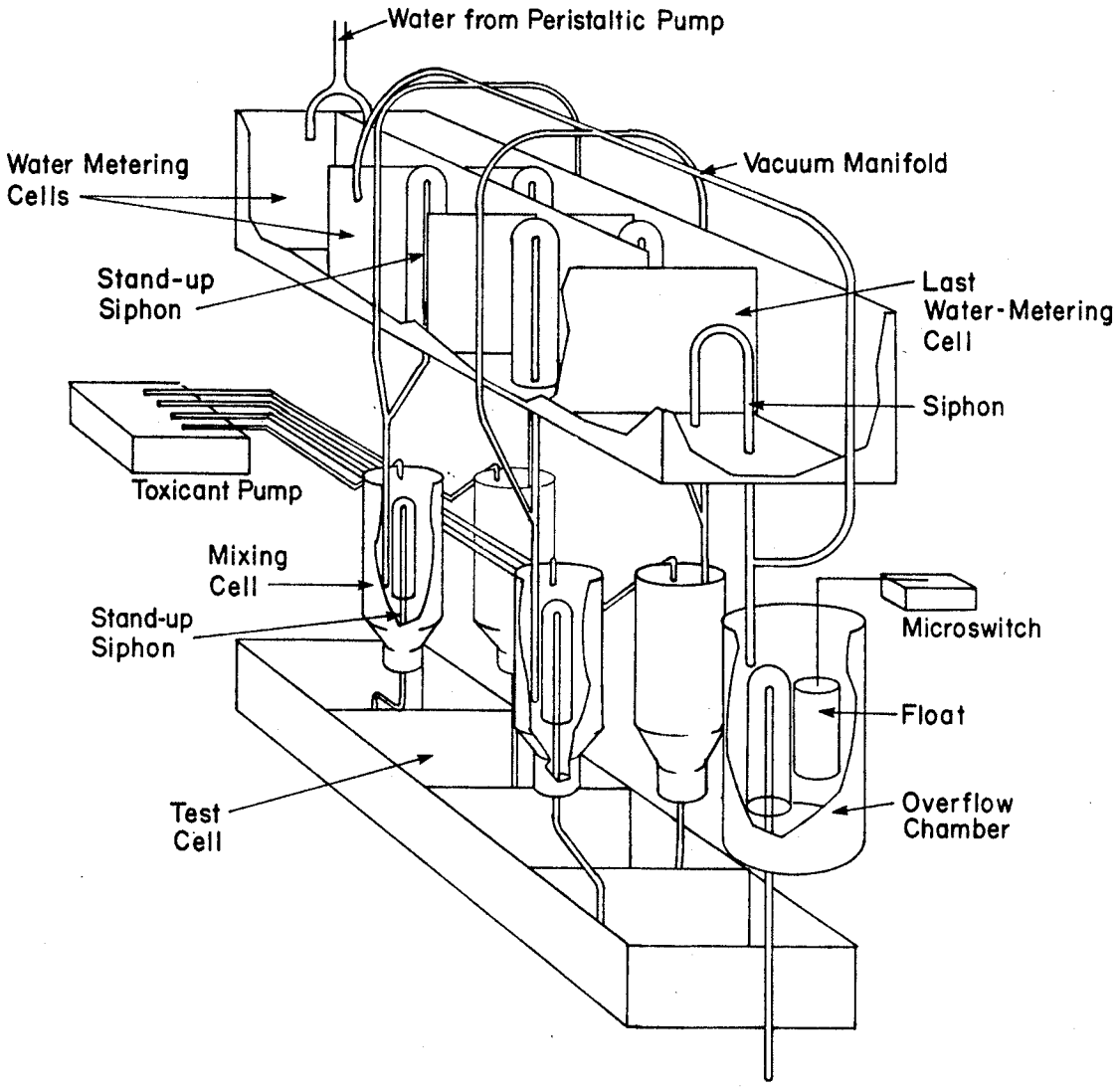

Figure 2. Schematic diagram of dosing apparatus.

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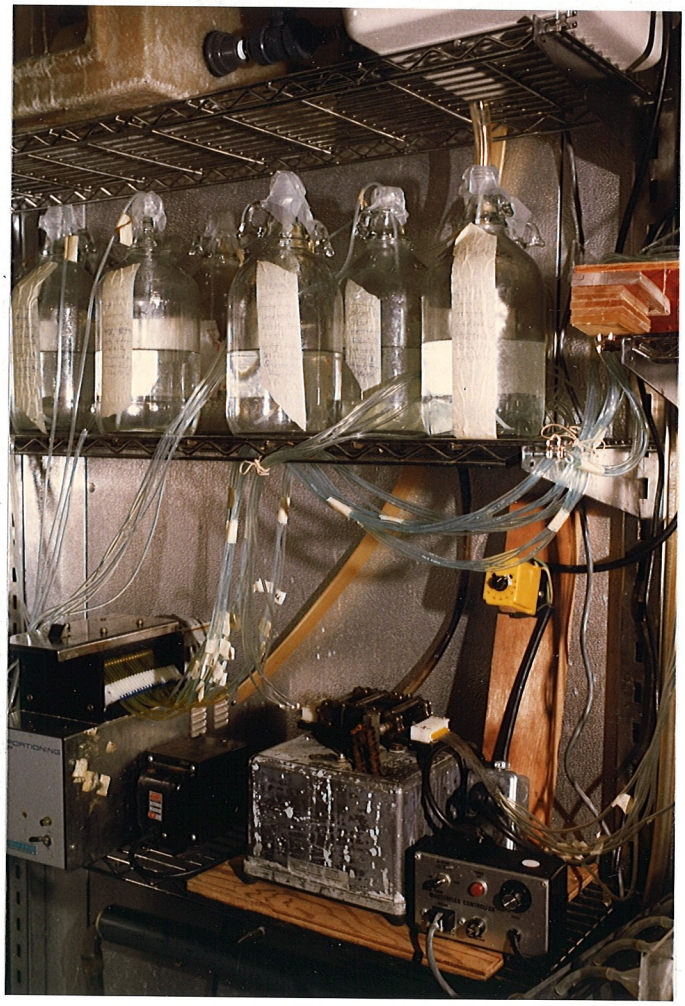

Plate 1. Left side of dosing apparatus, including peristaltic pumps and bottles of stock solustion.

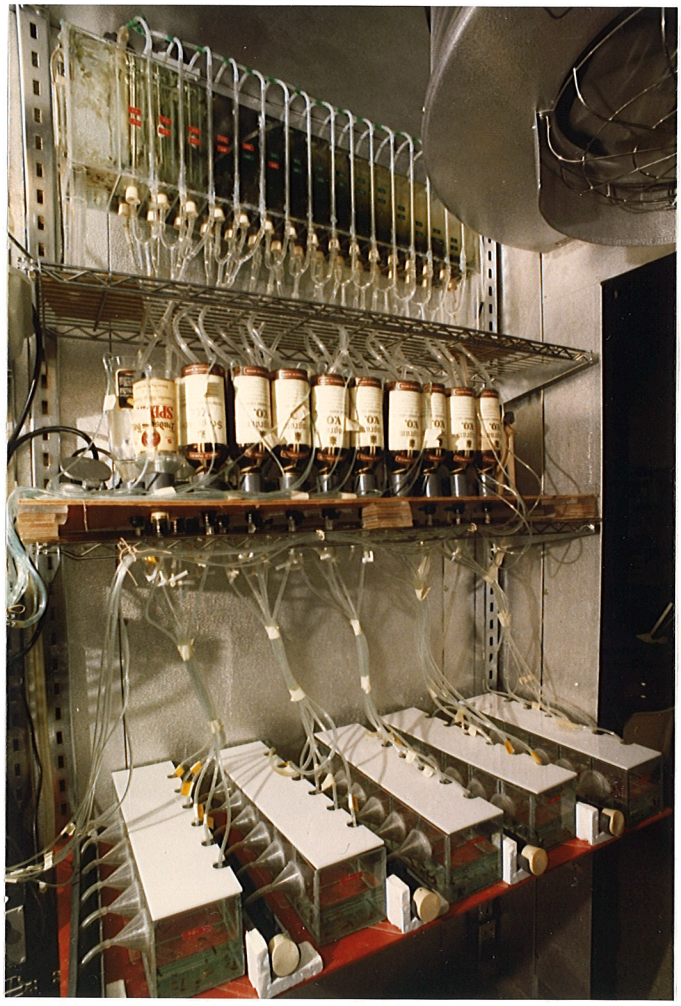

Plate 2. Right side of dosing apparatus, including water metering cells, mixing chambers, and test cells.

float went up, and the peristaltic pump stopped. At this point the overflow chamber, the metering-cells and then the mixing chambers emptied. When the float went down again, both the peristaltic pump dispensing water and the pump(s) dispensing toxicant started their cycles. The stock solutions were dispensed for an exact length of time with a Carlo-Erba multi-channel constant speed peristaltic pump, controlled with a Potter and Brumfield time delay interval-on adjustable timer.

Two-litre bottles of stock solutions, containing one or both of the ions examined in each experiment, were mixed to 30 times their desired strength before each experiment. The $MgSO_4$ used was a commercial Epsom salt that was weighed as a dry salt and mixed into the stock solution. Other ions were dispensed from stock solutions of higher measured concentrations. Tubing from the peristaltic pump was randomly inserted into stock solution bottles, hence the pattern of toxicants dispensed into the test chambers was also randomized. The final dilution rate depended on combined differences in the sizes of water-metering cells, the ages of the peristaltic tubing, the lengths and thus the resistance of tubing, and the order and speed in which the water-metering cells emptied. The dilution rates of the stock solution into each test cell were calibrated between experiments in the following manner. A salt solution of known concentration was dispensed into all test cells in the system for at least 5 cycles of the system, conductivities were measured in the test cells, and dilution was calculated by comparing the conductivities to a standard curve of conductivity vs. dilution.

Dilution generally ranged between 1/25 to 1/35, although 1/15 and 1/45 was possible when the peristaltic pump tubing had worn thin. Rates were consistent within cells within experiments.

Stock solutions of the metals, prepared before the 1 in 30 dilution, were prepared in a chemistry laboratory and were analyzed for exact concentrations. Chemical concentrations in the test cells were confirmed analytically on several occasions, and were correct to within 5% of the calculated concentrations. The concentrations of the metals and the exhange rates were believed to be sufficiently high that the thiosulphate was not a major factor in neutalizing their effects. pH changes were neglibible. Preliminary experiments with NaCl and $NaSO_4$ showed that the anions were not toxic even at considerably higher concentrations than used in the experiments.

Each possible two-chemical combination of the ions Cu^{++}, K^{+}, Cd^{++}, Mg^{++}, and Zn^{++} was tested in at least one experiment. Two-toxicant-combinations tested in the main part of the experimental series (Experiments Cu-K-1 to Zn-K-1, September 1983 to March 1984, Table 2) were tested in the following order: Cu + K; Cu + Cd; Cd + Zn; Zn + Mg; Mg + K; K + Cd; Cd + Mg; Mg + Cu; Cu + Zn; and Zn + K. This order allowed information from the previous experiment to be used in setting the ranges for the next, and also ensured that the tests of each chemical were spread out over time. Additional toxicity tests on individual toxicants were done several times in order to make decisions about experimental ranges or design. These are not tabulated or presented. Also, some of the experiments in the above series were

repeated if the chosen ranges did not produce a sufficient variation in responses or if there was too much or too little mortality.

It was initially attempted to use a central composite experimental design (Box and Hunter 1957), with additional central and control values, to allocate concentrations of the two toxicants tested. It was also attempted to use experimental concentrations which yielded between 0 and 80% mortality in 96 hours for each toxicant concentration. However, due to the mechanical complications explained above, an experimental design was not preserved. The experiments were far from balanced, but mixtures with different ratios of toxicant concentration ratios were still tested, thus avoiding the possible interpretational mistakes which can be made with experiments with equitoxic mixtures of concentrations. The resultant experimental designs can be seen on Tables D1 to D18 (Appendix D).

Gammaraus lacustris lacustris, the test organism (Plate 3), is a common freshwater amphipod which lives in permanent bodies of water in northern temperate climate of North America and Eurasia (Bousfield 1958). It is recommended as a toxicological test species by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). It has been used for a variety of toxicological tests (review in de March 1980).

Adult sized Gammarus lacustris were collected from Lake 101 (Nora Lake) near Erickson, Manitoba (50^{o} 30' W, 100^{o} 10' W) in the autumns of 1982 and 1983. Gammarus occurs at extremely high densities in this lake, so that simple dip-netting from the substrate or from the sides of a net

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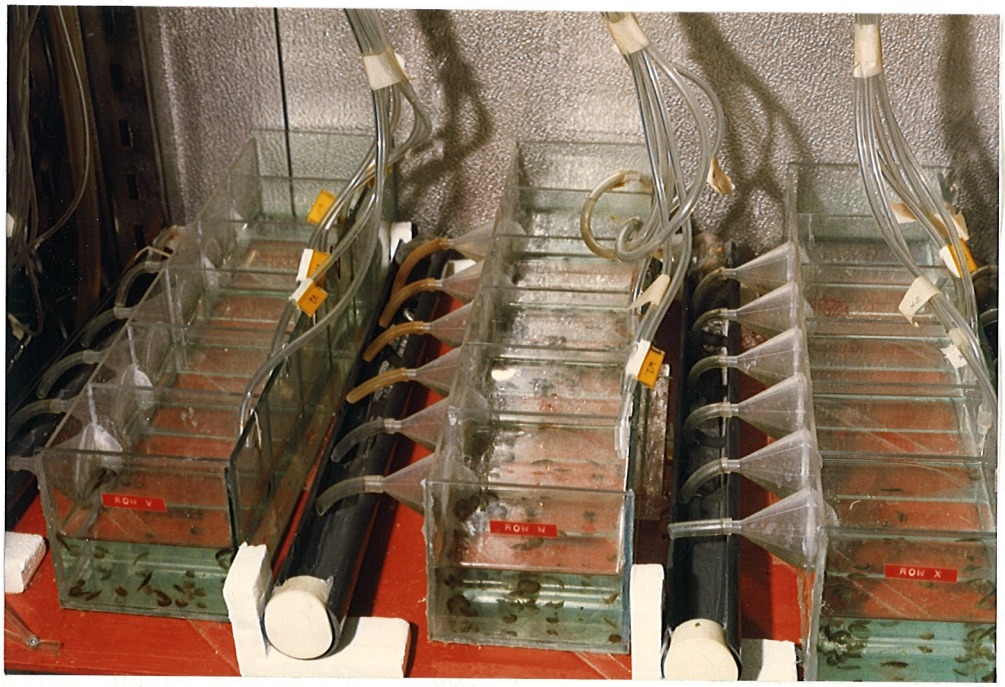

Plate 3. Test cells with Gammarus lacustris.

yielded thousands of animals with little effort. These animals were then kept in flow-through aquaria at temperatures near 5^{o} C, and at a 16 hour daylength to prevent sexual maturation of females (de March 1982). They were fed ad libitum three times a week. Details of normal culture conditions are described in de March (1980). In the spring of 1984, numerous electrical problems in the Freshwater Institute environmental chambers caused intermittent heating of the culture tanks, up to 20^{o} C, and periods of darkness, which induced reproduction. The increasing control mortality during the series of experiments done during that time is thus believed to be due to accelerated aging caused by exposure to warm temperatures. Adults had reproduced and died by one month after the last experiment. This amount of fluctuation is normal on the shores of shallow prairie lakes.

Animals were acclimated to the test temperature of 15^{o} C over 24 hours before each experiment. Animals were then spooned into beakers, five at a time, randomizing the order of the beakers after each five animals, up to a total of at approximately 30 animals per beaker in the main part of the experimental series. The contents of these beakers were then randomly distributed to the test chambers, in which the toxicant was already at the desired levels. Experiments were done with constant illumination to avoid diurnal effects.

Dead animals were counted and removed at regular intervals, at least once every 12 hours, for approximately five days, but sometimes only three, and sometimes as many as eight, depending on the mortality rates. Animals were counted more often, and the length of the experiment shortened, if animals died rapidly.

2.2. Statistical Methods

SAS programming statements (SAS Institute 1979) sufficient to demonstrate the exact statistical methods used are given in Appendix B.

Curves of cumulative % mortality against time in individual test cells in all experiments were generally S-shaped, due to a slight lag at the beginning of each experiment, a period of steady mortality, and then the levelling off to an asymptotic level after which no more mortality occurred. The logit transformation did not straighten out the response curve as expected, but produced an asymptotic curve. Thus, the results in each test cell could be described by the following equation.

$$\text{logit}(P) = a*HR + b*HR^2 + c + \epsilon, \qquad [17],$$

in which P = the cumulative % mortality, logit(P) = $\underline{\ln}(P/(1-P))$, HR is the elapsed time in hours, a, b, c, are fitted regression coefficients, and ϵ is the expected normal error. Points with 0 and 100% mortality were not included in the calculation of Equation [17]. If mortality was delayed or if all animals died rapidly, equations were often based on fewer data points than the number of observations. If no mortality occurred in a test cell, Equation [17] was assumed to have only an intercept representing the death of 1/2 animal, that is, logit(P) = c = $\underline{\ln}(0.5/(T-0.5))$, in which T was the total number of animals in the test cell (Ashton 1972).

A number of summary statistics describing the response(s) in each test cell could be calculated from Equation [17]. Among these were regression coefficients, expected responses at given times, mean mortality times, rates, asymptotes and inflection points. Two of these summary statistics, Q96, the predicted survival at 96 hours in a test cell, or Q48, the predicted survival at 48 hours for experiments shorter than 96 hours, and also the corresponding logits of either Q96 or Q48, were described in terms of toxicant concentrations in models describing various modes of joint action. All response surfaces describing the effects of two toxicants were of a form such that the effects of a single toxicant were described as follows:

$$Q96 \text{ or } Q48 = a*C + b*C^2 + k \text{ , and} \qquad [18]$$

$$\text{logit}(P96) \text{ or } \text{logit}(P48) = c*C + d*C^2 + e \text{ .} \qquad [19].$$

The response surfaces consistent with the definitions of various modes of joint action are shown in Table 1.

It would have been possible to use an equations other than [18] and [19] as underlying models. This was done in earlier exploratory stages, but two trends became obvious. 1) Any other three-parameter-models described results with a similar degree of fit as the above. 2) Any four-parameter-models described the results better, but when such models were extended to multivariate forms to incorporate definitions for various modes of joint action, similarly to Table 1, the models described data almost perfectly. It was felt that such models allowed

Table 1. Equations for different modes of joint action. R is the response, either Q96, Q48, logit(P48) or logit(P96). The coefficients a to k, r, r1 and r2 are constants. The codes for the equations are as in Table 4.

Code	Description and Equations
	RESPONSE TO TOXICANTS INDIVIDUALLY:
Tox1	R = a*C1 + b*C1*C1 + k.
Tox2	R = c*C2 + d*C2*C2 + k.
	JOINT EFFECTS MODELS:
	Additive effects:
5Par	R = a*C1 + b*C1*C1 + c*C2 + d*C2*C2 + k.
	Additive effects with 2 r's: r1 applies only if toxicant 1 is present and r2 only if toxicant 2 is present. Both can take on any values.
5Par+2r	R = (a*C1 + b*C1*C1) * (1 + r2) + (c*C2 + d*C2*C2) * (1 + r1) + k.
	Synergistic Effects:
6Par	R = a*C1 + b*C1*C1 + c*C2 + d*C2*C2 + e*C1*C2 + k.
9Par	R = a*C1 + b*C1*C1 + c*C2 + d*C2*C2 + e*C1*C2 + f*C1*C2*C2 + g*C1*C1*C2 + h*C1*C1*C2*C2 + k.
	SIMPLE SIMILAR ACTION:
SSA	$R = a*C1 + b*C2 + j*(a*C1 + b*C2)^2 + k.$
	Simple similar action with one class variable: r, between -1 and 1, applies only if both toxicants are present.
SSA+1r	$R = (a*C1+b*C2) + j*(a*C1+b*C2)^2 + k) * (1-r) + r*k.$
	INDEPENDENT JOINT ACTION:
IJA (r=0)	Q = (a*C1 + b*C1*C1 + 1) * (c*C2 + d*C2*C2 + 1) * k.
IJA (r=0)	logit(P) = ln(L1*L2/L3 - 1), in which L1 = EXP(a*C1 + b*C1*C1 + k) + 1, L2 = EXP(c*C2 + d*C2*C2 + k) + 1, and L3 = EXP(k) + 1. EXP specifies expression raised to the natural base of logarithms, e.
	Independent joint action with one class variable: (r, between -1 and 1, applies only if both toxicants are present).
IJA+1r	Q = (a*C1+b*C1*C1+1) * (c*C2+d*C2*C2+1) * k*(1-r) + r*k.
	COMPLETE CORRELATION OF SUSCEPTIBILITIES:
IJA (r=0)	Q = Minimum of (a*C1 + b*C1*C1 + k) and (c*C2 + d*C2*C2 + k).
	logit(P) = Maximum of (a*C1 + b*C1*C1 + k) and (c*C2 + d*C2*C2 + k).

several inflection points in a response surface, and thus did not necessarily describe monotonically increasing responses as expected with lethal levels of toxicants.

To test for simple similar action, models in which C1 was replaced by C1 + f* C2 were tested (SSA, Table 1). To test for completely correlated effects, the minimum of such two functions (if the response was Q), or the maximum of such two functions (if the response was logit(P)), whichever was appropriate at any point on the response surface, was fitted (IJA (r=1), Table 1). To test for independent joint action with no correlation of tolerances in the model describing Q, two equations describing Q were multiplied together to obtain an expression for joint survivorship (IJA (r=0), Table 1). To test for independent joint action in the model describing logit(P) a) equations describing the response to individual toxicants or describing the control response were solved for component Q's, that is for Q1, Q2, and Q0. b) These three Q's were multiplied together to obtain an expression for predicted joint survival, and c) an equation for $\underline{\ln}((1-Q1*Q2*Q0)/Q1*Q2*Q0)$, representing the logit of the overall response was found.

The applicability of class variables such as the correlation between susceptibilities "r" and Koenemann's (1981) Mixture Toxicity Index was examined for a limited number of models describing Q. In all three cases it was assumed that the component Q's , or a joint Q, could be modified by Q' = Q*(1-r) + r, as initially defined by Bliss (1939). In one case "r" was applied to the joint probability, Q1*Q2 (IJA+1r, Table 1). In another "r1" was applied to Q2, and "r2" to Q1 in an

additive effects model (5Par+2r, Table 1). In a third case, the entire equation describing simple similar action was modified by Q'=Q*(1-r) + r*Q0 (SSA+1r, Table 1).

All linear curve fitting was done with PROC GLM in SAS (SAS Institute 1979) (Appendix B). All non-linear curve fitting methods were done with PROC NLIN. In some cases the equations had to be expressed differently or the starting ranges had to be changed from those given in Appendix B to obtain convergence. Since nonlinear models are not necessarily expected to have an intercept, PROC NLIN does not print out R^2 values based on corrected sums of squares. Thus R^2 values comparable to those obtained from other linear procedures such as GLM were obtained by calculating (1-(sum of squares for error)/(corrected total sum of squares)).

All data were also examined as linear models to examine the applicability of joint effects models. A 5-parameter model with no interactive terms (5Par, Table 1), and 6- and 9-parameter models with either 1 or 4 interactive terms (6Par and 9Par, Table 1) were fitted to the results of each experiment. The significance of additional terms as a unit in the 6- and 9-parameter models compared to the 5-parameter model was tested with the likelihood ratio test statistic (Graybill 1976).

It was also desirable to obtain the simplest adequate equation with all coefficients significant at the alpha = 0.05 level to describe the results of each experiment. The STEPWISE procedure in the SAS Statistical Package (SAS Institute, 1979), was used in the following

manner: 1) The linear concentration values of both toxicants were initially forced into the equation, 2) additional terms were added by stepwise (forward and backward) selection, and 3) if after this selection, the initial forced concentration terms did not remain significant at the alpha <= 0.05 level, based on Type III sums of squares, then they were eliminated in the final expression of the equation.

The variance of Q48 or Q96 about predicted terms in most models was was sufficiently close to P*Q that the inverse of this term was used as a weighting factor for the error variance (Appendix B).

RESULTS AND DISCUSSION

General information about the 18 experiments including code names, dates, ions and concentration ranges used, number of test cells, and mean number of organisms per test cell, are given in Table 2.

3.1. Responses in Individual Test Cells

In most test cells, mortalities were not observed immediately, but after a time lag dependent on the strength of the toxicant. Then a period of intense mortality usually occurred, most often on days 2 and 3. The mortality curves also reached an upper asymptote, that is, relatively few mortalities were observed after 96 or 120 hours. Thus an s-shaped response curve, tapering off at the far end, was described, thus justifying Equation [17] as a description of the response.

Dead animals were easily recognized in test cells with Cu^{++}, Cd^{++}, and Zn^{++} because they became rigid and elongated near the point of death. Animals in K^{+} had to be prodded to determine whether or not they were still responsive to determine mortality. Dead animals were counted and removed frequently enough that very little cannibalism occurred. However, in some cases, only heads were counted.

The equations describing the S-shaped temporal response curve (Equation 17) in individual test cells were generally fit with a high degree of predictability, with R^2 values > 0.95 in 267 out of 402 fitted regression lines, and > 0.90 in 353 of 402. The errors about these

Table 2. General information about experiments.

Experiment	Date	Elements tested and ranges (mg/L)	No. Test Cells	Mean No. animals/cell
Cu-Cd-1	24/03/83	Cu 0.0520 - 0.140 Cd 0.0218 - 0.0610	21	28
Cu-Cd-2	11/04/83	Cu 0.0216 - 0.202 Cd 0.00970 - 0.0585	28	40
Cd-K-1	25/04/83	Cd 0.0121 - 0.0863 K 3.59 - 26.6	28	45
Cu-Zn-1	03/06/83	Cu 0.00992 - 0.0699 Zn 0.140 - 1.34	30	33
Cu-Zn-2	16/06/83	Cu 8.00E-5 - 8.69E-4 Zn 0.00111 - 0.00688	26	33
Cu-K-1	20/09/83	Cu 0.0134 - 0.0811 K 4.25 - 35.23	21	19
Cu-Cd-3	02/10/83	Cu 0.0271 - 0.127 Cd 0.00542 - 0.0681	22	13
Cd-Zn-1	12/10/83	Cd 0.00980 - 0.0542 Zn 0.0245 - 0.431	23	15
Cd-Zn-2	24/10/83	Cd 0.00943 - 0.0542 Zn 0.0148 - 0.431	22	23
Zn-Mg-1	22/11/83	Zn 0.0433 - 1.52 Mg 19.5 - 178	24	38
Zn-Mg-2	08/12/83	Zn 0.0730 - 1.06 Mg 21.8 - 164	23	33
Mg-K-1	03/01/84	Mg 18.4 - 122 K 2.53 - 12.1	23	30
Mg-K-2	11/01/84	Mg 20.3 - 150 K 7.60 - 31.3	24	28
Cd-K-2	16/01/84	Cd 0.00189 - 0.0406 K 4.46 - 39.0	22	30
Cd-Mg-1	25/01/84	Cd 0.00889 - 0.110 Mg 34.4 - 190	23	29
Cu-Mg-1	03/02/84	Cu 0.0540 - 0.228 Mg 46.5 - 283	22	29
Cu-Zn-3	10/02/84	Cu 0.155 - 0.388 Zn 0.411 - 1.70	24	32
Zn-K-1	23/02/84	Zn 0.0529 - 0.233 K 12.9 - 36.9	23	31

predicted lines were smaller than the errors in the models relating summary statistics to toxicant concentrations, and were therefore ignored from this point.

Matrices of correlations coefficients between several of the calculated summary statistics and toxicant concentrations are shown in Tables C1 to C18 in Appendix C. A high degree of correlation between the three regression coefficients within experiments suggested that response curves with the same slopes or intercepts generally had the same overall shape. This fact, as well as the results of exploratory work relating these alternative summary statistics from these curves to toxicant concentrations, led to the conclusion that one summary statistic from each test cell would sufficiently describe the overall results of that test cell.

Two summary statistics, the predicted survival at 96 hours (Q96), or at 48 hours (Q48) in three experiments which were terminated before 96 hours, and the predicted time to 50% mortality (T50), were in general more strongly correlated with test concentrations than other summary statistics (Tables C1 to C18, Appendix C). Q48 or Q96 and the corresponding logit transformation, $\ln((1-Q)/Q)) = \text{logit}(P)$, were chosen as summary statistics to be used to examine the applicability of alternative models. T50 was not used since it was not available for test cells in which 50% mortality was not reached.

3.2. Acute Toxicity of Individual Ions

Table 5 has simple descriptive equations, the choice of which will be explained in Section 3.33, for the variables Q48 or Q96 for each experiment in terms of concentrations of both toxicants. The interpolated 96- or 48- hour LC50's for the ions Cu^{++}, Cd^{++}, Zn^{++}, K^{+}, and Mg^{++} obtained from these equations are given in Table 3. All LC50 values are close to average values reported in the literature. The between-experiment variability between LC50's for the same ions is small compared to ranges in the literature and reported within-laboratory variability.

3.3. Modelling Results for Mixtures

R^2 values describing the goodness of fit of Q96 or Q48, and the corresponding logits, to the different chosen models in Table 1 are given in Table 4. These R^2 values are relative measures indicating the extent to which the raw data can be described in terms of different models. The actual values of Q48 or Q96 used in these models are shown plotted against toxicant concentrations in Figures D1 to D18 (Appendix D).

The models describing "Q" generally had slightly larger R^2 values that those describing "logit(P)" (first and second page, Table 4). Nevertheless the two parts of Table 4 generally have similar patterns of R^2 values. The two different dependent variables are expected to have a

Table 3. Predicted 96- or 48- hour LC50's (mg/L) for individual chemicals estimated from the simple equations on Table 5. Extrapolations are marked with an asterisk (*). The first tabulated LC50 is estimated by setting Q=0.5, the second, by setting Q=1/2 of control survivorship (the intercept), this correction equivalent to Abbott's (1925) formula for correcting mortality.

Chemical	48- or 96- hour		LC50's		Obtained from Experiment
Cu++	96		0.0938	0.128	Cu-Cd-1
	96		0.0272	0.197	Cu-Cd-2
	96		0.0479	0.0589	Cu-Cd-3
	96			* 0.00215	Cu-Zn-2
	96		0.261	0.411	Cu-Zn-3
	48	*	0.0953	0.166	Cu-Zn-1
Cd++	96		0.0314	0.0586	Cu-Cd-1
	96		0.00971	* 0.0705	Cu-Cd-2
	96		0.0264	0.0326	Cu-Cd-3
	96		0.0314		Cd-Zn-1
	96	*	0.0663	0.0690	Cd-Zn-2
	96		0.0431	0.0773	Cd-K-1
	96	*	0.0414	0.0445	Cd-K-2
Zn++	96			* 0.127	Cu-Zn-2
	96		0.872	1.38	Cu-Zn-3
	96		0.422	0.507	Cd-Zn-1
	96		0.403	0.419	Cd-Zn-2
	96	*	0.461	0.448	Zn-Mg-1
	48		0.575	1.00	Cu-Zn-1
	48	*	0.233	0.368	Zn-K-1
K+	96		15.0	26.8	Cd-K-1
	96	*	20.2	21.6	Mg-K-1
	48		6.68	11.2	Zn-K-1
Mg++	96	*	414	461	Cu-Mg-1

Table 4. R^2 values obtained by describing the results of 18 experiments by the models in Table 1. If 6- and 9-parameter models account for a significantly larger sums of squares than the 5-parameter model at the alpha <= 0.05 level, then they are marked with an asterisk (*).

Experiment	Model Q =										
Code:	Tox1	Tox2	9Par	6Par	5Par	5Par +2r	SSA	SSA +1r	IJA (r=0)	IJA +1r	IJA (r=1)
parameters:	3	3	9	6	5	7	4	5	5	6	5
Cu-Cd-1	.213	.228	.815*	.739	.677	.801	.670	.730	.633	.746	.427
Cu-Cd-2	.213	.138	.715*	.598	.596	.611	.590	.598	.595	.595	.486
Cu-Cd-3	.669	.762	.964*	.944*	.918	.977	.938	.964	.960	.965	.957
Cu-Zn-1	.012	.488	.712*	.661*	.593	.663	.602	.656	.612	.665	.680
Cu-Zn-2	.090	.276	.606*	.415	.405	.533	.387	.429	.405	.424	.334
Cu-Zn-3	.543	.768	.959*	.924	.922	.934	.911	.936	.875	.937	.767
Cd-Zn-1	.695	.652	.879	.871	.870	.889	.816	.822	.865	.869	.842
Cd-Zn-2	.458	.726	.836	.812	.785	.827	.809	.818	.799	.821	.844
Cu-K-1	.363	.485	.744	.709	.655	.688	.703	.710	.628	.676	.558
Cd-K-1	.327	.259	.683	.648	.643	.693	.408	.703	.643	.677	.623
Cd-K-2	.484	.105	.786*	.696*	.596	.717	.489	.520	.589	.614	.737
Zn-K-1	.016	.937	.974	.957*	.938	.969	.947	.960	.937	.964	.937
Mg-K-1	.365	.597	.738	.698	.688	.716	.662	.675	.681	.684	.623
Mg-K-2	.642	.467	.858*	.752	.725	.824	.658	.758	.671	.798	.649
Cu-Mg-1	.173	.676	.772	.735	.734	.759	.734	.743	.733	.749	.740
Cd-Mg-1	.231	.096	.489	.364	.342	.406	.231	.407	.340	.406	.231
Zn-Mg-1	.542	.006	.824*	.707*	.554	.803	.694	.809	.552	.797	.552
Zn-Mg-2	.599	.248	.843*	.733*	.653	.815	.610	.772	.651	.779	.658

Table 4, continued. Table of R^2 values obtained by describing the results of all experiments in terms of models described in Table 1.

Experiment	Model logit(P)=							
Code:	Tox1	Tox2	9Par	6Par	5Par	SSA	IJA (r=0)	IJA (r=1)
parameters:	3	3	9	6	5	4	5	5
Cu-Cd-1	.209	.221	.804*	.725	.663	.650	.621	.443
Cu-Cd-2	.196	.148	.698*	.576	.576	.548	.573	.196
Cu-Cd-3	.367	.667	.914*	.855*	.828	.801	.865	.667
Cu-Zn-1	.009	.455	.700*	.648*	.566	.576	.594	.455
Cu-Zn-2	.091	.278	.614*	.422	.409	.391	.409	.091
Cu-Zn-3	.415	.670	.942*	.911	.886	.897	.826	.568
Cd-Zn-1	.550	.528	.806	.789	.787	.706	.789	.764
Cd-Zn-2	.223	.538	.831*	.713*	.640	.712	.790	.538
Cu-K-1	.274	.385	.605	.557	.511	.542	.467	.274
Cd-K-1	.332	.252	.673	.641	.636	.414	.635	.330
Cd-K-2	.332	.277	.681*	.518	.462	.342	.464	.578
Zn-K-1	.134	.833	.898	.858	.833	.834	.841	.860
Mg-K-1	.332	.572	.717	.704	.701	.666	.704	.572
Mg-K-2	.407	.357	.651	.547	.546	.448	.490	.357
Cu-Mg-1	.101	.595	.678	.637	.637	.637	.630	.101
Cd-Mg-1	.188	.060	.435	.335	.307	.189	.188	.188
Zn-Mg-1	.579	.007	.690	.688	.630	.679	.592	.579
Zn-Mg-2	.361	.078	.534	.465	.383	.424	.453	.361

different variance structure hence a difference such as this is not surprising. In the models describing Q, both large and small values are more heavily weighted in the analysis through the weighting factor. Since the experiments were not balanced, the response surface was possibly drawn through outlying values, resulting in an overall better fit.

Differences in the magnitude of R^2 values between experiments can be attributed primarily to the range of responses obtained in different experiments. There was more variability in experiments in which a smaller range of responses was observed. Response ranges were often smaller or larger than expected because final concentrations were not as expected.

Differences between R^2 values for diffferent models within experiments related to the number of fitted parameters in the models and to a lesser extent, the ability of models to describe the data. It is obvious in Table 4 that models with more parameters and with fewer restrictions on the ranges of the parameters described results better. Thus comparisons between the applicability of different models can be made only between models of approximately the same complexity or between models which can be compared using statistical criteria.

3.31. Traditional Models

In the joint effects models described in the next Section 3.32, an average R^2 change of 0.07 was associated with statistically significant improvement (alpha <= 0.05) in the predictive capacity of a larger model over the 5-parameter model. This value can be used as a very rough guide for the significance of differences between two non-linear models such as those for SSA and IJA.

R^2 values for the two most commonly scrutinized modes of action, simple similar and independent joint action (Columns SSA and IJA (r=0), Table 4) are similar in 14 of 18 experiments. The R^2 value for IJA is often slightly larger than that for SSA, possibly due to more parameters in the model. Neither SSA nor IJA can be rejected as a significant model describing the data in any of these 14 experiments. These data support what might have been expected given previous theoretical considerations - that the two modes of action defined in this model are similar. None of the four experiments in which the two R^2 values differ more strongly (Experiments Cd-K-1, Cd-K-2, Cd-Mg-1, and Zn-Mg-1) were experiments with two metals.

R^2 values for models describing complete correlation of tolerances (Column IJA (r=1), Table 4) are generally lower than those for other modes of action. It was shown in Appendix A that this model is not as similar to the above two modes of action as they are to each other. It is thus possible that the applicability of this model compared to others can be recognized. Independent joint action with complete correlation of

tolerances is thus not strongly supported as a mode of action by these experimental results.

The applicability of the constant "r", ranging from -1 to 1, was tested in the two models labelled SSA+1r and IJA+1r (Tables 1 and 4). Slight increases in the R^2 values showed that such parameters improved the fit to the models. If this change were significant, it would describe the case in which the presence or absence of a compound, as well as the concentration, is important in describing the response surface. The applicability of two class variables not restricted to the -1 to 1 range was tested in the 5Par+2r models (Tables 1 and 4). Again, increases in R^2 values showed that two such indices described results better than one or none. The R^2 values in these columns were not larger than those for other models with the same number of parameters, hence the applicability of class variables to describe modes of joint action is not demonstrated.

3.32. Joint Effects Models

The R^2 values for the additive effects models (5Par, Table 4) are comparable to those describing simple similar or independent joint action in the same experiments. The fact that the additive effects models are as applicable as the other two commonly scrutinized modes of action suggests that "similar action" in general, as defined by Bliss (1939), that is, the case in which the effects of chemicals can be described in terms of each other in any manner, can be considered as a

basic mode of action in models describing the joint effects of toxicants. In the particular additive effects models used here, the relative effects of $a*C1 + b*C1^2$ and $c*C2 + d*C2^2$ can be described as a ratio.

"Simple similar action" and "complex similar action" could thus be subcategories of "additive effects" or "similar action" for a given reponse variable in an overall classification system.

The 6- and 9-parameter models (6Par, 9Par, Tables 1 and 4) with a significant increase in the sums of squares accounted for at the alpha = 0.05 level compared to the 5-parameter (5Par) model are marked with an asterisk (*). The significant addition of any other terms containing both C1 and C2 would indicate more-than- or less-than-additive effects for the response variable in the model. The 6-parameter and(or) the 9-parameter joint effects models describing Q have a significantly better predictive capacity than the 5-parameter models in 11 out of 18 experiments (Table 4). Eight of these 11 are metal-metal experiments if Mg is considered to be as a metal. In the 7 of 18 cases in which the additive effects model is sufficient, 5 of 7 are experiments with K or Mg, and 2 are with Zn-Cd mixtures. A similar pattern exists in models describing logit(P). Seven of 8 cases in which the expanded models are a significant inprovement are metal-metal experiments. The R^2 values associated with these expanded joint effects models are conspicuously larger than those associated with the other common modes of action previously discussed.

In 8 of 12 models describing the effects of K, the additive effects model appears sufficient. In 5 of 12 models with Mg, the other ion accounts for a large part of the variability, and it can be argued that no mode of joint action is strongly supported. In the remaining 6 models describing Q, the 9-parameter model is significantly better than the 5-parameter model in 3 cases; in 6 models describing logit(P), the additive effects model seems to be an adequate description of experimental results, but not different from simple similar action and independent joint action.

Of 16 models describing the effects of Zn, the expanded models have a significantly better predictive capacity than the 5-parameter model in 10 cases. The exceptions are the two Cd-Zn experiments, which are adequately described by the additive effects models.

Expanded models seem to be more applicable in describing the joint effects of metals than in describing combinations with only one or no metals.

3.33. Simple Descriptive Equations

In the above models, any number of estimated coefficients may not be significant even though the model in general describes the data well. Because of this, it was desirable to determine whether simpler equations would adequately describe data, this perhaps giving information about which models are a better basis for expansion. Also, it is possible that some of the models examined were over-parameterized in view of the

unbalanced experimental design. The examination of efficient descriptive models, containing only fitted coefficients significantly different from 0, and containing independent variables of a simple structure in preference to more complex ones if their predictive capacities were equal, was a desirable objective at this point. The final simple equations chosen to describe Q, using the stepwise selection procedure described in the Section 2.2 are shown in Table 5. Figures D1 to D18 in Appendix D show the raw data as well as isolines of expected responses obtained from these response curves plotted against toxicant concentrations.

In 6 of 8 experiments with combinations of Cu, Cd, and Zn, additive effects are suggested (Table 5). Examination of the graphs for these (Figures D1 to D18, Appendix D) shows that that the isolines are almost straight even when interactive terms are in the descriptive equation. Thus additive effects for the three metals are supported by these models.

Four of six mixture experiments with K are best described by less-than- or more-than-additive effects, as can be seen from cross-products in equations in Table 5. In three of these six experiments (Cu-K-1, Cd-K-2, and Mg-K-2) the data either have excessive variability, or the right types of models defining complex interactive effects, have not been examined. The corresponding Figures D9, D11, and D14 (Appendix D) show that there are similar types of outliers in all three experiments, namely very low survivorship in ranges where it is not predicted. Only further research would show whether this is a real

Table 5. Best simple equations describing Q48 or Q96, the probability of survival at 48 or 96 hours in terms of toxicant concentrations (mg/L ion). MSE = means square for error in the regression model, n = sample size, df = degrees of freedom for the Chi-square test. In the Chi-square test of goodness of fit, if (1-alpha) > 0.95 then then hypothesis that deviations are due to chance can be rejected.

Experiment	Equation Q96 =	R^2	n
Cu-Cd-1	= 0.683 - 20.8*Cu^2 - 5.83*Cd.	0.667	21
Cu-Cd-2	= 0.537 - 1.36*Cu - 3.81*Cd.	0.590	28
Cu-Cd-3	= 0.840 - 7.13*Cu - 12.9*Cd + 111*Cu*Cd.	0.919	22
Cu-Zn-1	Q48 = 0.702 - 2.12*Cu - 0.351*Zn.	0.586	30
Cu-Zn-2	= 0.439 - 102*Cu - 17.3*Zn.	0.345	26
Cu-Zn-3	= 0.732 - 0.890*Cu - 0.266*Zn.	0.912	24
Cd-Zn-1	= 0.857 - 19.0*Cd + 243*Cd^2 -0.846*Zn.	0.860	23
Cd-Zn-2	= 0.963 - 6.98*Cd - 1.15*Zn + 20.9*Cd*Zn.	0.802	22
Cu-K-1	= 0.926 - 0.279*Cu*K.	0.636	21
Cd-K-1	= 0.592 - 49.6*Cd^2 - 0.000411*K^2.	0.633	28
Cd-K-2	= 0.925 + 14.8*Cd - 605*Cd^2.	0.485	22
Zn-K-1	Q48 = 0.731 - 0.992*Zn - 0.0376*K + 0.000453*K^2 + 0.0407*Zn*K.	0.956	23
Mg-K-1	= 0.939 - 0.0217*K - 0.000805*Mg.	0.662	23
Mg-K-2	Q48 = 1.03 - 0.0127*K -0.0129*Mg + 0.0000727*Mg^2.	0.723	24
Cu-Mg-1	= 0.907 - 0.348*Cu -0.000984*Mg.	0.734	22
Cd-Mg-1	= 0.956 -0.435*Cd.	0.220	23
Zn-Mg-1	= 0.982 - 0.127*Zn^2 + 0.000696*Zn*Mg.	0.668	24
Zn-Mg-2	= 1.00 - 0.265*Zn + 0.00124*Mg - 0.0000123*Mg^2 + 0.00153*Zn*Mg.	0.733	23

Table 5, continued. Best simple equations describing Q48 or Q96, the probability of survival at 48 or 96 hours in terms of toxicant concentrations (mg/L ion).

Experiment	MSE	Chi-square	df	1 - alpha
Cu-Cd-1	0.02686	16.996	19	0.410
Cu-Cd-2	0.02015	19.485	26	0.185
Cu-Cd-3	0.1284	23.676	19	0.791
Cu-Zn-1	0.04438	37.964	28	0.901
Cu-Zn-2	0.01483	10.786	24	0.00954
Cu-Zn-3	0.03515	18.662	22	0.334
Cd-Zn-1	0.07806	24.118	20	0.763
Cd-Zn-2	0.05906	23.488	19	0.783
Cu-K-1	0.1424	73.883	20	1.000
Cd-K-1	0.02492	27.146	26	0.598
Cd-K-2	0.1479	61.136	20	1.000
Zn-K-1	0.03302	17.317	19	0.432
Mg-K-1	0.04425	27.688	21	0.850
Mg-K-2	0.7823	185.59	21	1.000
Cu-Mg-1	0.02359	14.327	20	0.186
Cd-Mg-1	0.01655	16.798	22	0.226
Zn-Mg-1	0.01941	19.576	22	0.391
Zn-Mg-2	0.03982	12.690	19	0.146

effect or experimental flaws.

The joint effects of Cu or Cd with Mg on the probability of survival are additive, while the joint effects of Zn and Mg are less-than-additive.

In 7 experiments with Zn, 3 describe less-than-additive effects, once with Cd and twice with Mg. Additive effects are described in other mixtures.

These observations are consistent with the physiological events described in Section 1.5. Only copper and cadmium and perhaps magnesium are expected to act similarly, and these in fact have additive effect on the probability of survival in several experiments. Magnesium is associated with both increased and decreased mortality, perhaps competing with other heavy metals at low concentrations and exerting toxic effects at high ones. Zinc is expected to act as a metal, competing with other metal ions at active sites, but also affect the blood buffer system. In this study, the joint effects of zinc and other metals on the probability of response are additive, while the effects of zinc and other ions are variable. No results are inconsistent with previously reported results.

The modes of action suggested by these descriptive equations are not the same as suggested in the last section. In the last section, metal-metal effects were best described by expanded models, while in this section, they are best described by additive effects models. The latter is the more conservative approach and may be more valid in view of the experimental design.

3.34 Chi-square Tests of Goodness of Fit

Chi-square tests are more stringent criterion of goodness of fit than the testing of model variances if data are quantal. Chi-square tests were done only with the most conservative models describing the data, namely those given in Table 5. These models are usually subsets of the more complex models in Table 4, hence if excess scatter is not demonstrated with these, it would also not be demonstrated with a more complex model. The "observed" value was assumed to be a rounded value of the prediction from the logit(p) vs. time curve (Equation 17) predicted from each test cell. One degree of freedom was removed from the total for each regression coefficient in the equation (Snedecor and Cochran 1967, decision made on the basis of developments on page 247). The Chi-square values for these models (Table 5) show that data did not have excessive scatter except for three mixtures with K. Most other major modes of action could have been confirmed in the other 15 experiments.

3.35. Experimental Results and the Toxic Units Model

This research was motivated by the need to assess the applicability of the toxic units model or similar models suitable for setting standards. In Section 1.41, the relationship between response surfaces and tolerance models was demonstrated, this mathematical relationship being sufficient justification for the use of a tolerance model. The response surfaces described here can be used to demonstrate the

applicability of an expanded toxic units model.

Most of the equations in Table 5 contain simple linear terms, hence in such cases the toxic units model as traditionally used is justified. Other equations have more complex terms, and are therefore associated with more complex tolerance models. The applicability of extended forms of the toxic units model can be assessed by evaluating the credibility of other parameters besides the LC50's estimated by the equations.

Either upper or lower asymptotic values can be calculated from equations which contain both linear and quadratic terms of concentration values. The lower asymptote represents the highest concentration at which no mortality is expected, and the higher asymptote, the lowest concentration causing 100% mortality. These were obtained by solving for inflection points of the response curve. Low asymptotes of Cd^{++} estimated from Experiments Cd-Zn-1 and Cd-K-2 are 0.0391 and 0.0122 mg/L. The latter estimate, obtained with healthier animals, is lower than all reported LC50 values. The upper asymptote for K^{+} is estimated as 41.50 mg/L from Experiment Zn-K-1, also a reasonable estimate given reported ranges of LC50's. Lower asymptotes for Mg, obtained from Experiments Mg-K-2 and Zn-Mg-2 are 88.72 and 50.41 respectively, just below reported toxic ranges. Knowledge of these critical values would merit the use of more complex tolerance models.

The tolerance equation for Experiment Cd-K-2, a model with a lower asymptote, with reference to Equations [10] and [13] would be:

$$1 = Cd/Cd' + (K/K')* (1-K/(2*0.0122))/(1-K'/(2*0.0122)),$$

in which 0.0122 mg/L is the lower tolerance limit.

Less-than-additive effects for the response Q are suggested by the cross-products in experiments Cu-Cd-3 and Cd-Zn-2. The tolerance equation for Experiments Cu-Cd-3, obtained by reference to Equation [15], is:

$$1 = Cu/Cu' + Cd/Cd' - 11.6*Cu*Cd/(SQRT(Cu'*Cd')).$$

Likewise, the constant analogous to -11.6 above, for Experiment Cd-Zn-2 is -7.38. The applicability of these constants cannot be judged since the deviation from additivity has not been quantified in any manner by other authors.

The model for Experiment Cu-K-1 contains only a cross-product, thus only a joint LC50 which is a cross-product, (Cu*K)' can be calculated. The tolerance equation is:

$$1 = (Cu*K)/(Cu*K)'.$$

The value of (Cu*K)' is approximately 1.5 (calculated from Table 5). This is considerable larger than the cross-product of Cu' and K' (Table 3), thus the model estimates lower toxicity than the toxic units model.

The tolerance equations for experiments Zn-K-1, Zn-Mg-1, and Zn-Mg-2 are more complex. A tolerance equation for Experiment Zn-K-1, for example, might be:

$$1 = (Zn/Zn') + (K/K')*(1-K/(2*41.5)/(1-K'/(2*41.5) - (0.0410*Zn*K/Zn'),$$

this particular form of J as in Equation [16].

4.0. SUMMARY AND CONCLUSIONS

The development of satisfactory working models in any scientific discipline is often a long and convoluted process. The field of joint effects modelling is no exception, but perhaps more confused than most due to recent external demand for development of the field.

It is shown in this research that existing theory and models consist of intuitively appealing ideas about variables or about mathematical relationships, but are not logical alternatives. The three modes of action generally cited as reference points, as well as modes of joint action expressed as statistical linear models, are among these incomplete models. None of the underlying assumptions in any of the described modes of action, such as the normal distribution of tolerances, relationships between probabilities of response, or the existence of class variables such as the correlation between susceptibilities "r", have been critically examined. Different modes of action are generally defined within different response surfaces, this fact making comparisons and classifications of modes of joint action extremely difficult. It is shown that the degree of similarity between modes of joint action is dependent on the response surface in which the modes of action are defined. No one response surface has been adopted as one which can be expanded to describe a diversity of types of responses. If one were adopted, some of the traditional modes of action would not be practical classifications. In view of these problems, it is not surprising that data in the past have served only to confirm

preconceived modes of action or demonstrate mathematical methods. Thus decisions about joint action models for acute toxicity data may depend entirely on choices between several intuitively appealing concepts and the practicality of alternative models.

The analysis of data from 18 experiments supports the theoretical arguments that it is difficult to discriminate between modes of action defined within any of the common models. Hence a choice of model depends on intuition and practical considerations.

The linear model decided on in this study is as good as others in terms of predictive capacity, and has practical advantages as well. Types of joint effects can be classified by ratios of terms in the model. The presence or absence of significant terms constitute a number of alternative hypotheses about modes of joint action or joint effects. Linear modelling techniques are easily implemented on many statistical packages. These offer a variety of options which may be of value in particular situations, among these being contrasts, simultaneous models, flexibility in experimental designs which combine analysis of variance and regression techniques, and exploratory techniques such as stepwise regression. The direct relationship of the linear model to tolerance models such as the toxic units concept may be a primary consideration for future work.

The problems encountered in this study are the same as encountered in any modelling exercise. The balances between the complexity, structure, basis in data, and the applicability of existing models; the accuracy, balance, structure, and volume of existing data; and the power

of alternative experimental designs in relation to the theory, must all be considered in choosing models to describe a particular set of events. In taking all of the above into account, the use of simple efficient descriptive models rather than existing models, seemed to be a better alternative. Their main advantage, besides their simplicity, would be that they would allow the evolution of joint effects concepts.

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Appendix A. Similarities between four modes of joint action.

The four most commonly examined modes of joint action are simple similar action (SSA), independent joint action with no correlation in susceptibilities (IJA, r=0), independent joint action with complete correlation of susceptibilities (IJA, r=1) and additive effects for a defined response variable. In the following developments it is assumed that one of the four modes of joint action is the true mode while another is the observed mode, and then correlation between the two is determined.

It is assumed that responses to individual toxicants can be described by:

$$\underline{f}(p_1) = a*C1 + b*C1^2 \quad \text{and} \quad \underline{f}(p_2) = c*C2 + d*C2^2,$$

in which $\underline{f}(p_1)$ and $\underline{f}(p_2)$ are positive non-decreasing functions of the probability of response, C1 and C2 are the concentrations of the toxicants 1 and 2, and a, b, c, and d are numerical constants describing the response. There are no constants in the equations because the control response is assumed to be part of the functions.

Comparisons were made in the following manner. It was assumed that both toxicants were tested individually at a range of concentrations which yielded 40 evenly spaced responses between 1.25 and 98.75 percent response. Expressions in terms of the original probabilities representing the joint responses for the four modes of joint action were

then obtained. The expression for independent joint action with no correlation of susceptibilities (IJA, r=0) is:

$$\underline{f}(p_1+p_2+p_1*p_2) = \underline{f}(1-q_1*q_2).$$

Complete correlated tolerances (IJA, r=1) can be described by:

$$\text{Maximum of } (\underline{f}(p_1), \underline{f}(p_2)),$$

since the functions increases with concentration. The expression for additive effects (ADD) for the response $f(p_j)$ is:

$$\underline{f}(p_j) = \underline{f}(p_1) + \underline{f}(p_2).$$

These three joint responses can be easily calculated from the original probability values.

Simple similar action describes the joint response only if if $b/d=(a/c)^2$ in the Eqaution on the last page. A constant, $z=b/(a^2)=d/(c^2)$, occurs in the equation for the joint response, which has the following form:

$$\begin{aligned}\underline{f}(p_j) &= a*C1 + c*C2 + z*(a*C1 + c*c2)^2 \\ &= a*C1 + c*C2 + z*a^2*C1^2 + z*c^2*C2^2 + 2*a*c*z*C1*C2. \\ &= \underline{f}(p_1) + \underline{f}(p_2) + 2*a*c*z*C1*C2. \\ &= \underline{f}(p_1) + \underline{f}(p_2) \\ &\quad + (z^2/2)*(-1+(1+(4*\underline{f}(p_1)/z))^{1/2})*(-1+(1+(4*\underline{f}(p_2)/z))^{1/2}).\end{aligned}$$

The last expression is obtained by expresssing C1 and C2 from the original two equations in terms of $\underline{f}(p_1)$ and $\underline{f}(p_2)$. The positive root of the quadratic equation solving for C1 and C2 was used because $\underline{f}(p_1)$ and $\underline{f}(p_2)$ increase with concentration. Joint responses for different values

of z can be calculated from the initial probabilities. If z = 0 then simple similar action and additive effects are described by the same model.

Thus there were 1600 (=40*40) predicted joint responses for the two toxicants for each mode of action. Any of the predicted joint responses are equivalent to the simple responses above if the other toxicant is absent.

There are several methods of comparing the responses from the different models. The actual magnitude of the differences can be compared if it is known that one of the modes of action is the true response or if the parameters from the response equations describing $\underline{f}(p_1)$ and $\underline{f}(p_2)$ are exact. Graphs showing the actual magnitudes are shown in de March (in prep.) for the probit(p) vs ln(dose) models. This type of comparison was not believed to be the most realistic for the analyses performed in this study. In experiments in which both individual and joint responses are observed, all data would most likely be described by one response surface representing one mode of action. Thus the actual correlation between predicted values for the four modes of action is of interest.

The matrices of R^2 values obtained by correlating predictions for two response variables, p and logit(p) in the above manner are shown. $\underline{F}(P)$ represents the response to one toxicant, that is either $\underline{f}(p_1)$ or $\underline{f}(p_2)$ above. IJA represents independent joint action with r=0, MAX represents independent joint action with r=1, ADD represents additive effects, SSA(z) describes simple similar action with a given z,

described above.

Matrix of R^2 values.
Response = f(p) = p.

	F(P)	IJA	MAX	ADD	SS z=-50	SS z=-10	SS z=-5	SS z=5	SS z=10	SS z=50
F(P)	1.000				0.479	0.474	0.461	0.486	0.484	0.481
IJA	0.429	1.000			0.687	0.650	0.620	0.723	0.710	0.696
MAX	0.375	0.926	1.000		0.580	0.564	0.533	0.609	0.597	0.586
ADD	0.500	0.857	0.750	1.000	0.958	0.948	0.923	0.972	0.967	0.962

Matrix of R^2 values.
Response = f(p) = ln(p/(1-p)).

	F(P)	IJA	MAX	ADD	SS z=-50	SS z=-10	SS z=-5	SS z=5	SS z=10	SS z=50
F(P)	1.000				0.469	0.466	0.457	0.473	0.472	0.470
IJA	0.451	0.000			0.796	0.801	0.807	0.789	0.792	0.795
MAX	0.364	0.915	1.000		0.557	0.560	0.565	0.553	0.554	0.556
ADD	0.500	0.903	0.727	1.000	0.938	0.931	0.914	0.947	0.944	0.940

It was also desirable to determine which z gave the highest correlation with the other modes of joint action. This was determined with non-linear curve fitting methods using PROC NLIN in SAS (SAS Institute 1979). In fact the best z was always "0", this making the descriptions of SSA and ADD equivalent. In other words, if any mode of action other than SSA was the true mode, then the results were most similar to those of a description of SSA with z=0.

Whether or not a data set or an experimental design is capable of discriminating between modes of action would depend on the total variance, error variance, sample size, the number of parameters, the covariance between predicted values in alternative models, the range of responses expected, the orthogonality of the experimental design, and whether or not models have independent error variances. The simplest method of determining the ability of an experiment to discriminate between models would be to generate hypothetical data and then determine whether or not the design is sufficient. An example of this process is given in de March (in prep). It is generally concluded that discrimination between models is very difficult even in experiments more exact than most reported ones and is perhaps not worthwhile.

Appendix B.

Simplified and combined SAS computer programs (SAS Institute 1979). Calculations involving class variables are not shown. TP = cumulative number of animals observed dead out of a total of T animals at time HR hours in the test cell identified by TANK. LHR is the time in hours of the last observation in any test cell. C1 and C2 are the concentration of the two toxicants used in an experiment. The calculation of all other variables is shown in the program.

```
---------------------------------------------------------------------------
* DATA ENTERED AND TRANSFORMATIONS CALCULATED;
  DATA EXPT1;
  INPUT TANK$ TP T HR LHR C1 C2;
       P=TP/T;  Q=1-P;   IF P>0 AND P<1 THEN LOGIT=LOG(P)-LOG(Q);
       HR2=HR*HR;
  PROC SORT; BY TANK;

* PERTINENT SUMMARY STATISTICS FROM EACH TANK KEPT FOR MERGING;
  PROC MEANS; VAR C1 C2 T LHR; BY TANK;
  OUTPUT OUT=MEANS MAX=C1 C2 T LHR;

* CURVE FOR EACH TEST CELL (TANK) CALCULATED. VARIABLES HR, HR2
  AND INTERCEP WILL BE REGRESSION COEFFICIENTS IN A FILE NAMED SUMMARY;
  PROC REG DATA=EXPT1 OUTEST=SUMMARY; BY TANK;
       M1: MODEL LOGIT=HR HR2;

* FOLLOWING DATA SET USED FOR ALL MODELLING STATEMENTS;
  DATA SUMMARY; MERGE SUMMARY MEANS; BY TANK; DROP _MODEL_;
* RESPONSE AT 96 (OR 48) HOURS CALCULATED;
       PLOGIT=HR*96 + HR2*96*96 + INTERCEP;
       PP=(1/(EXP(-PLOGIT)+1));   PQ=1-PP;   WEIGHT=1/(PQ*PP);
* RATES AT 96 HOURS CALCULATED;
       LRATE=HR + 2*HR2*96;        * INSTANTEOUS RATE CHANGE OF LOGIT;
       RATE=LRATE/WEIGHT;          * INSTANTANEOUS RATE CHANGE OF PP;
* PREDICTED TIME TO 50% MORTALITY CALCULATED;
        IF HR2=0 THEN GO TO PASS;
        INFLECT=-HR/(2*HR2);   LASYM=((-HR*HR)/(4*HR2))+INTERCEP;
        ASYM==1/(EXP(-LASYM)+1);  ROOT=HR*HR-4*HR2*INTERCEP;
        IF ROOT<0 THEN ROOT=.;  ROOT=SQRT(ROOT);
        T50=(-HR+ROOT)/(2*HR2);
  PASS: IF HR2=0 AND HR NE 0 THEN T50=INTERCEP/HR;
        IF T50>LHR THEN T50=.;
* TRANSFORMATIONS OF TOXICANT CONCENTRATIONS CALCULATED;
        C12=C1*C1; C22=C2*C2; C1C2=C1*C2;
        C1C22=C1*C2*C2;  C2C12=C2*C1*C1; C12C22=C1C2*C1C2;

* CORRELATION MATRIX FOR RELEVENT VARIABLES;
  PROC CORR; VAR C1 C2 HR HR2 INTERCEP T50 LRATE RATE PLOGIT PQ ASYM T;
```

```
* INDIVIDUAL RESPONSES TO TOXICANTS;
  PROC GLM; MODEL PQ = C1 C12; WEIGHT WEIGHT;
  PROC GLM; MODEL PQ = C2 C22; WEIGHT WEIGHT;
* 5 PARAMETER MODEL FOR PQ;
  PROC GLM; MODEL PQ = C1 C2 C12 C22; WEIGHT WEIGHT;
* 9 PARAMETER FOR PQ;
  PROC GLM; MODEL PQ = C1 C2 C12 C22 C1C2 C1C22 C2C12 C12C22;
        WEIGHT WEIGHT;

* SIMPLE SIMILAR ACTION TO DESCRIBE PQ;
  PROC NLIN; PARMS A=0 B=0 J=0 K=1;
        _WEIGHT_ = WEIGHT;
        COMBO = A*C1 + B*C2;
        MODEL PQ = COMBO + J*COMBO*COMBO + K;
        DER.A = 2*J*COMBO*C1 + C1;      DER.B = 2*J*COMBO*C2 + C2;
        DER.J - COMBO * COMBO;          DER.K = 1;

* INDEPENDENT JOINT ACTION TO DESCRIBE PQ;
  PROC NLIN; PARMS A=0 B=0 C=0 D=0 K=1;
        _WEIGHT_ = WEIGHT;
        T1 = A*C1 + C*C12 + 1;          T2=B*C2 + D*C22 + 1;
        MODEL PQ = K*T1*T2;
        DER.A = K*T2*C1;                DER.B = K*T1*C2;
        DER.C = K*T2*C12;               DER.D = K*T1*C22;
        DER.K = T1*T2;

* COMPLETE CORRELATION OF TOLERANCES DESCRIBING PQ;
  PROC NLIN; PARMS A=0 B=0 C=0 D=0 K=1;
        _WEIGHT_ = WEIGHT;
        T1 = A*C1 + C*C12;              T2 = B*C2 + D*C22;
    IF T1=T2 THEN DO;
        MODEL PQ = T1 + T2 + K;
        DER.A=C1;  DER.B=C2;   DER.C=C12;   DER.D=C22;  DER.K=1;   END;
    IF T1<T2 THEN DO;
        MODEL PQ = T1 + K;
        DER.A=C1;  DER.B=0;    DER.C=C12;   DER.D=0;    DER.K=1;   END;
    IF T1>T2 THEN DO;
        MODEL PQ = T2 + K;
        DER.A=0;   DER.B=C2;   DER.C=0;     DER.D=C22;  DER.K=1;   END;


* INDIVIDUAL RESPONSES TO TOXICANTS FOR PLOGIT;
  PROC GLM; MODEL PLOGIT = C1 C12;
  PROC GLM; MODEL PLOGIT = C2 C22;
* 5 PARAMETER MODEL FOR PLOGIT;
  PROC GLM; MODEL PLOGIT = C1 C2 C12 C22;
* 9 PARAMETER MODEL FOR PLOGIT;
  PROC GLM; MODEL PLOGIT = C1 C2 C12 C22 C1C2 C1C22 C2C12 C12C22;

* SIMPLE SIMILAR ACTION FOR PLOGIT;
```

```
  PROC NLIN; A=0 B=0 J=0 K=-10,-1,0,1;
       COMBO = A*C1 + B*C2;
       MODEL PLOGIT = COMBO + J*COMBO*COMBO + K;
       DER.A = 2*J*COMBO*C1 + C1;        DER.B = 2*J*COMBO*C1 + C2;
       DER.J = COMBO*COMBO;              DER.K = 1;

* INDEPENDENT JOINT ACTION IN LOGISTIC MODEL;
* THE ACTUAL PROGRAM HAD BYPASSES FOR EXPONENT UNDER- AND OVER-FLOWS;
  PROC NLIN; PARMS A=0 B=0 C=0 D=0 K=-10;
       T1 = A*C1 + C*C12 + K;      L1 = EXP(T1) + 1;
       T2 = B*C2 + D*C22 + K;      L2 = EXP(T2) + 1:
       T3 = K;                     L3 = EXP(K) + 1;
       RQ = (L1*L2)/L3; * RQ=RECIPROCAL OF PRODUCT OF 3 PROBABILITIES;
       MODEL PLOGIT = LOG(RQ-1);
       DER.A = C1*(L1*L2-L2)/(L1*L2-L3);
       DER.B = C2*(L1*L2-L1)/(L1*L2-L3);
       DER.C = C12*(L1*L2-L2)/(L1*L2-L3);
       DER.D = C22*(L1*L2-L1)/(L1*L2-L3);
       DER.K = (L1*L2*L3-L1*L3-L2*L3+L1*L2)/(L3*L3*(L1*L2-L3);

* COMPLETE CORRELATION OF TOLERANCES DESCRIBING PLOGIT;
  PROC NLIN; PARMS A=0 B=0 C=0 D=0 K=-10,-1,0,1;
       T1 = A*C1 + C*C12;       T2 = B*C2 + D*C22;
      IF T1=T2 THEN DO;
       MODEL PLOGIT = T1 + T2 + K;
       DER.A=C1;   DER.B=C2;   DER.C=C12;   DER.D=C22;   DER.K=1;  END;
      IF T1>T2 THEN DO;
       MODEL PLOGIT = T1 + K;
       DER.A=C1;   DER.B=0;    DER.C=C12;   DER.D=0;    DER.K=1;   END;
      IF T2>T1 THEN DO;
       MODEL PLOGIT = T2 + K;
       DER.A=0;    DER.B=C2;   DER.C=0;    DER.D=C22;   DER.K=1;   END;

* CHI-SQUARED TEST FOR DESCRIPTIVE MODEL PREVIOUSLY DETERMINED;
  PROC GLM; MODEL Q = C12 C2; WEIGHT WEIGHT;
       OUTPUT OUT=TEST PREDICTED=PRED;
  DATA TEST; SET TEST;
*OUT OF RANGE PREDICTIONS AND PREDICTED VALUES < 1 ANIMAL ELIMINATED;
       IF PRED>0 AND PRED<1 THEN DEN = PRED*(1-PRED); ELSE DEN=1;
       IF (PRED*TT)<1 OR (PRED*TT)>(TT-1) THEN DEN=1;
       CHI = ((ROUND(Q*TT)/TT-PRED)**2;   CHI=CHI*TT/DEN;
  PROC MEANS NOPRINT; VAR CHI;
       OUTPUT OUT = TEST2 SUM=SUMSQ N=NSAMPLE;
  DATA TEST2; SET TEST2;
       DF=NSAMPLES-2;    PROB=PROBCHI(SUMSQ,DF); ALPHA=1-PROB;
  PROC PRINT; VAR SUMSQ NSAMPLES DF PROB ALPHA;
```

Appendix C.

Tables C1 to C18. Matrices of correlation coefficients between summary stastistics describing the response and toxicant concentration values in 18 experiments. A, B, and C are regression coefficients in the Equation logit(p) = A*HR + b*HR*HR + C. From this equation are calculated: T50, the predicted time to 50% mortality; Q96 and Q48, the predicted survivorships at 96 and 48 hours respectively; LOGIT, the logit of the probability of response = ln(p/q); and LRATE, the instantaneous rate of change of the logit at 96 or 48 hours. See Appendix B for calculations.

Table C1. Matrix of correlation coefficients between variables in Experiment Cu-Cd-1. Maximum n=21.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	-0.255 ns	1.000							
A	-0.095 ns	-0.366 ns	1.000						
B	0.178 ns	0.484 **	-0.939 ****	1.000					
C	0.245 ns	0.410 *	-0.890 ****	0.835 ****	1.000				
T50	-0.497 **	-0.542 **	0.244 ns	-0.418 *	-0.560 ***	1.000			
Q96	-0.448 **	-0.469 **	0.249 ns	-0.425 *	-0.582 ***	0.965 ****	1.000		
LOGIT	0.446 **	0.468 **	-0.255 ns	0.432 ns	0.586 ***	-0.963 ****	-1.000 ****	1.000	
LRATE	0.189 ns	0.412 *	-0.062 ns	0.362 ns	-0.083 ns	-0.344 ns	-0.272 ns	0.600	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C2. Matrix of correlation coefficients between variables in Experiment Cu-Cd-2. Maximum n=28.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.465 **	1.000							
A	0.222 ns	0.047 ns	1.000						
B	0.135 ns	0.195 ns	-0.862 ****	1.000					
C	0.025 ns	-0.025 ns	-0.740 ****	0.493 ***	1.000				
T50	0.434 **	-0.313 ns	-0.395 **	0.230 ns	-0.168 ns	1.000			
Q96	0.429 **	-0.352 *	-0.484 ***	0.280 ns	-0.069 ns	0.971 ****	1.000		
LOGIT	0.439 ns	0.337 *	0.480 ***	-.286 NS	0.085 NS	-0.963 ****	-0.998 ****	1.000	
LRATE	0.171 ns	0.458 **	0.296 ns	0.228 ns	-0.492 ***	-0.326 **	-0.403 **	0.383 **	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C3. Matrix of correlation coefficients between variables in Experiment Cu-Cd-3. Maximum n=22.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.282 ns	1.000							
A	0.522 **	0.310 ns	1.000						
B	0.328 ns	-0.023 ns	-0.906 ****	1.000					
C	0.020 ns	0.475 **	-0.488 **	0.589 ***	1.000				
T50	0.569 ***	-0.659 ***	-0.619 ***	0.449 **	-0.297 ns	1.000			
Q96	0.662 ****	-0.697 ****	-0.538 ***	0.218 ns	-0.221 ns	0.944 ****	1.000		
LOGIT	0.601 ***	0.803 ****	0.567 ***	-0.192 *	0.237 ns	-0.822 ****	-0.895 ****	1.000	
LRATE	0.089 ns	0.422 *	-0.423 **	0.767 ****	0.521 **	0.092 ns	-0.351 ns	0.450 *	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C4. Matrix of correlation coefficients between variables in Experiment Cu-Zn-1. Maximum n=30.

	C1	C2	A	B	C	T50	Q48	LOGIT	LRATE
C1	1.000								
C2	0.342 *	1.000							
A	0.399 **	0.516 ***	1.000						
B	0.442 **	-0.491 ***	-0.945 ****	1.000					
C	0.412 **	-0.119 ns	-0.766 ****	0.623 ****	1.000				
T50	0.304 ns	-0.555 ***	-0.565 ***	0.601 ***	0.069 ns	1.000			
Q48	0.071 ns	-0.669 ****	-0.476 ***	0.445 **	-0.125 ns	0.951 ****	1.000		
LOGIT	0.075 ns	0.674 ****	0.476 ***	-0.445 **	0.125 ns	-0.950 ****	-1.000 ****	1.000	
LRATE	0.100 ns	0.111 ns	0.231 ns	0.100 ns	-0.479 ***	0.074 ns	-0.125 ns	0.124 ns	1.000
	C1	C2	A	B	C	T50	Q48	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C5. Matrix of correlation coefficients between variables in Experiment Cu-Zn-2. Maximum n=26.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.245 ns	1.000							
A	0.024 ns	-0.007 ns	1.000						
B	0.015 ns	0.034 ns	-0.942 ****	1.000					
C	0.042 ns	0.177 ns	-0.846 ****	0.679 ****	1.000				
T50	0.180 ns	-0.411 **	-0.040 ns	0.138 ns	-0.447 **	1.000			
Q96	0.189 ns	-0.485 **	-0.095 ns	0.080 ns	-0.307 ns	-0.932 ****	1.000		
LOGIT	0.182 ns	0.498 ***	0.100 ns	-0.080 ns	0.299 ns	-0.926 ****	-0.999 ****	1.000	
LRATE	0.020 ns	0.080 ns	-0.201 ns	0.518 ***	-0.175 ns	0.302 ns	-0.010 ns	0.021 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C6. Matrix of correlation coefficients between variables in Experiment Cu-Zn-3. Maximum n=24.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.216 ns	1.000							
A	0.334 ns	0.619 ***	1.000						
B	0.279 ns	-0.451 **	-0.948 ****	1.000					
C	0.424 **	0.208 ns	-0.119 ns	0.023 ns	1.000				
T50	0.531 **	-0.572 **	-0.680 ***	0.691 ***	-0.606 ***	1.000			
Q96	0.644 ****	-0.804 ****	-0.624 ***	0.507 **	-0.537 ***	0.980 ****	1.000		
LOGIT	0.613 ***	0.819 ****	0.702 ****	-0.593 ***	0.494 ***	-0.955 ****	-0.985 ****	1.000	
LRATE	0.065 ns	0.062 ns	-0.501 ***	0.750 ****	-0.185 ns	0.592 ***	0.082 ns	-0.153 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C7. Matrix of correlation coefficients between variables in Experiment Cd-Zn-1. Maximum n=23.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.308 ns	1.000							
A	0.060 ns	0.366 *	1.000						
B	0.317 ns	-0.070 ns	-0.844 ****	1.000					
C	0.065 ns	-0.028 ns	-0.780 ****	0.639 ***	1.000				
T50	0.446 *	-0.587 *	-0.447 **	0.214 ns	0.025 ns	1.000			
Q96	0.642 ****	-0.712 ****	-0.359 *	-0.070 ns	-0.074 ns	0.897 ****	1.000		
LOGIT	0.635 ***	0.716 ****	0.372 *	0.057 ns	0.071 ns	-0.901 ****	-0.993 ****	1.000	
LRATE	0.686 ****	0.445 **	-0.002 ns	0.538 ***	-0.036 ns	-0.419 *	-0.696 ****	0.691 ****	1.000

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C8. Matrix of correlation coefficients between variables in Experiment Cd-Zn-2. Maximum n=22.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.288 ns	1.000							
A	0.265 ns	0.292 ns	1.000						
B	0.178 ns	-0.259 ns	-0.949 ****	1.000					
C	0.078 ns	0.082 ns	-0.849 ****	0.782 ****	1.000				
T50	0.308 ns	-0.503 **	-0.541 **	0.545 **	0.200 ns	1.000			
Q96	0.424 **	-0.696 ****	-0.640 ***	0.561 ***	0.182 ns	0.929 ****	1.000		
LOGIT	0.465 **	0.661 ****	0.625 ***	-0.494 **	-0.180 ns	-0.930 ****	-0.957 ****	1.000	
LRATE	0.341 ns	0.210 ns	0.550 ***	-0.258 ns	-0.532 ***	-0.214 ns	-0.474 **	0.602 ***	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C9. Matrix of correlation coefficients between variables in Experiment Cu-K-1. Maximum n=21.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.278 ns	1.000							
A	0.199 ns	-0.222 ns	1.000						
B	0.173 ns	0.261 ns	-0.945 ****	1.000					
C	0.007 ns	0.395 *	-0.917 ****	0.836 ****	1.000				
T50	0.652 **	-0.696 ***	-0.619 **	0.497 *	-0.068 ns	1.000			
Q96	0.587 ***	-0.638 ***	0.025 ns	-0.032 ns	-0.373 *	0.941 ****	1.000		
LOGIT	0.491 **	0.613 ***	-0.083 ns	0.087 ns	0.436 **	-0.908 ****	-0.966 ****	1.000	
LRATE	0.185 ns	-0.040 ns	0.712 ****	-0.483 **	-0.761 ****	-0.515 *	-0.002 ns	-0.040 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns at alpha > 0.10 level.

Table C10. Matrix of correlation coefficients between variables in Experiment Cd-K-1. Maximum n=28.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.380 **	1.000							
A	0.311 ns	0.468 ns	1.000						
B	0.698 ****	-0.517 ***	-0.841 ****	1.000					
C	0.190 ns	-0.172 ns	-0.760 ****	0.511 ***	1.000				
T50	0.344 **	-0.257 ns	-0.331 *	0.143 ns	-0.215 ns	1.000			
Q96	0.461 **	-0.227 ns	-0.300 ns	0.016 ns	-0.183 ns	0.972 ****	1.000		
LOGIT	0.461 **	0.224 ns	0.303 ns	-0.016 ns	0.178 ns	-0.969 ****	-1.000 ****	1.000	
LRATE	0.768 ****	-0.175 ns	0.108 ns	0.447 **	-0.317 ns	-0.301 ns	-0.466 **	0.470 **	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C11. Matrix of correlation coefficients between variables in Experiment Cd-K-2. Maximum n=22.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.250 ns	1.000							
A	0.144 ns	0.171 ns	1.000						
B	0.255 ns	-0.051 ns	-0.809 ****	1.000					
C	0.153 ns	-0.024 ns	-0.910 ****	0.702 ****	1.000				
T50	0.579 *	-0.547 ns	0.340 ns	-0.398 ns	-0.544 ns	1.000			
Q96	0.613 ***	-0.418 *	-0.192 ns	-0.239 ns	-0.045 ns	0.980 ****	1.000		
LOGIT	0.459 **	0.392 *	0.310 ns	0.109 ns	-0.019 ns	-0.984 ****	-0.932 ****	1.000	
LRATE	0.610 ***	0.217 ns	0.543 ***	0.053 ns	-0.543 ***	0.064 ns	-0.669 ****	0.683 ****	1.000

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C12. Matrix of correlation coefficients between variables in Experiment Zn-K-1. Maximum n=23.

	C1	C2	A	B	C	T50	Q48	LOGIT	LRATE
C1	1.000								
C2	0.383 *	1.000							
A	0.700 ****	0.275 ns	1.000						
B	0.512 **	-0.093 ns	-0.942 ****	1.000					
C	0.000 ns	-0.203 ns	-0.512 **	0.513 **	1.000				
T50	0.842 ****	-0.363 *	-0.806 ****	0.654 ***	0.116 ns	1.000			
Q48	0.913 ****	-0.461 **	-0.774 ****	0.592 ***	0.014 ns	0.956 ****	1.000		
LOGIT	0.899 ****	0.365 *	0.703 ****	-0.500 ***	0.131 ns	-0.921 ****	-0.952 ****	1.000	
LRATE	0.282 ns	0.435 **	-0.213 ns	-0.528 ***	0.199 ns	-0.136 ns	-0.236 ns	0.323 ns	1.000
	C1	C2	A	B	C	T50	Q48	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C13. Matrix of correlation coefficients between variables in Experiment Mg-K-1. Maximum n=23.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.107 ns	1.000							
A	0.106 ns	0.008 ns	1.000						
B	0.189 ns	-0.097 ns	-0.984 ****	1.000					
C	0.327 ns	0.258 ns	-0.823 ****	0.737 ****	1.000				
T50	0.344 ns	-0.133 ns	-0.242 ns	0.184 ns	-0.448 ns	1.000			
Q96	0.712 ****	-0.368 *	-0.348 ns	0.433 **	-0.221 ns	0.785 *	1.000		
LOGIT	0.724 ****	0.383 *	0.310 ns	-0.398 *	0.271 ns	-0.718 ns	-0.981 ****	1.000	
LRATE	0.420 *	-0.475 ***	0.322 ns	-0.149 ns	-0.657 ****	-0.346 ns	0.371 *	-0.400 *	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table B14. Matrix of correlation coefficients between variables in Experiment Mg-K-2. Maximum n=24.

	C1	C2	A	B	C	T50	Q48	LOGIT	LRATE
C1	1.000								
C2	0.439 **	1.000							
A	0.183 ns	0.112 ns	1.000						
B	0.040 ns	-0.051 ns	-0.917 ****	1.000					
C	0.092 ns	-0.040 ns	-0.827 ****	0.665 ****	1.000				
T50	0.796 ****	-0.344 ns	-0.339 ns	0.072 ns	0.138 ns	1.000			
Q48	0.586 ***	-0.397 *	0.058 ns	-0.386 *	-0.109 ns	0.871 ****	1.000		
LOGIT	0.579 ***	0.465 **	-0.081 ns	0.416 **	0.122 ns	-0.859 ****	-0.982 ****	1.000	
LRATE	0.134 ns	0.229 ns	-0.631 ***	0.888 ****	0.338 ns	-0.322 ns	-0.686 ****	0.717 ****	1.000

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C15. Matrix of correlation coefficients between variables in Experiment Cu-Mg-1. Maximum n=22.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.104 ns	1.000							
A	0.216 ns	0.378 *	1.000						
B	0.085 ns	-0.444 **	-0.958 ****	1.000					
C	0.214 ns	0.081 ns	-0.820 ****	0.683 ****	1.000				
T50	0.896 ***	-0.262 ns	0.011 ns	-0.300 ns	-0.030 ns	1.000			
Q96	0.294 ns	-0.811 ****	-0.427 **	0.463 **	-0.097 ns	0.532 ns	1.000		
LOGIT	0.316 ns	0.760 ****	0.440 **	-0.433 *	0.050 ns	-0.536 ns	-0.960 ****	1.000	
LRATE	0.389 *	-0.338 ns	-0.147 ns	0.425 **	-0.235 ns	-0.559 ns	0.251 ns	-0.102 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C16. Matrix of correlation coefficients between variables in Experiment Cd-Mg-1. Maximum n=23.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.455 **	1.000							
A	0.029 ns	-0.255 ns	1.000						
B	0.151 ns	0.232 ns	-0.942 ****	1.000					
C	0.043 ns	0.297 ns	-0.964 ****	0.845 ****	1.000				
T50	0.292 ns	0.620 **	-0.459 *	0.264 ns	0.515 **	1.000			
Q96	0.383 *	-0.057 ns	-0.242 ns	0.236 ns	0.052 ns	0.346 ns	1.000		
LOGIT	0.427 **	0.174 ns	0.232 ns	-0.215 ns	-0.040 ns	-0.310 ns	-0.953 ****	1.000	
LRATE	0.207 ns	-0.217 ns	0.783 ****	-0.530 ***	-0.871 ****	-0.726 ***	-0.173 ns	0.188 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C17. Matrix of correlation coefficients between variables in Experiment Zn-Mg-1. Maximum n=24.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.168 ns	1.000							
A	0.117 ns	-0.148 ns	1.000						
B	0.037 ns	0.170 ns	-0.981 ****	1.000					
C	0.066 ns	0.120 ns	-0.991 ****	0.975 ****	1.000				
T50	0.852 ***	0.667 **	-0.362 ns	0.268 ns	0.324 ns	1.000			
Q96	0.738 ****	0.211 ns	-0.193 ns	0.058 ns	0.102 ns	0.782 ***	1.000		
LOGIT	0.729 ****	-0.086 ns	0.022 ns	0.104 ns	0.089 ns	-0.889 ****	-0.897 ****	1.000	
LRATE	0.398 *	-0.092 ns	0.928 ****	-0.838 ****	-0.915 ****	-0.546 ns	-0.429 **	0.261 ns	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Table C18. Matrix of correlation coefficients between variables in Experiment Zn-Mg-2. Maximum n=23.

	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE
C1	1.000								
C2	0.230 ns	1.000							
A	0.112 ns	-0.190 ns	1.000						
B	0.175 ns	0.202 ns	-0.988 ****	1.000					
C	0.216 ns	0.219 ns	-0.980 ****	0.957 ****	1.000				
T50	0.873 ****	0.086 ns	-0.492 *	0.397 ns	0.381 ns	1.000			
Q96	0.655 ****	-0.015 ns	0.113 ns	-0.133 ns	-0.259 ns	0.695 ***	1.000		
LOGIT	0.584 ***	0.277 ns	-0.662 ****	0.641 ***	0.794 ****	-0.691 ***	-0.696 ****	1.000	
LRATE	0.023 ns	-0.165 ns	0.979 ****	-0.937 ****	-0.974 ****	-0.636 **	0.082 ns	-0.666 ****	1.000
	C1	C2	A	B	C	T50	Q96	LOGIT	LRATE

**** denotes correlation significant at the alpha <= 0.001 level; ***, at 0.001 < alpha < 0.01; **, at 0.01 < alpha < 0.05 level; *, at 0.05 < alpha < 0.10 level; ns, at alpha > 0.10 level.

Appendix D.

Figures D1 to D18. Percent survival at either 96 or 48 hours, shown on the graphs as actual numbers, and the response isolines, obtained from Equations in Table 5, plotted against concentrations of two toxicants, in each of 18 experiments.

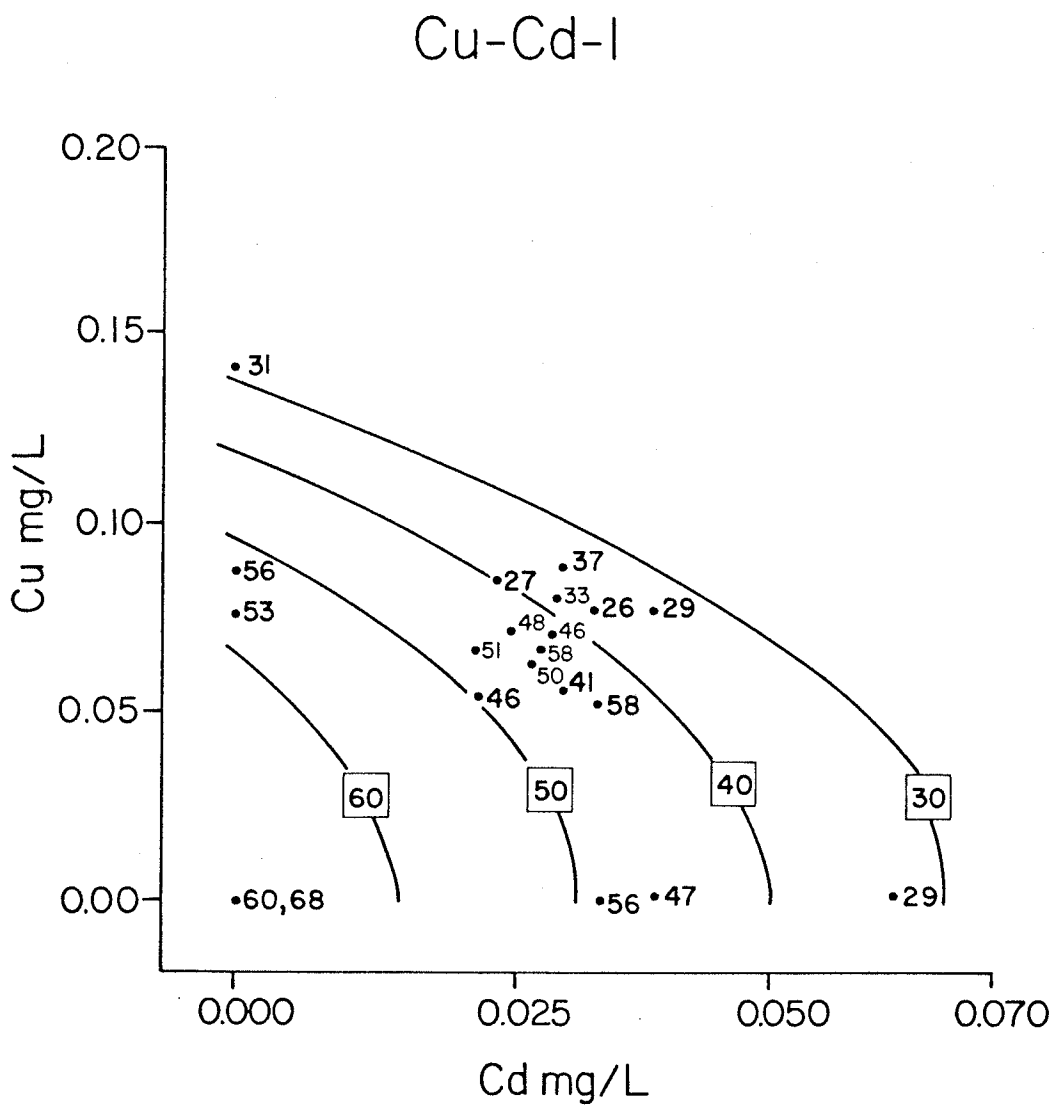

Figure D1. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Cd, in Experiment Cu-Cd-1.

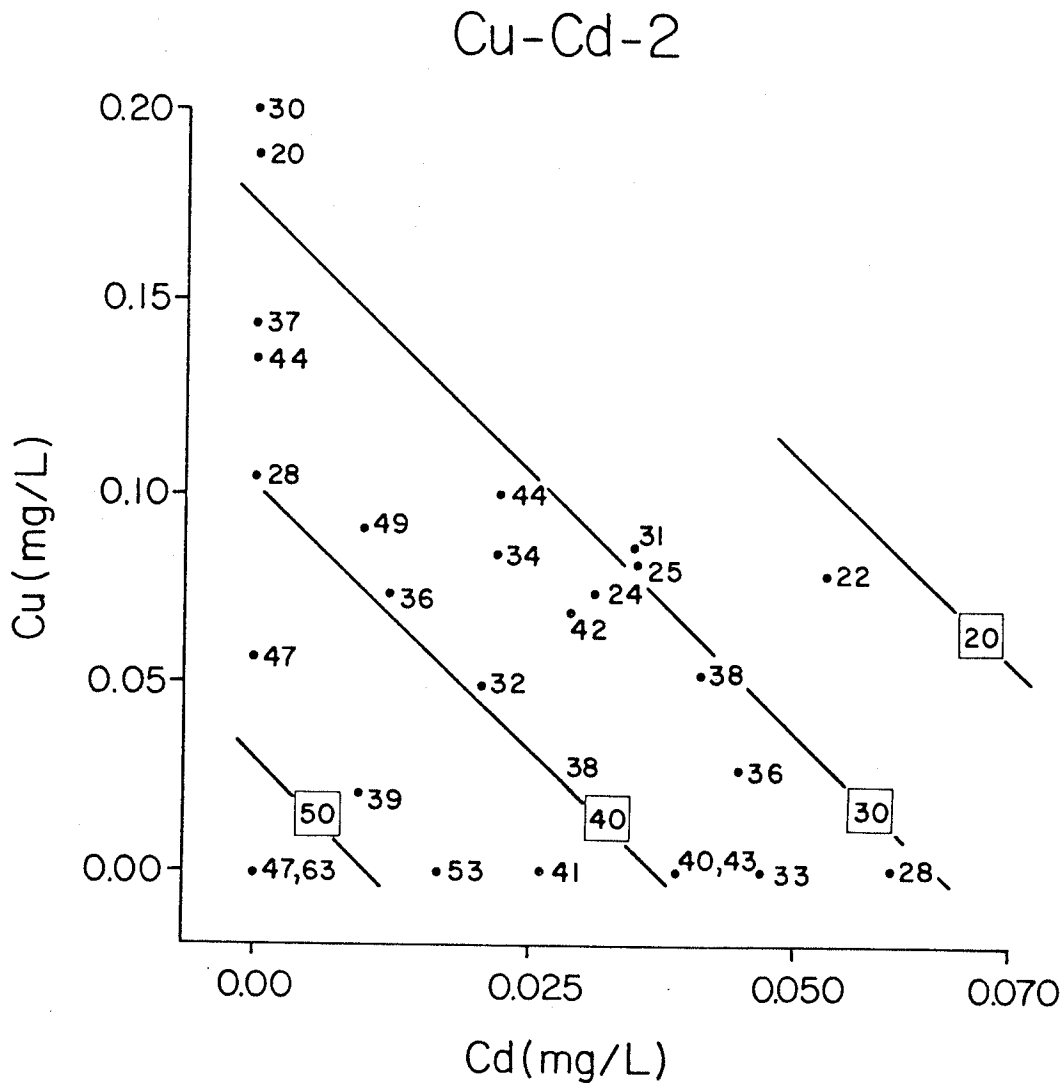

Figure D2. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Cd, in Experiment Cu-Cd-2.

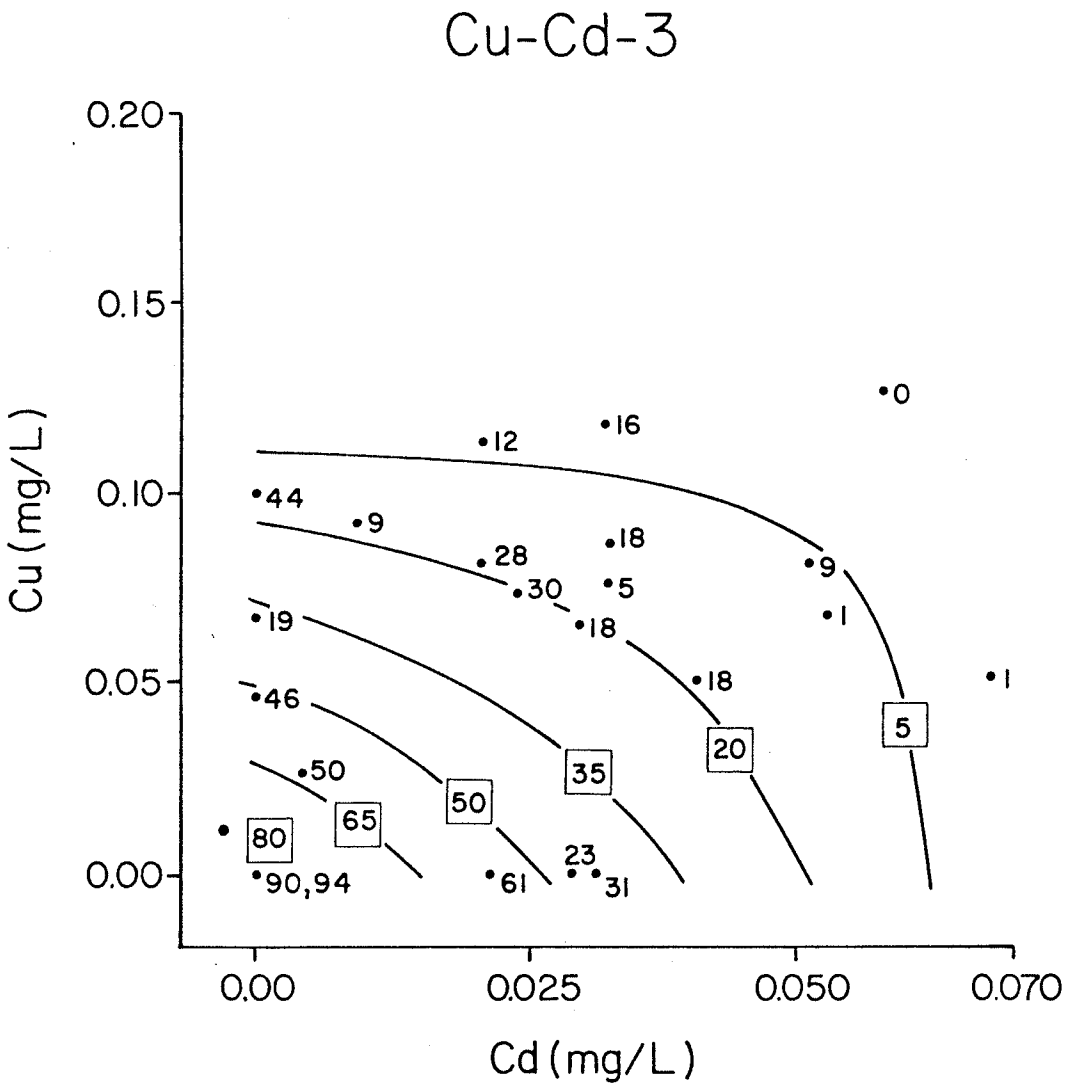

Figure D3. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, in Experiment Cu-Cd-3.

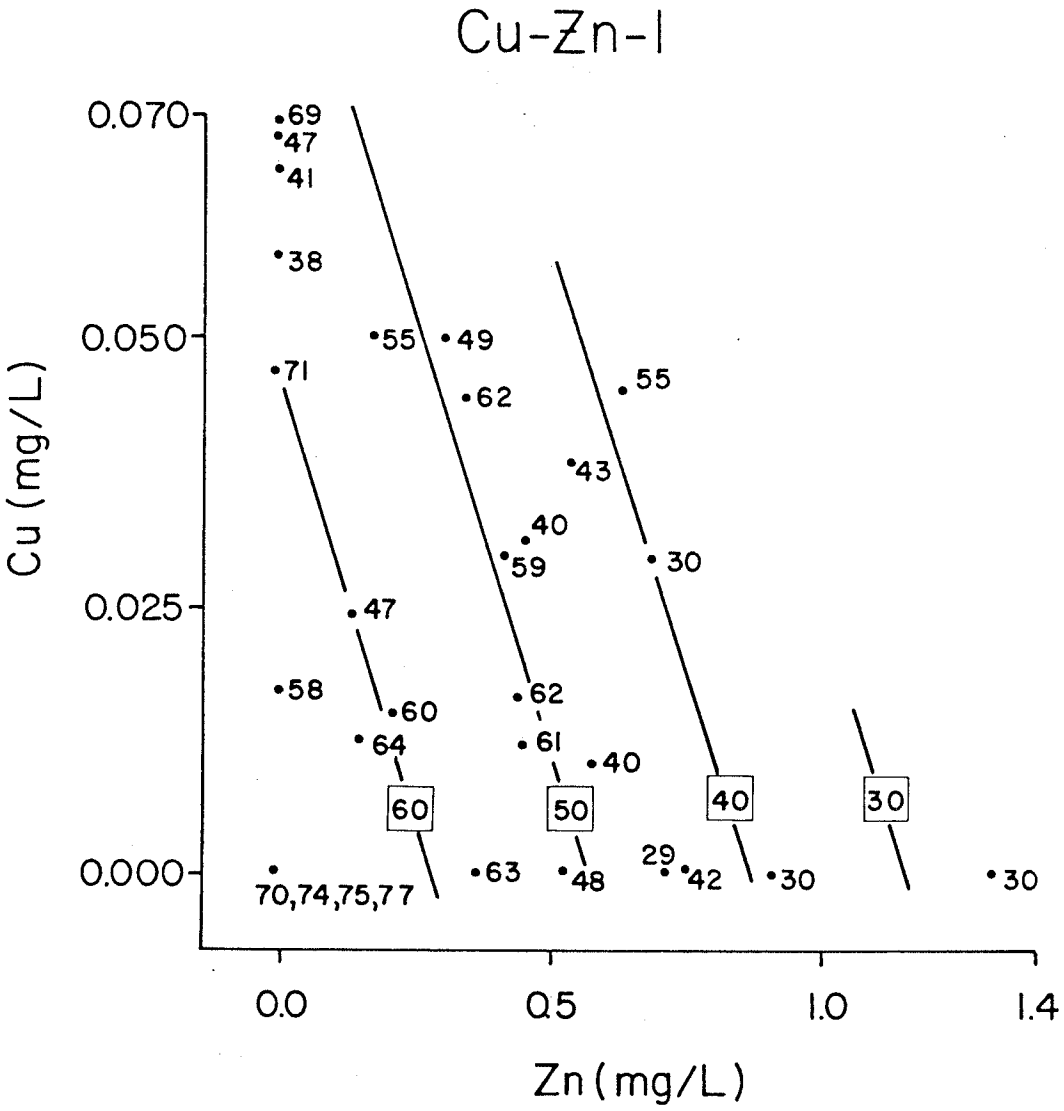

Figure D4. Percent survival at 48 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Zn, in Experiment Cu-Zn-1.

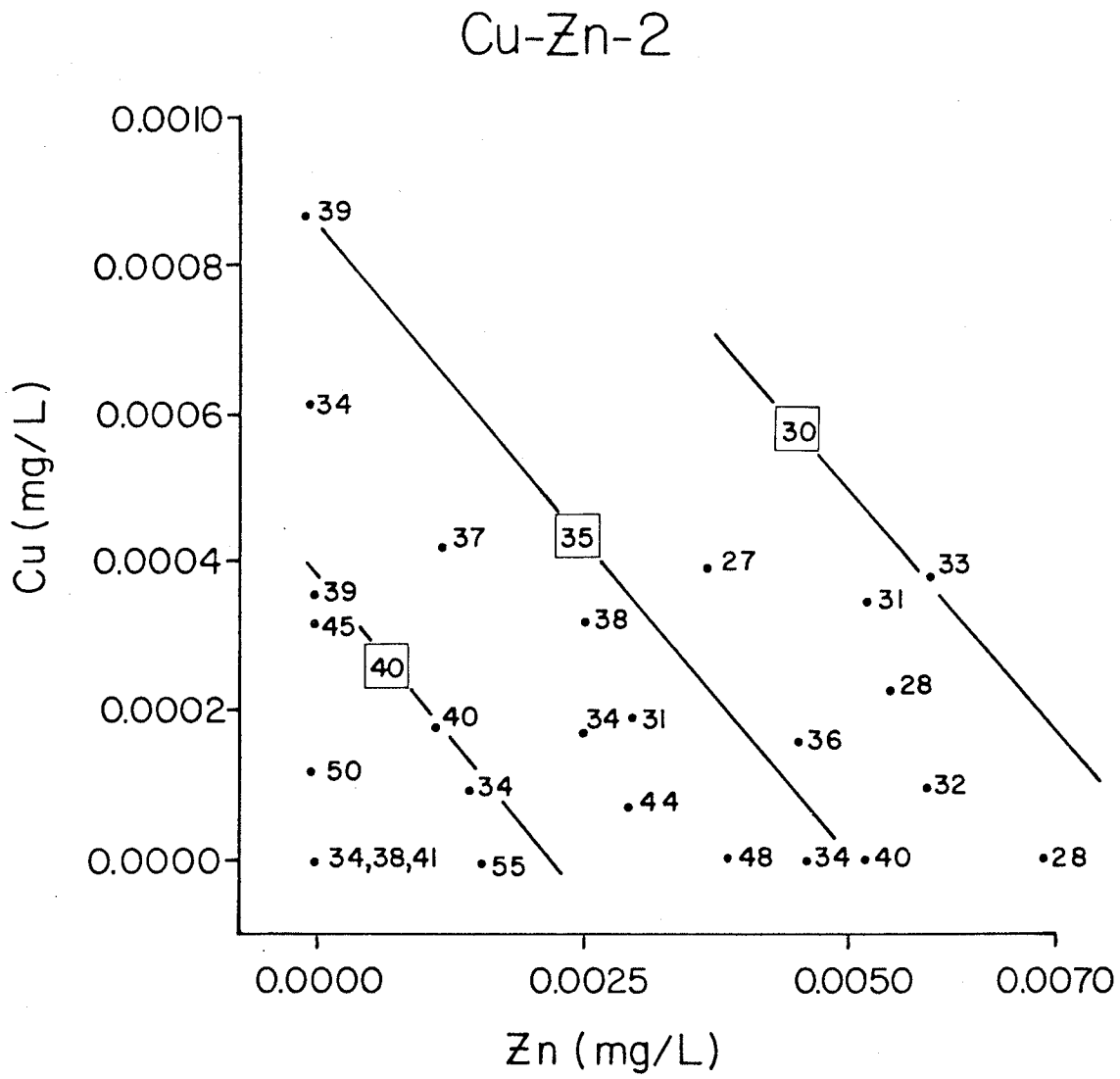

Figure D5. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Zn, in Experiment Cu-Zn-2.

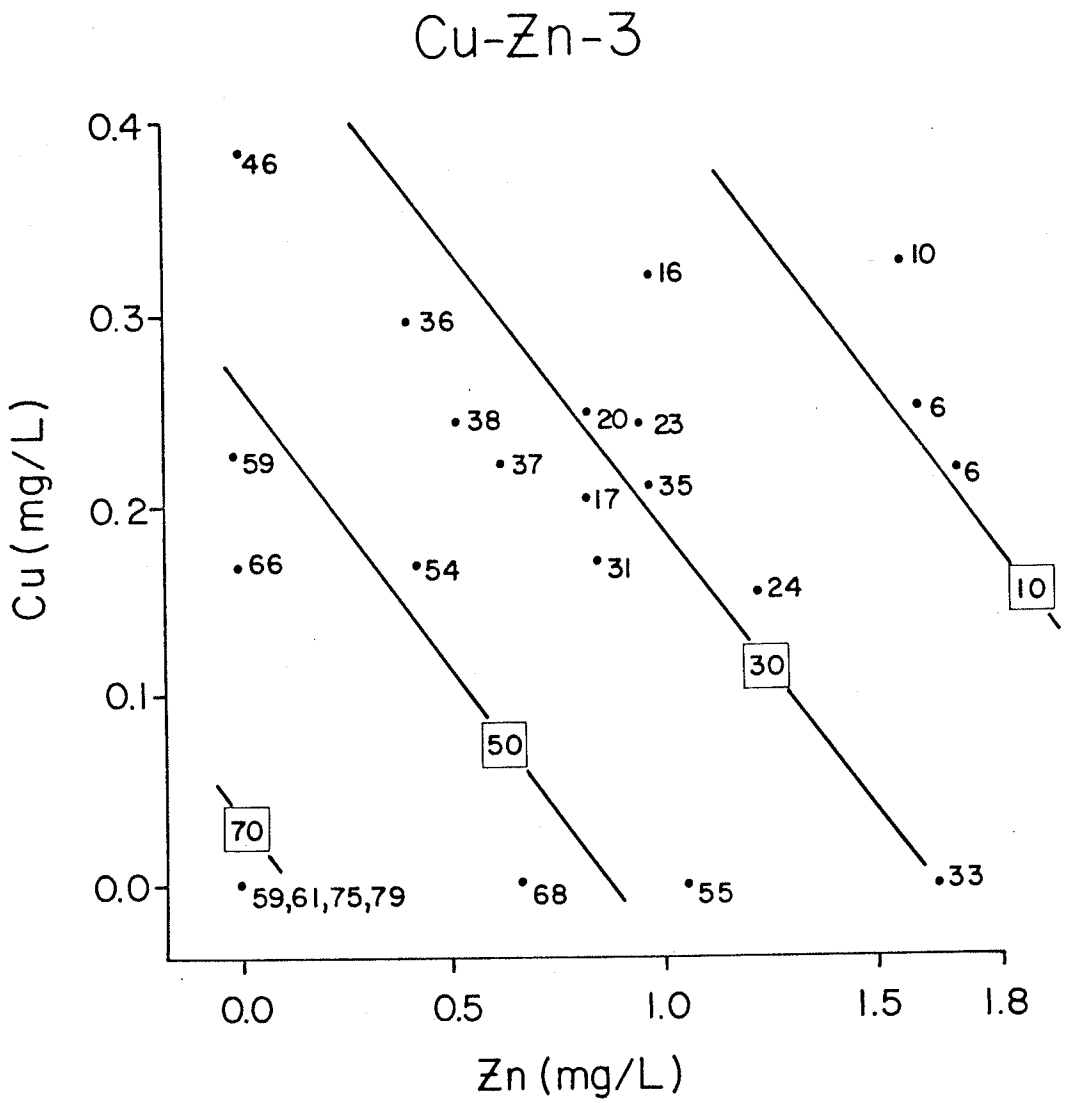

Figure D6. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Zn, in Experiment Cu-Zn-3.

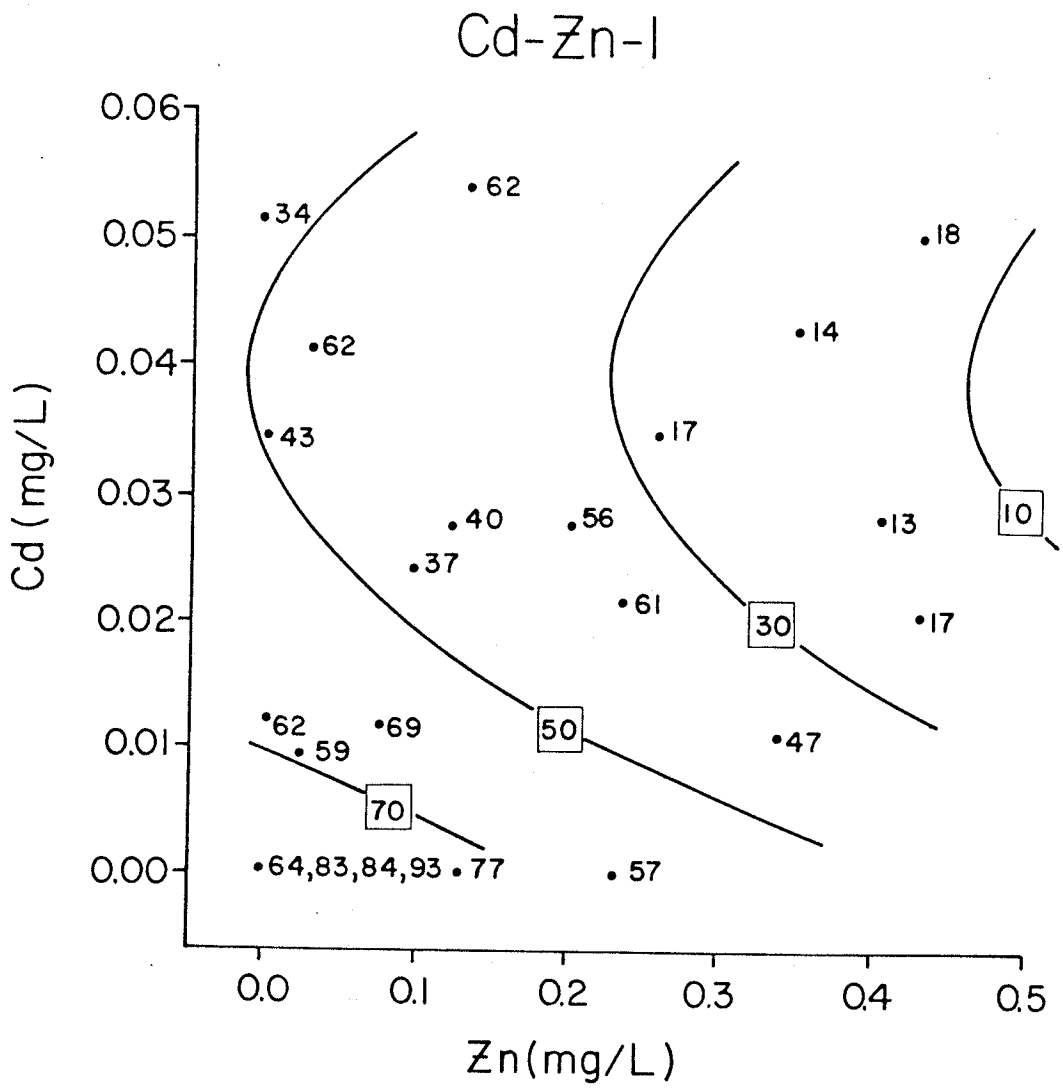

Figure D7. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cd and Zn, in Experiment Cd-Zn-1.

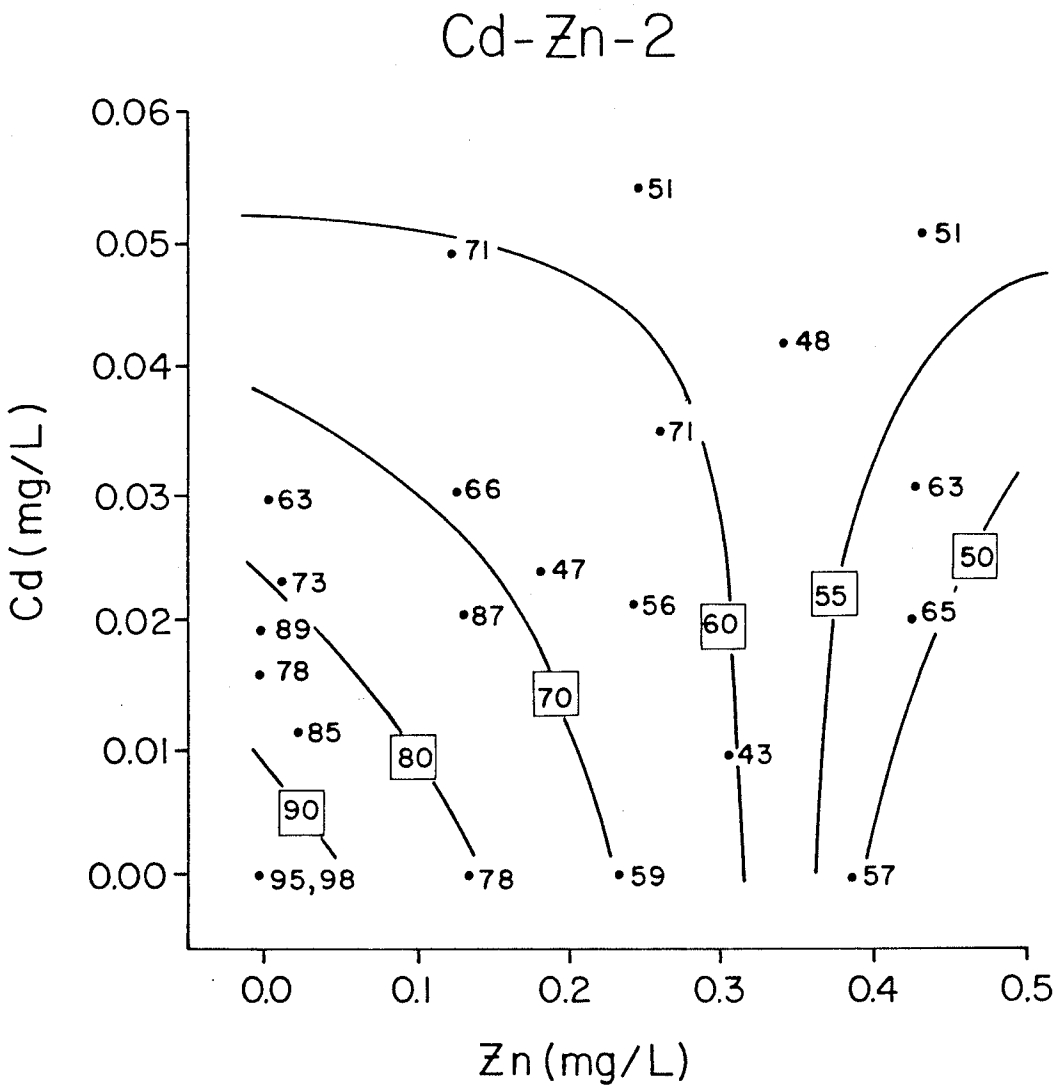

Figure D8. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cd and Zn, in Experiment Cd-Zn-2.

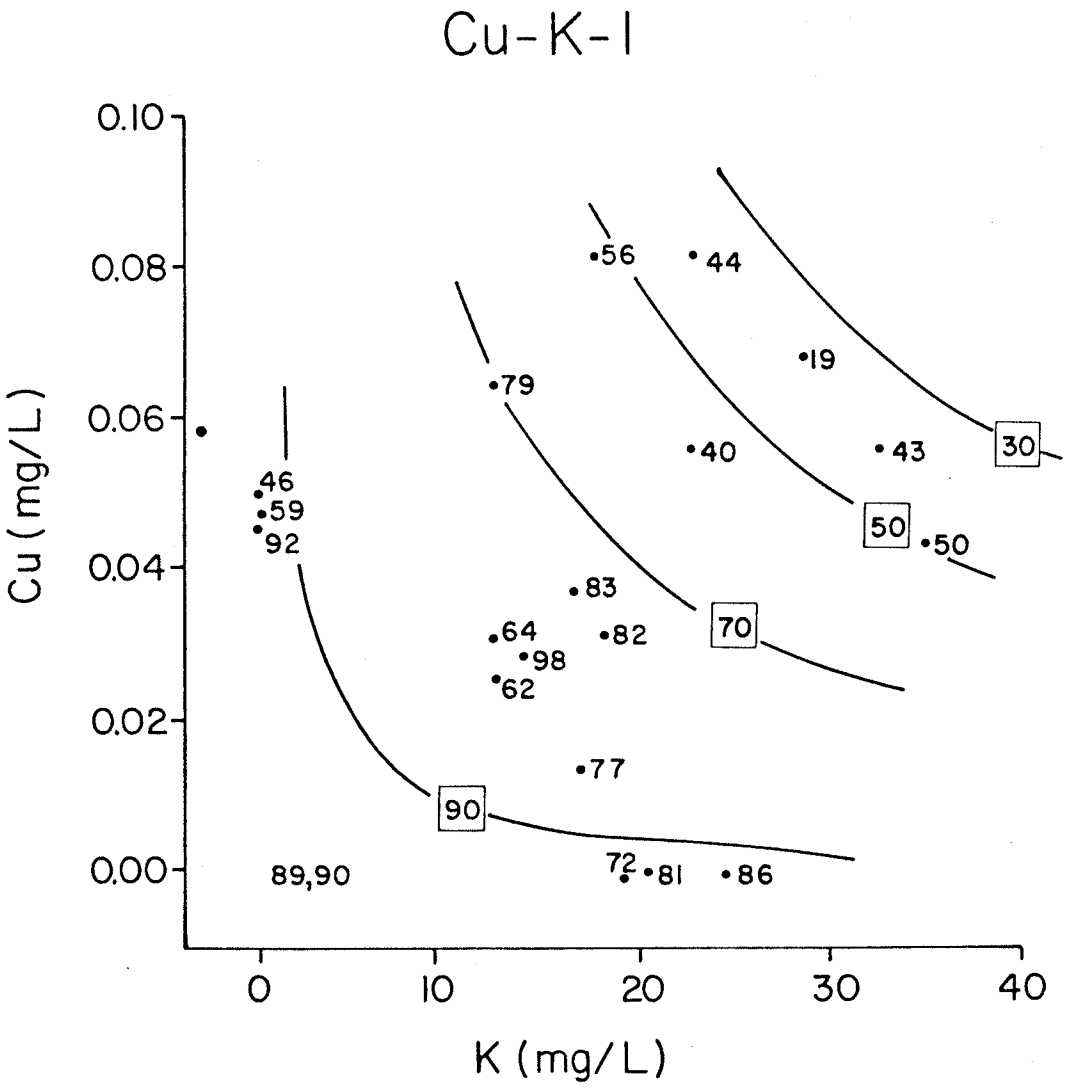

Figure D9. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and K, in Experiment Cu-K-1.

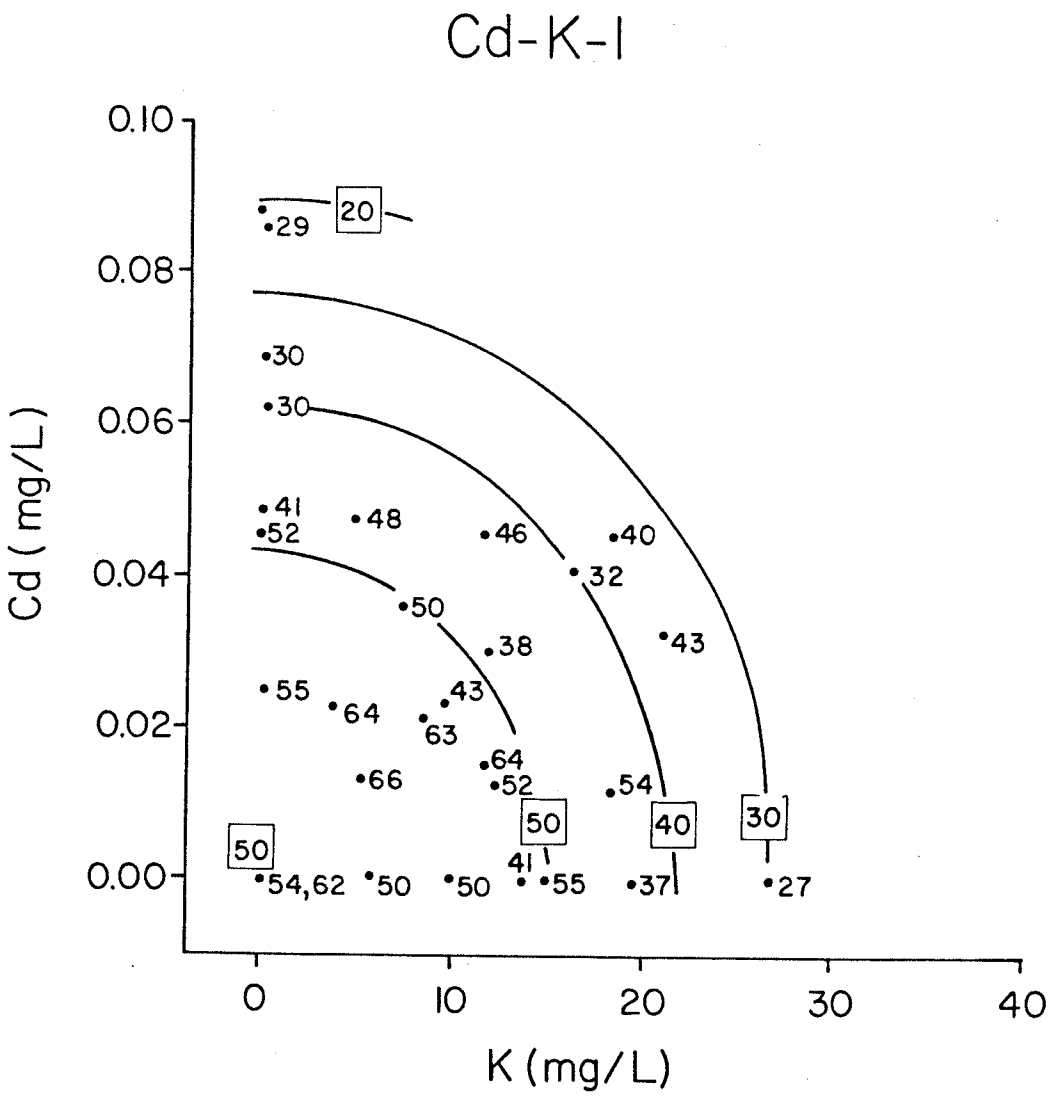

Figure D10. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cd and K, in Experiment Cd-K-1.

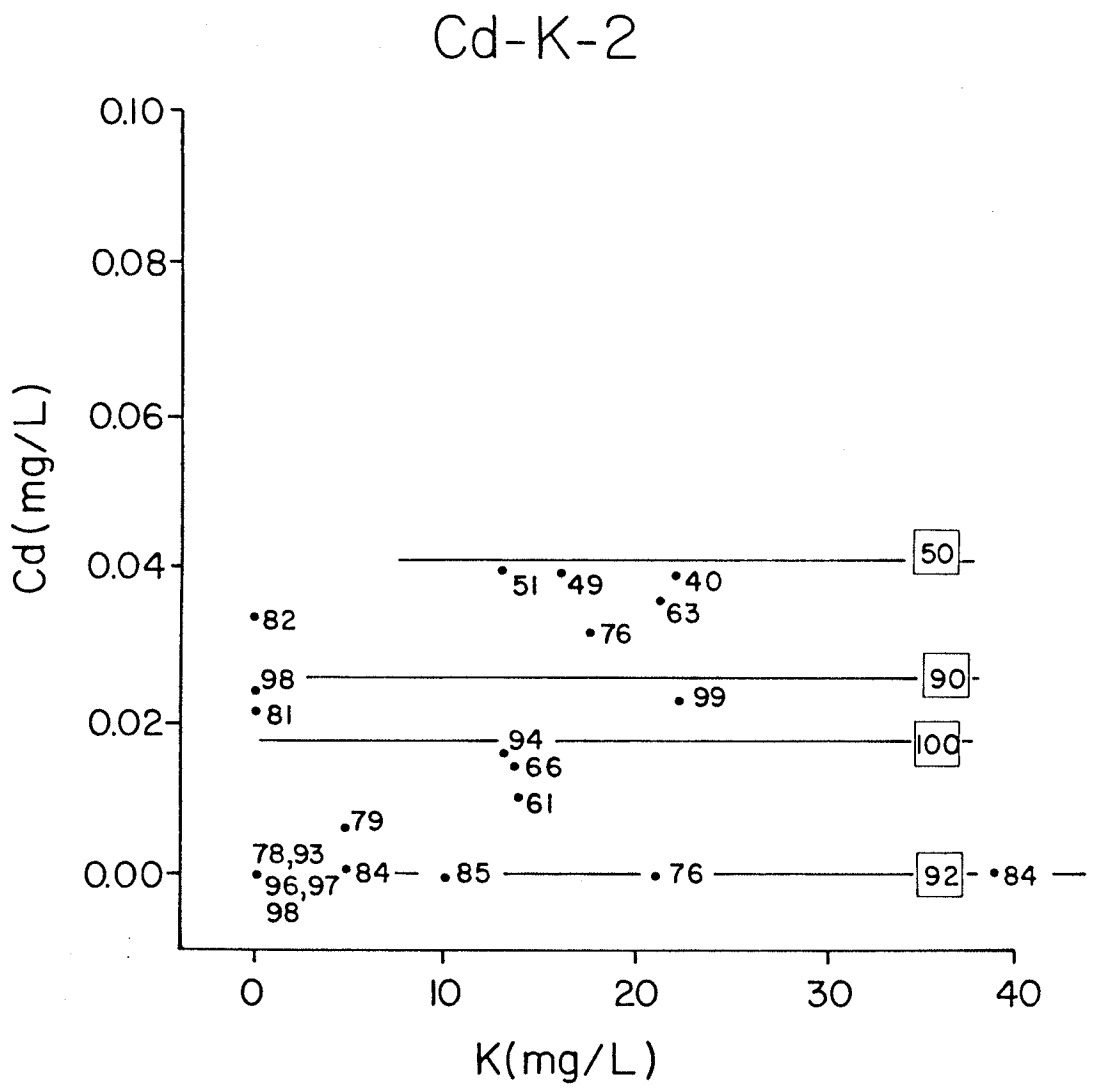

Figure D11. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cd and K, in Experiment Cd-K-2.

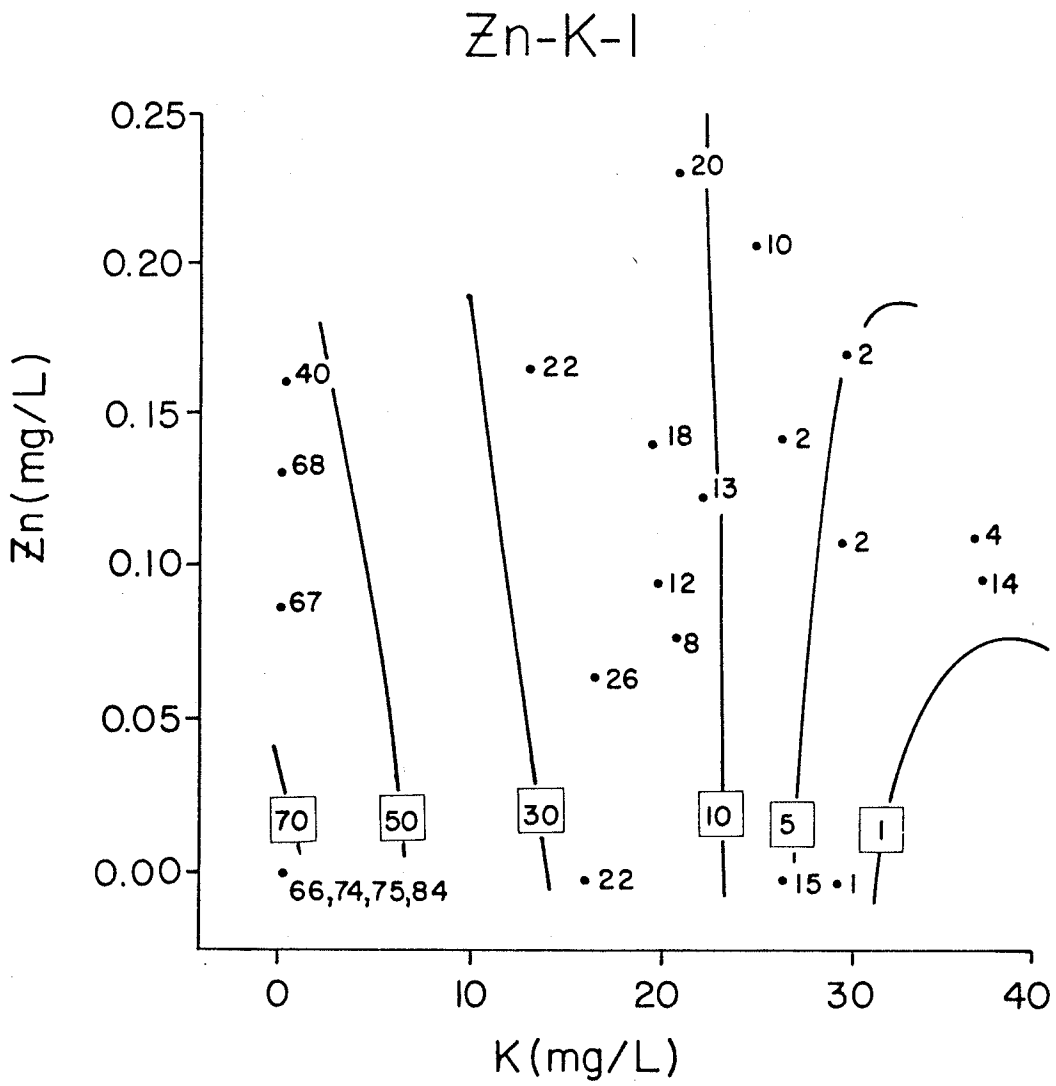

Figure D12. Percent survival at 48 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Zn and K, in Experiment Zn-K-1.

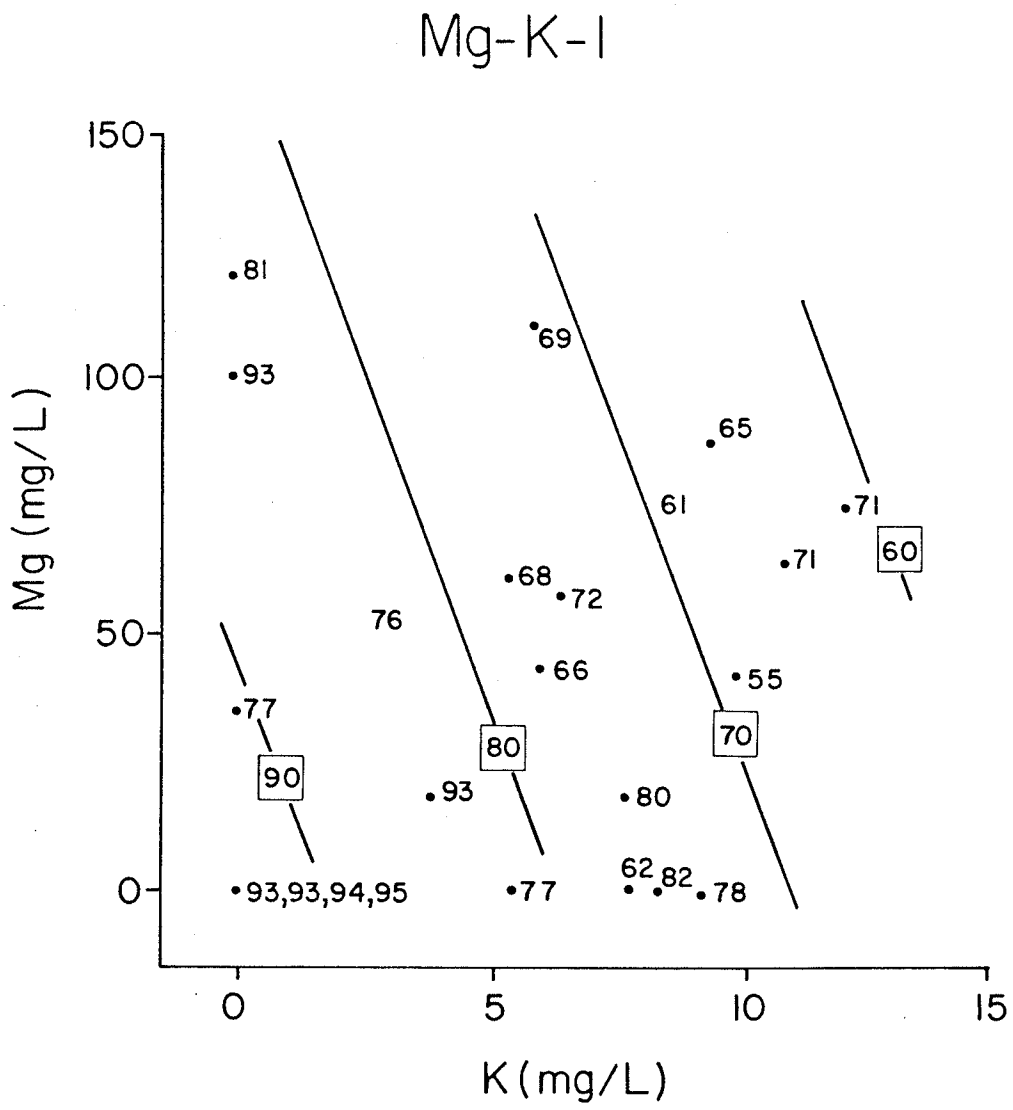

Figure D13. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Mg and K, in Experiment Mg-K-1.

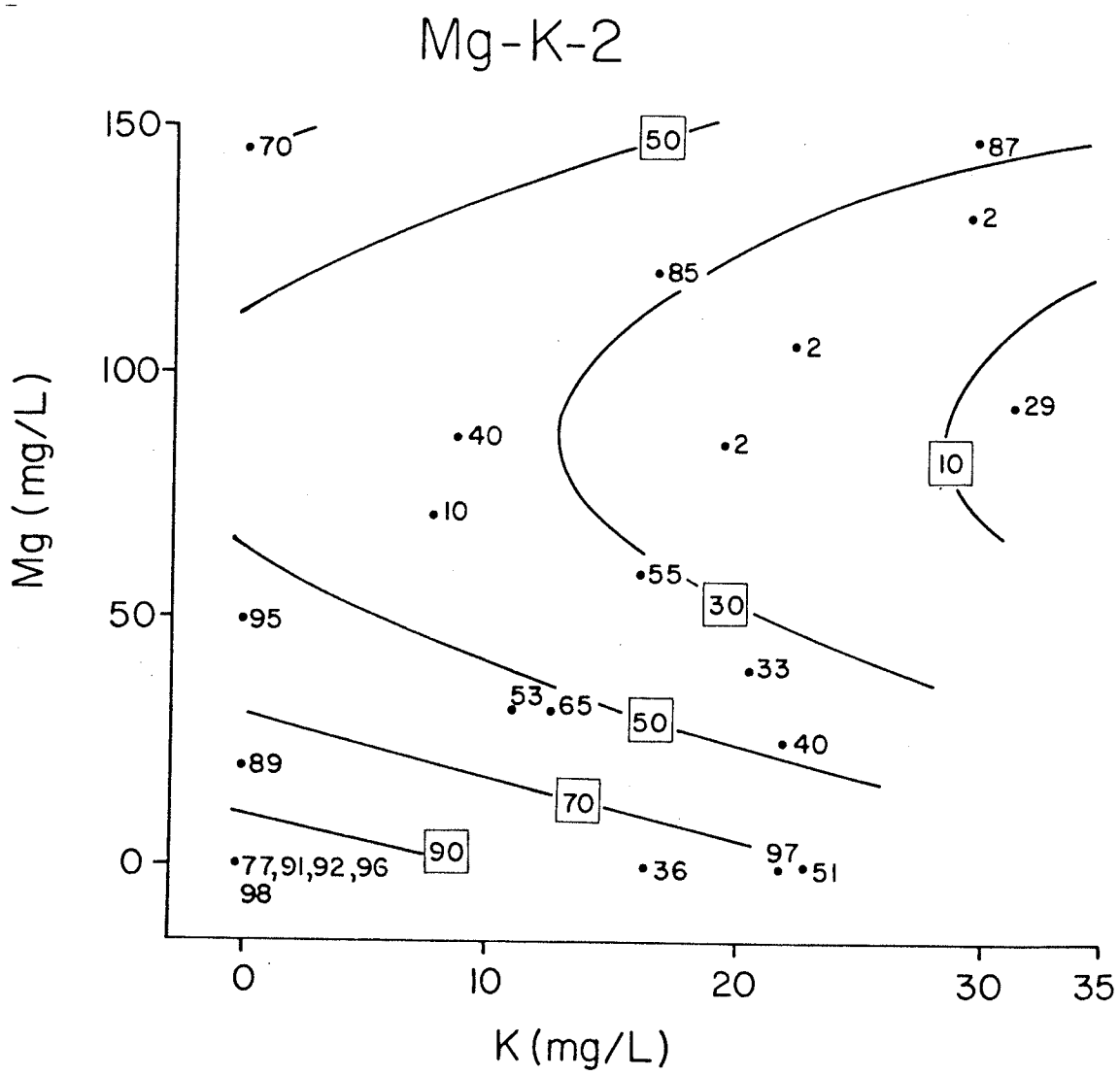

Figure D14. Percent survival at 48 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Mg and K, in Experiment Mg-K-2.

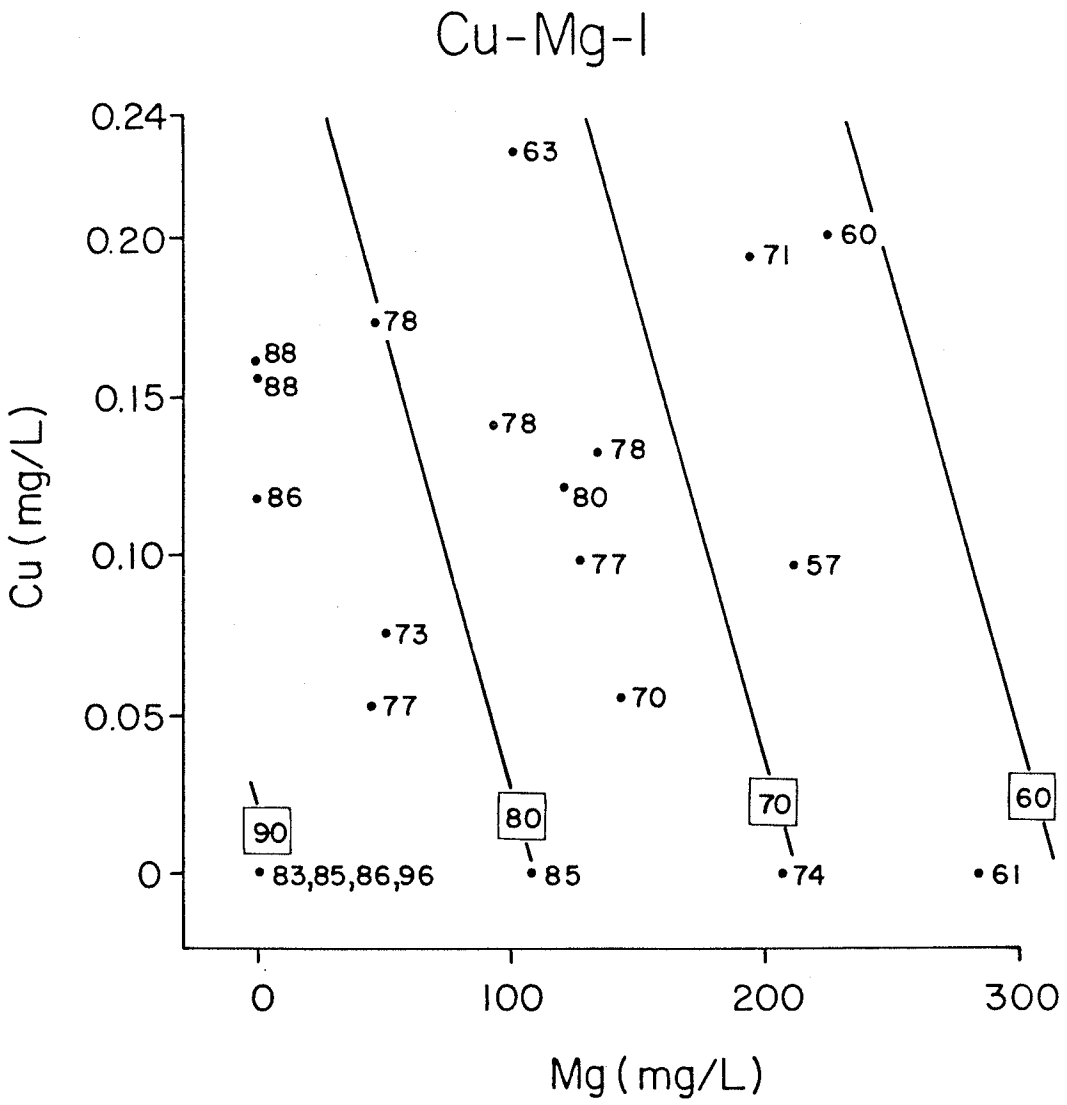

Figure D15. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cu and Mg, in Experiment Cu-Mg-1.

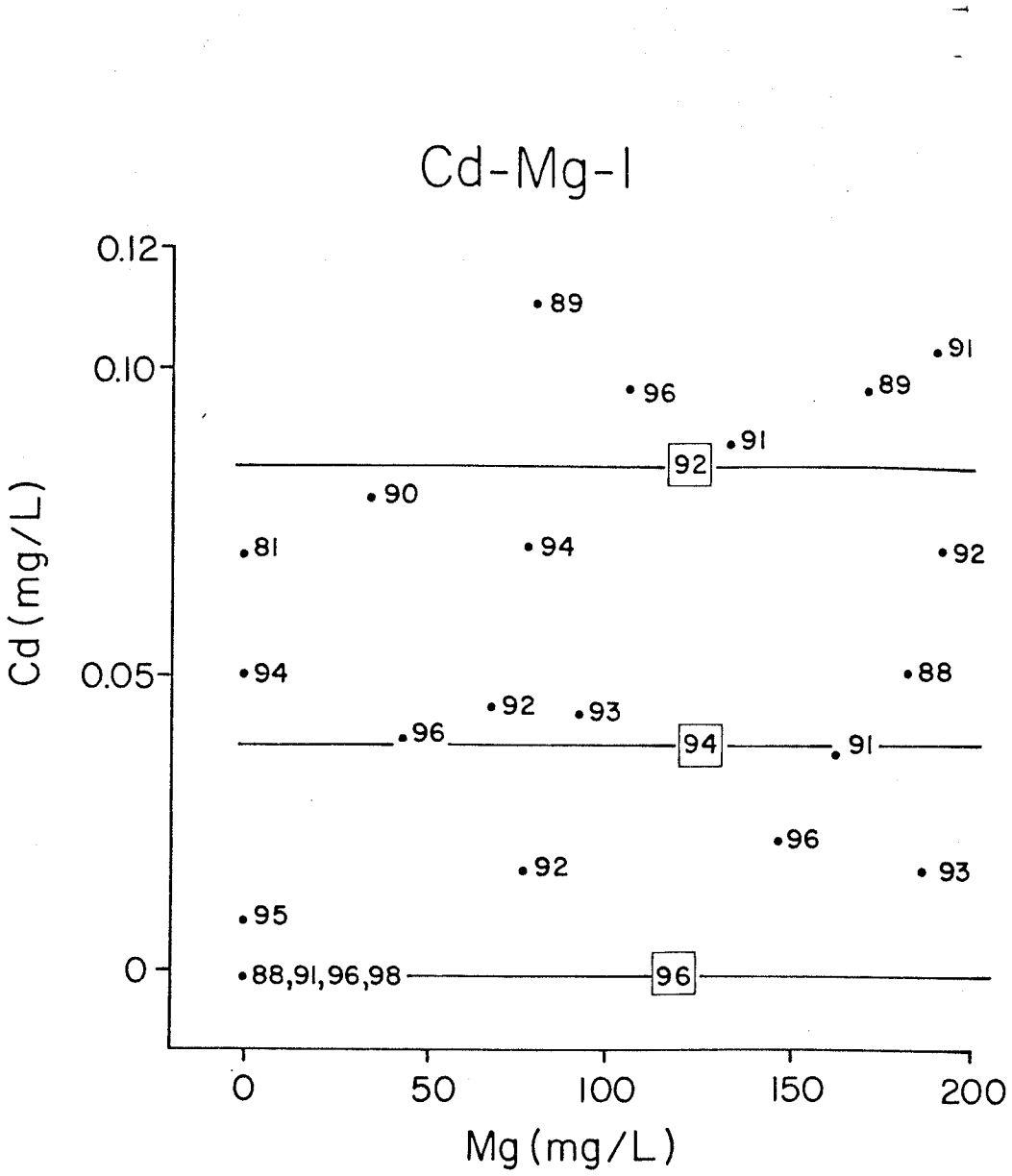

Figure D16. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Cd and Mg, in Experiment Cd-Mg-1.

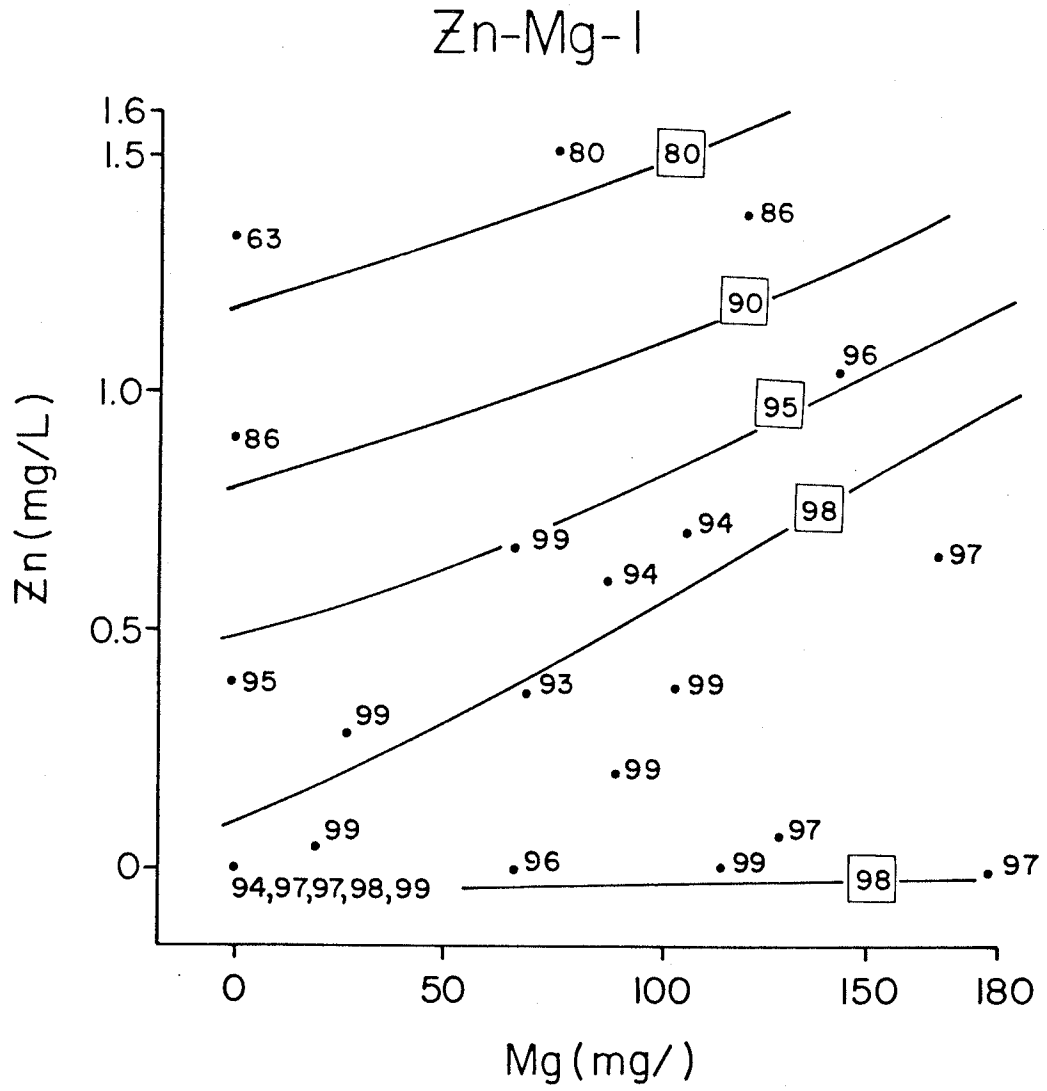

Figure D17. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Zn and Mg, in Experiment Zn-Mg-1.

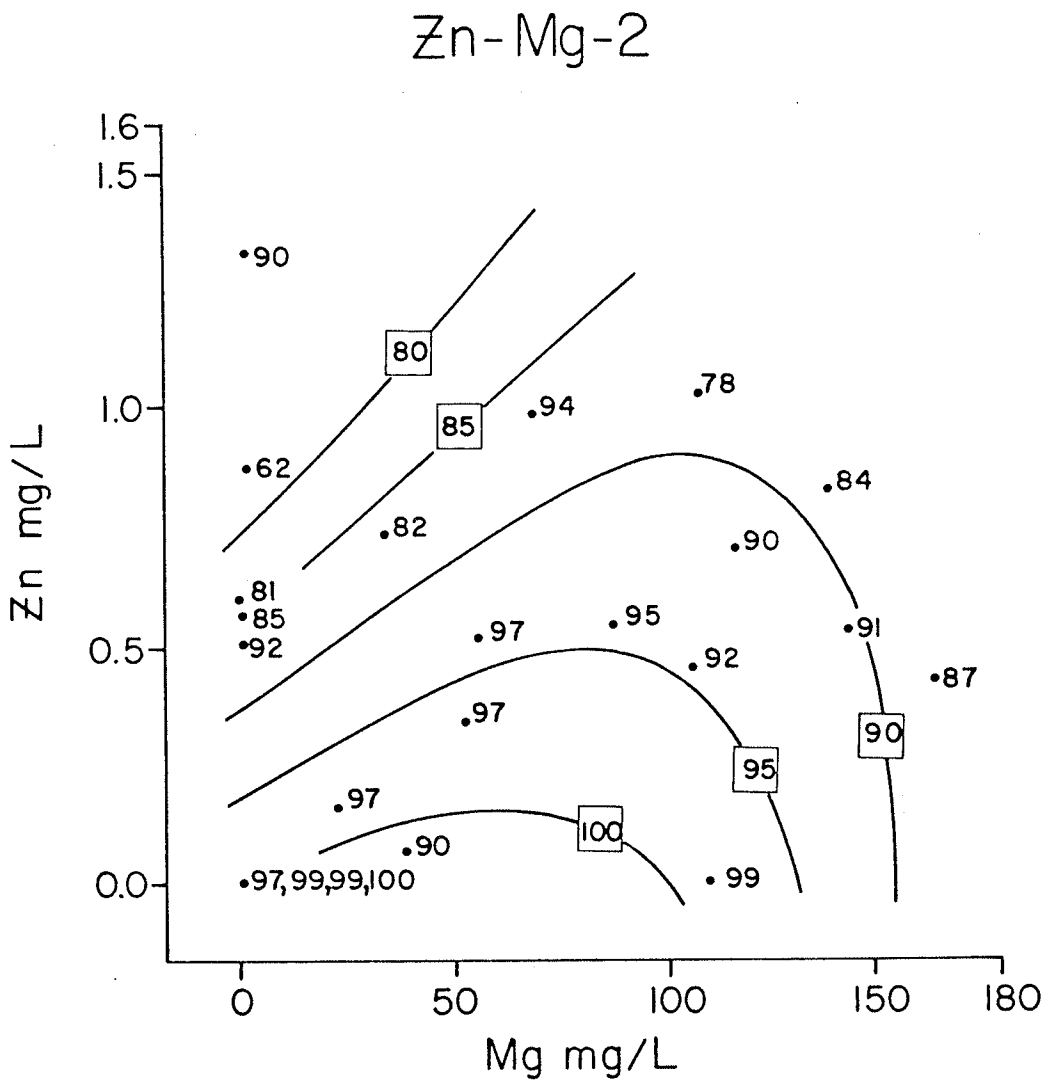

Figure D18. Percent survival at 96 hours and response isolines, predicted from equations in Table 5, plotted against the concentrations of Zn and Mg, in Experiment Zn-Mg-2.