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MULTIPLE TOXICITY ASSESSMENT FOR MIXTURES OF AQUATIC POLLUTANTS

Norman L. Weinstein

A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirements
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ABSTRACT

MULTIPLE TOXICITY ASSESSMENT FOR MIXTURES OF AQUATIC POLLUTANTS

Norman L. Weinstein

An approach to assessing quantitatively, the potencies of mixtures of toxic pollutants was employed for studying the multiple toxicity of copper and nickel both at lethal and sublethal levels to the zebrafish, *Brachydanio rerio*.

Experiments were performed in which groups of zebrafish were exposed to various fixed concentrations of copper and nickel as discrete toxicants and in mixtures. Exposure periods were ninety-six hours for lethal bioassays and ten days for sublethal bioassays. Reproduction was studied as an indicator of sublethal toxicity.

The results of lethal bioassays showed that mixtures of copper and nickel evoked responses which exceeded those predicted by theoretical models of CONCENTRATION-ADDITION and RESPONSE-ADDITION. A similar pattern of multiple toxicity was observed in sublethal bioassays, during which reproduction was almost completely inhibited. The interaction of these heavy metals in lethal and sublethal mixtures was therefore classified as SUPRA-ADDITIVE SYNERGISM. A quantitative model for assessing the potential hazards of mixtures of SUPRA-ADDITIVE toxicants is proposed.

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INTRODUCTION

Natural waters have become "mixing zones" for a diverse array of chemical pollutants which are released or mobilized in the environment as a consequence of man's activities. This pattern is promoted by water's effectiveness as a general chemical solvent, its cyclic movement through the geosphere, and by the tendency of many anthropogenic agents to persist. The convergence of pollutants within natural waters often results in chemical mixtures which are toxic in composition and therefore hazardous to the well-being of aquatic organisms. The insidious nature of such unpredictable mixtures circumvents traditional methods of risk assessment which rely on the screening of individual pollutants and point-source effluents. Consequently, the incidental accumulations of various anthropogenic agents in receiving waters pose a serious problem to water quality management agencies.

The poisonous actions and detrimental effects which are uniquely attributable to chemical mixtures are identified categorically by the phrase "multiple toxicity". Forms of multiple toxicity which are considered to be hazardous to aquatic organisms are characterized by: (1) potencies greater than those of the discrete constituents of a mixture; and/or (2) toxic effects different from and more harmful than the respective toxicities of the constituents of a mixture (Anderson and d'Apollonia, in press).

Because, as mentioned previously, traditional methods of risk assessment are not applicable for hazards related to mixing

zones, water quality management agencies must be provided with rationales that consider specifically, the unique problems posed by the multiple toxicities of incidental mixtures. Aspects that must be considered in such rationales are the diversity of incidental mixtures in terms of the identities and levels of the constituents, and the ubiquity of these mixtures in the environment. This information can only be derived through extensive environmental monitoring programs which survey receiving waters and aquatic organisms for contaminating chemical residues. Conceivably, the identification of those mixtures which are potentially detrimental to aquatic organisms may be based on epidemiological studies. However, it is virtually impossible to screen by means of such studies, all existing and potential combinations of pollutants for hazardous forms of multiple toxicity. Thus, it is essential that water quality management agencies understand the mechanisms of multiple toxicity and develop, through this understanding, rationales that effectively estimate the risk of toxic combinations.

Authorities in the field of water pollution control concede that the hazards of contaminant mixtures should be assessed not only in terms of the immediate survival of exposed organisms, but also in terms of the abilities of these organisms to continue the normal functioning of vital biological processes such as growth and reproduction. Studies have demonstrated that such vital processes may be impeded by sublethal mixtures of chemical pollutants (Weiss, 1959; Sprague et al., 1965; Negilski, 1973; d'Agostino and Finney, 1974; Muska and Weber, 1977). In general, sublethal stresses on the

organism may be ultimately reflected by ecological perturbations at the population level (Rosenthal and Alderdice, 1976). Yet in spite of this, there has been little concerted effort to develop rationales that effectively estimate the potential hazards of sublethal mixtures.

The primary purpose of this study was to investigate whether an approach which has been used to predict the potencies of lethal mixtures may be adapted to predict the potencies of sublethal mixtures. This approach is based on the premise that in many cases, the potencies of mixtures may be estimated from a knowledge of the respective potencies of the discrete constituents. The underlying models of multiple toxicity are tested against empirical demonstrations of the lethal and sublethal toxicities of mixtures of copper and nickel to the zebrafish, *Brachydanio rerio* (Hamilton-Buchanon). Reproduction, one of the most sensitive and ecologically meaningful indicators of sublethal toxicity (Brungs, 1969; Sprague, 1971), was studied as a response parameter.

Copper and nickel were selected as the toxicants to be studied because the multiple toxicity of these heavy metals at lethal levels to fish has already been shown to conform to a predictive model (Anderson and Weber, 1975a). Furthermore, nickel in its principle ores is found in combination with copper (Beliles, 1975). Consequently, these heavy metals frequently concur in surface waters, primarily as a result of dust emissions from mining operations and leachates from mine tailings. Such sources of copper and nickel pollution are apparently responsible for the contaminated watersheds in the mining areas of Northeastern Minnesota (USEPA, 1978) and Sudbury, Ontario

(Hutchinson and Stokes, 1975; Hutchinson et al., 1976; Whitby et al., 1976), where the high prevailing levels of these heavy metals are a cause for concern.

The zebrafish was chosen as the test organism for this study for the following reasons. The zebrafish is small and therefore easy to maintain in the laboratory, it is a continuous spawner that breeds well under laboratory conditions, it produces large numbers of demersal, non-adherent, and transparent eggs, and the timing of egg-laying in the laboratory may be controlled by adjusting the onset of light during the photoperiod (Laale, 1977; P. Anderson[1], personal communication). Furthermore, this species has been recommended by the International Standards Organization (1975) as a standard bioassay organism for the toxicity testing of aquatic pollutants.

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THEORY

In assessing the effects of toxicants on whole organisms it is convenient to correlate measures of an observable response with the intensity of an applied toxic stimulus. To this end, toxicity bioassays are conducted in which groups of organisms are exposed for an arbitrary time period to various fixed concentrations of a toxicant or toxicants. Reactions are measured in terms of either the proportions of animals demonstrating an all-or-none (quantal) effect, the exposure time required to elicit a particular response, or the magnitude of a graded effect.

To explain the relationship between the concentration of a toxicant and the observable response that it evokes in exposed organisms, one would wish to know the specific sequence of metabolic events which begins with exposure and ultimately leads to the response. However, such mechanistic knowledge is generally unattainable. To compensate, hypothetical models have been proposed to represent the dose-response[1] relationships which may be observed in toxicity

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[1]The words "dose" and "concentration" both refer to the quantity of a specific drug or toxicant within a unit volume or weight of medium, i.e. water, tissue, etc. However, pharmacology distinguishes between "dose", which is the precise level of a drug or toxicant fed to or injected into an organism, and "concentration", which is the level of a drug or toxicant to which an organism is exposed in its ambient environment. In the present study fish were exposed to toxicants which were dissolved in surrounding water. Thus, while the expression "concentration-response relationship" might in this case, be semantically more appropriate than the phrase "dose-response relationship", the latter phrase, which is conventionally used in toxicology, was adopted in this study in order to avoid confusion.

bioassays. From these models are deduced mathematical formulations which, when applied to limited empirical data, estimate the entire dose-response relationship across a wide range of toxicant intensity.

The type of statistical model which may be applied to toxicity data generally varies according to the kind of response parameter measured. For example, quantal response data are commonly subjected to probit analysis, a technique introduced by Bliss (1935) and refined by Finney (1971). This method prescribes the "maximum likelihood" fitting of a weighted linear regression to data in which the proportion of test organisms responding and concentration are transformed to the metameters of probit (normal equivalent deviate plus five) and common logarithm respectively. When the probit of response is plotted against the common logarithm of concentration, two relationships which are useful in toxicology are realized: (1) the reciprocal of the slope estimates the standard deviation within the sample's tolerance distribution; (2) the concentration which brings about a standard response level (such as 50%) may be used as a relative measure of the toxicant's potency for the sample of test organisms.

Statistical treatment of graded response data can be approached in two ways. When a graded effect is measured for each test animal in a bioassay the proportion of organisms whose reactions exceed an arbitrarily selected magnitude may be recorded and interpreted as quantal data. Conveniently, the statistical treatment of such data may then proceed according to the method of probit analysis as cited above. However, the reduction of graded data to a quantal form

results in a loss of information. Such a conversion conceals not only the absolute magnitude of each organism's reaction, but also the spread of the graded response distribution at each exposure concentration (Hewlett and Plackett, 1956).

For some graded responses it is impractical to collect precise measurements for each organism in a bioassay. In such cases another statistical approach may be adopted for the data analysis. The graded parameter is defined arbitrarily as the magnitude of the reaction of a single test organism, or either as the total or average reaction for a group of animals at each exposure concentration. By means of a least squares linear regression, the magnitude of the observed response is expressed as a function of the common logarithm of concentration. According to Bliss (1957), this model is usually suitable for quantifying graded dose-response data and is preferable to deriving a linear function based on the arithmetic values of toxicant concentrations. The former representation allows the researcher to determine, as in quantal data analysis, the relative potency of a toxicant in terms of the concentration which corresponds to a standard response level. If it is assumed that for each organism within a sample there exists a tolerance threshold which, in terms of concentration, denotes the highest level of a toxicant below which a particular graded response does not occur, then one may conceive of a distribution of such tolerance thresholds within the sample. In contrast to the relation between a quantal response curve and the corresponding tolerance distribution, the standard deviation of a tolerance

distribution for graded responses is not estimated by the reciprocal of the graded response curve's slope. Nevertheless, it is conceivable that the slope of a graded response curve depends to some extent on the distribution of tolerances for the observed response. This relationship however, has not yet been elucidated.

The interpretation of dose-response relationships for mixtures of toxicants is generally more involved. Two possible approaches have been identified (Plackett and Hewlett, 1952; Schild, 1961). The first approach is to account for empirical data by deriving an arbitrary function which relates observed responses to the test concentrations of the constituents. The second approach is to postulate theoretical models of the physiological mechanisms of toxic interactions, to derive the corresponding predictions of response for the mixtures tested, and to determine which of these predictions best accord with empirical data. Although Marubini and Bonanomi (1970) claimed that the first approach for evaluating the toxicities of mixtures involves a simpler experimental design, Plackett and Hewlett (1952) considered the second approach to be more rationale and ultimately more useful. As is apparent in the literature, most multiple toxicity studies are based on the application of theoretical models of toxic interaction.

A variety of hypothetical models have been proposed and discussed with reference to the quantal responses of organisms to mixtures of toxicants (Bliss, 1939; Plackett and Hewlett, 1948, 1952, 1963, 1967; Hewlett and Plackett, 1952, 1957, 1959, 1964; Lowe, 1953; Ashford and Smith, 1964, 1965; Sprague and Ramsay, 1965; Brown, 1968; Marubini and Bonanomi, 1970; Finney, 1971;

Abt et al., 1972; Anderson and d'Apollonia, in press; Anderson and Weber, 1975a; Muska and Weber, 1977; Seba, 1975). Most of these models relate to certain pharmacological assumptions regarding drug action and distinguish in concept between those toxic constituents which, within mixtures, act similarly on the same type of physiological receptor[1] in organisms and those which act independently on different types of receptors. Bliss (1939), whose influential work in this field is frequently cited, assumed that the tolerances of individual organisms to lethal levels of similarly acting toxicants are completely and positively correlated, a feature which is reflected by parallel quantal response curves for the toxicants. On the other hand, Bliss (1939) stated that the tolerances of individual organisms to lethal levels of independently acting toxicants are not necessarily correlated; thus, the corresponding quantal response curves may or may not be parallel. These assumptions have generally been accepted by others who study toxic mixtures. However, Hewlett and Plackett (1959) have presented an opposing argument which asserts that the tolerances of individual organisms to poisons which act on the same type of receptor need not be completely and positively correlated, and that parallelism of quantal curves may, in many instances, not not be a valid criterion for similar action. In the same article Hewlett and Plackett proposed a "unified theory for quantal responses

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[1]Receptor, in this content, is defined as a toxophilic chemical group which evokes a physiological response by combining with a sterically complementary toxicant.

to mixtures of drugs" which, in its rationale, considers the possibility of non-parallel dose-response curves for similarly acting toxicants. However, the practicality of their mathematical model is limited in that its equations require the quantification of certain parameters which generally cannot be measured in bioassays using whole animals.

Although any quantal response of an organism to a toxicant is presumed to be a result of an underlying graded reaction (Hewlett and Plackett, 1956), most of the proposed multiple toxicity models have been limited to the assessment of quantal effects. Consequently, only a few statistical procedures have been suggested for exploring multiple toxicity in terms of graded reactions (Lowe, 1953; Zipf and Hamacher, 1966; Marubini and Bonanomi, 1970; Fedeli et al., 1972; Ashford and Cobby, 1974; Muska and Weber, 1977). Fedeli and his associates (1972) commented on some of the statistical treatments which have been applied. Of those procedures considered, a response surface methodology was recommended as the most rewarding. However, this technique is not suitable for many studies in that it requires, as Fedeli and his colleagues (1972) pointed out, a large quantity of empirical data.

Rather than developing new multiple toxicity models specifically for graded effects, a more practical approach might be to modify existing multiple toxicity models which have been adapted for quantal studies. This latter suggestion seems reasonable because many of the basic pharmacological assumptions upon which quantal models are based, pertain to graded dose-response phenomena

as well.

In the present study theoretical models of multiple toxicity based on those developed by Bliss (1939) and Plackett and Hewlett (1948) for quantal data, are applied both in quantal and in graded response experiments. Once it is demonstrated that empirical data are statistically represented by a particular model, it is inferred that the presumptive physiological mechanism of toxic interaction upon which the model is derived, applies to the dose-response system studied.

The literature is replete with a confounding array of terms used to define (often inconsistently) the various forms of multiple toxicity according to whether empirical responses are equal to, less than, or greater than those predicted by hypothetical models (see Ariens, 1972). In the present study a terminology employed by Anderson and Weber (1975a) has been used to classify the various forms of multiple toxicity.

Quantal Responses

1. RESPONSE-ADDITION:

The model of RESPONSE-ADDITION is derived on the postulate that individual toxicants within a mixture may stimulate different types of receptors in exposed organisms and contribute to a common effect. Such toxicants are described as "independently acting". The model assumes that an independently acting toxicant within a mixture contributes to the overall response of an exposed organism only if the concentration of that constituent exceeds its particular

reaction threshold[1]. Furthermore, exposure to any one constituent does not alter the reactions of an animal to other constituents in a mixture.

According to the model of RESPONSE-ADDITION, the potency of a mixture may be predicted from a knowledge of the potencies of the separate constituents. In this context, potencies are expressed in terms of the proportions of individuals demonstrating the response which is being studied.

Predictive formulations for this model consider that within any population or sample of organisms, the tolerances of individuals to different independently acting toxicants may or may not be correlated. If for a particular sample of organisms there is no correlation of tolerances to two RESPONSE-ADDITIVE toxicants, A and B, then the proportion of animals which will respond to a binary mixture is predicted by:

$$P_{AB} = P_A + P_B(1-P_A) \qquad (1)$$

where,

P_{AB} = proportion responding to mixture of A and B

P_A = proportion responding to A alone

P_B = proportion responding to B alone.

When the correlation in tolerances is completely positive, i.e., those animals which are most tolerant to A are also most tolerant to B, the response in a mixture is evoked by the more

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[1] A reaction threshold is the minimum concentration of toxicant which is sufficient to evoke a particular observable reaction in an organism.

toxic constituent. In this instance the multiple toxocity is given by:

$$P_{AB} = \begin{cases} P_A, \text{ if } P_A > P_B \\ P_B, \text{ if } P_B > P_A \end{cases} \quad (2)$$

If the correlation in tolerances is completely negative, i.e. those animals most tolerant to A are least tolerant to B, and vice versa, then the proportion of animals which will respond in a mixture is predicted by:

$$P_{AB} = \begin{cases} P_A + P_B, \text{ if } P_A + P_B < 1 \\ 1, \quad \text{if } P_A + P_B \geq 1 \end{cases} \quad (3)$$

2. CONCENTRATION-ADDITION:

The model of CONCENTRATION-ADDITION is based on the postulate that individual toxicants within a mixture can stimulate the same type of receptor in exposed organisms. Such toxicants are described as "similarly acting". The number of receptors which are occupied is therefore cumulative for all similarly acting poisons in a mixture. Therefore, in contrast to RESPONSE-ADDITIVE toxicants, a CONCENTRATION-ADDITIVE constituent does contribute to the overall response of an organism in a mixture even if the concentration of that constituent is below its particular reaction threshold.

According to the model of CONCENTRATION-ADDITION, the potency of a mixture may be predicted from a knowledge of the potencies of the separate constituents. This involves the empirical determination of a "relative potency factor" which denotes the

ratio of equipotent concentrations for the poisons in a mixture. If the individual dose-response curves for the single toxicants J and K are defined by the "maximum likelihood" probit regressions as follows:

$$Y_J = a_J + b_J \log_{10} C_J \tag{4}$$

$$Y_K = a_K + b_K \log_{10} C_K \tag{5}$$

where,

Y = response in probits

C = concentration of toxicant

a = intercept

b = slope,

then the relative potency factor $R_{K \cdot J}$, which compares equipotent concentrations of K and J at a given level of response Y* is:

$$R_{K \cdot J} = \text{antilog}_{10}\left[\frac{Y^* - a_K}{b_K} - \frac{Y^* - a_J}{b_J}\right] \tag{6}$$

When the individual dose-response curves for J and K (Equations 4 and 5) are parallel, Equation (6) may be simplified to define a constant $R_{K \cdot J}$ as follows:

$$R_{K \cdot J} = \text{antilog}_{10}\left[\frac{a_J - a_K}{b}\right] \tag{7}$$

where,

$$b = b_J = b_K$$

Thus, multiplying any concentration C_J by its relative potency factor, $R_{K \cdot J}$, mathematically converts C_J to an equipotent concentration C_K' (note that if $R_{K \cdot J}$ is calculated by Equation 6, Y* is the response level corresponding to C_J; see Equation 4). If J and K are CONCENTRATION-ADDITIVE, then the total concentration of a mixture may be expressed as C_K' plus C_K. If this sum is substituted for the independent variable C_K in Equation (5), then the potency of the mixture is predicted as follows:

$$Y = a_K + b_K \log_{10}(R_{K \cdot J} C_J + C_K) = a_K + b_K \log_{10}(C_K' + C_K) \quad (8)$$

3. SUPRA-ADDITIVE SYNERGISM:

Toxicants in mixtures that evoke responses which exceed predictions based on models of RESPONSE-ADDITION and CONCENTRATION-ADDITION are defined as SUPRA-ADDITIVE in their multiple toxicity. SUPRA-ADDITIVE SYNERGISM may result from interactions with pharmacokinetic processes which determine the actual quantity of toxicant(s) available at receptors, or from pharmacodynamic processes which mediate the observed reaction. Theoretically, this form of multiple toxicity may occur in mixtures whose constituents act similarly on the same type of receptor or independently on different types of receptors; however, a distinction between these possibilities cannot be deduced on the basis of whole organism studies. Unlike the multiple toxicities of RESPONSE-ADDITIVE or CONCENTRATION-ADDITIVE poisons, the potency of a mixture of SUPRA-ADDITIVE toxicants cannot be predicted solely

from a knowledge of the dose-response relationships for the constituents. Thus, SUPRA-ADDITIVE SYNERGISM which often results in unpredictably high levels of response, must be considered a particularly hazardous form of multiple toxicity.

4. INFRA-ADDITIVE ANTAGONISM:

INFRA-ADDITIVE toxicants in mixtures elicit responses which are less than those predicted based on models of RESPONSE-ADDITION and CONCENTRATION-ADDITION. Interactions with pharmacokinetic or pharmacodynamic processes presumably account for INFRA-ADDITIVE ANTAGONISM, which may be induced by mixtures whose constituents act on the same or on different types of receptors. Although the potency of a mixture of INFRA-ADDITIVE toxicants cannot be predicted solely on the basis of the dose-response relationships for the constituents, this form of multiple toxicity, which often results in low levels of response, is not considered to be particularly hazardous.

Graded Responses

The statistical criteria as outlined for the various multiple toxicity models which are described under the heading "Quantal Responses" do not apply to graded multiple toxicity data unless the measured responses are converted to a quantal form. The following discussion describes a theoretical basis upon which statistical criteria for multiple toxicity models may be derived specifically for graded responses. The rationale pertains to data which express the effect of a toxicant or toxicants in terms of

the resultant decrease in the capacity of a quantifiable biological function such as egg production or growth. The baseline capacity of such a function is measured under control conditions.

A plausible explanation for the observation that an individual organism's graded reaction to a toxicant generally increases with concentration may be based on the Simple Occupation Theory of pharmacodynamics (Albert, 1973) and the Law of Mass Action. According to these principles the graded effect of a drug is related proportionally to the fraction of receptors occupied by drug molecules, while the number of occupied receptors is itself dependent on the quantity of drug available at the site of action. It follows logically therefore, that the graded effect of a drug or toxicant is directly related to the concentration at the site of action.

The same theoretical principles may explain the observed relationship between the concentration of a toxicant and the total graded effect that it evokes in a group of organisms. Within any such group there is presumably, a critical cumulative number of specific receptors which, if all occupied, results in the total blocking or 100% reduction in the capacity of the biological function studied. The toxicant concentration which is sufficient to occupy this critical number of receptors may therefore be determined empirically. Conceivably, lower concentrations would stimulate fewer than this critical number of receptors and would consequently result in less drastic effects. The adaptation of multiple toxicity models for graded response data is based on the

assumption that the decrease in a function's capacity, as measured for a group of animals, is proportionally related to the fraction of the critical number of receptors occupied, which itself is directly dependent on the concentration of toxicant.

1. RESPONSE-ADDITION:

If the response of a group of organisms to each of two RESPONSE-ADDITIVE toxicants, A and B, is a particular reduction in the capacity of a specific biological function, then the response to a mixture of A and B is given by:

$$P_{AB} = P_A + P_B(1-P_A) \qquad (9)$$

where,

P_{AB} = reduction in capacity of a biological function as a result of exposure to mixture of A and B

P_A = reduction in capacity of a biological function as a result of exposure to A alone

P_B = reduction in capacity of a biological function as a result of exposure to B alone.

The factor "$1-P_A$" is necessary in Equation (9) because B can only reduce the capacity of a function in terms of what has not already been reduced by A.

2. CONCENTRATION-ADDITION:

If the individual dose-response curves for the single toxicants J and K are given by the following least squares linear regressions:

$$Y_J = a_J + b_J \log_{10} C_J \tag{10}$$

$$Y_K = a_K + b_K \log_{10} C_K \tag{11}$$

where,

Y = reduction in capacity of a biological function

C = concentration of toxicant

a = intercept

b = slope,

then the relative potency factor $R_{K \cdot J}$ which compares equipotent concentrations of K and J at a given level of response Y* is:

$$R_{K \cdot J} = \text{antilog}_{10}\left[\frac{Y^* - a_K}{b_K} - \frac{Y^* - a_J}{b_J}\right] \tag{12}$$

When the individual dose-response curves for J and K (Equations 10, 11) are parallel, Equation (12) may be simplified to define a constant $R_{K \cdot J}$ as follows:

$$R_{K \cdot J} = \text{antilog}_{10}\left[\frac{a_J - a_K}{b}\right] \tag{13}$$

where,

$$b = b_J = b_K$$

Thus, multiplying any concentration C_J by its relative potency factor $R_{K \cdot J}$, mathematically converts C_J to an equipotent concentration C_K'. If J and K are CONCENTRATION-ADDITIVE with respect to their effects on a given graded response parameter, then the potency of a mixture of J and K is given by:

$$Y = a_K + b_K \log_{10}(R_{K \cdot J} C_J + C_K) = a_K + b_K \log_{10}(C_K' + C_K) \quad (14)$$

3. SUPRA-ADDITIVE SYNERGISM; INFRA-ADDITIVE ANTAGONISM:

Toxicants comprising mixtures in which the reduction in capacity of a biological function exceeds predictions based on models of RESPONSE-ADDITION and CONCENTRATION-ADDITION are defined as SUPRA-ADDITIVE in their graded multiple toxicity. When empirical observations of multiple toxicity are less than predicted the constituents of mixtures are defined as INFRA-ADDITIVE.

MATERIALS AND METHODS

Test Organism

Zebrafish (Figure 1) were purchased as required from Aquaking and Tropicarium, tropical-fish distributors in Montreal. Upon arrival at the laboratory, fish were quarantined in a 352-liter fibreglass tank for several weeks during which time they were observed for apparent signs of disease, i.e. curvature of trunk, emaciation, etc.

Acclimation

Following the quarantine period, test organisms were transferred to twelve 51-liter glass aquaria (60.1 cm X 30.8 cm X 27.5 cm) where they were acclimated to control conditions for at least two weeks. These aquaria were supplied with a continuous flow of filtered, dechlorinated, and degassed water at a temperature of 24 or 27 C (acclimation temperatures for lethal and sublethal experiments respectively). A diurnal photoperiod of twelve hours light (8 AM - 8 PM) was maintained by overhead fluorescent fixtures which were controlled by a time switch.

During acclimation, fish were fed according to the following regimes. Test organisms which were to be exposed in lethal bioassays received Tetramin Staple tropical fish food each morning and Ewos trout and salmon food each afternoon. Fish which were designated for sublethal bioassays were fed Tetramin each morning and a prepared blend of food each afternoon. This blend consisted of minced, dried, and pulverized beef liver, calf heart, and Ewos trout and salmon

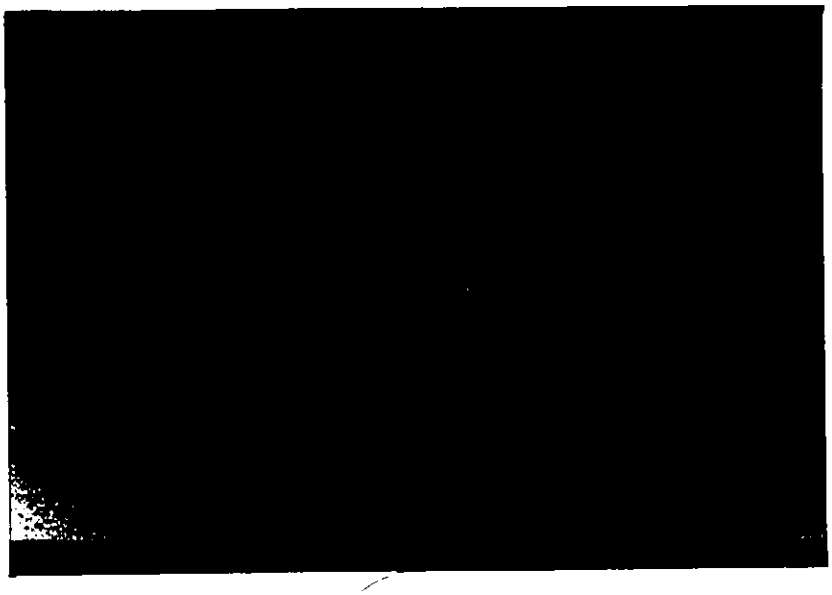

Figure 1. Photograph of the zebrafish, *Brachydanio rerio*

food, in a 2:1:1 ratio by weight (Kruzynski, 1972). The feeding of zebrafish according to this particular regime resulted in the production of large numbers of eggs through time. In contrast, previous diets of Tetramin and Ewos trout and salmon food, alone or in combination, resulted in low fecundity.

Preparations for Exposure of Test Organisms

Several days prior to experimentation, acclimating fish which appeared to be healthy were individually wet-weighed and distributed into lots representing discrete weight classes. These weight classes were arbitrarily defined by dividing the total weight-range of test organisms into several narrower and equal weight-ranges. The mean weight of fish within each lot varied not more than ±0.06 mg.

Only those fish which were designated for sublethal experiments were sorted according to gender. The criterion for differentiating the sexes was the presence of a distinct genital papillum in the female. This sexual dimorphism, which is apparent both in mature and immature fish, was considered to be a more reliable distinguishing feature than the secondary sex characteristics of body size, fin size, and colour, which may be variable in nature.

Because handling stress might influence the sensitivities of test organisms to toxicants, fish were returned in their discrete weight classes to the acclimating aquaria where they were maintained and observed for a few days prior to experimentation.

Before onset of the sublethal experiments the separate, discrete weight classes of males and females were paired such that the lots

representing the smallest, intermediate, and largest males were combined with the corresponding lots representing the smallest, intermediate, and largest females.

Toxicants

Toxicant stock solutions were prepared by dissolving reagent grade cupric sulfate ($CuSO_4 \cdot 6H_2O$; Anachemia) or industrial grade nickelous sulfate ($NiSO_4 \cdot 5H_2O$; Canadian Industries Ltd.) in glass-distilled water. Industrial grade nickelous sulfate was considerably more economical than the reagent grade of this salt for the large quantity required. In order to compare the purities of these grades, atomic absorption analyses were performed for a few selected heavy metals which might have been present. Based on the results of these analyses (Table 1), the levels of contaminating heavy metals in the industrial grade nickel salt were considered to be negligible. Stock solutions were stored in 27-liter glass Mariotte bottles (Grenier, 1960) for the duration of each experiment.

Exposure System

Stock solutions of copper and nickel were dripped at constant rates from their respective Mariotte bottles into a toxicant-diluting apparatus. This "diluter", which was constructed of five plexiglass chambers, was designed to continuously dilute, mix, and deliver precise concentrations of toxicant solutions either discretely or in mixtures to twelve 51-liter glass, flow-through aquaria (Figures 2, 3a, 3b). In each of the top four chambers a glass standpipe served to maintain a constant height of water or toxicant solution. Diluent

Table 1. Atomic absorption analyses of nickelous sulfate salts

Assay	Reagent grade (% of nickel)	Industrial grade (% of nickel)
Copper	0.0024	0.0012
Cadmium	0.0005	0.0005
Lead	0.0035	0.0079

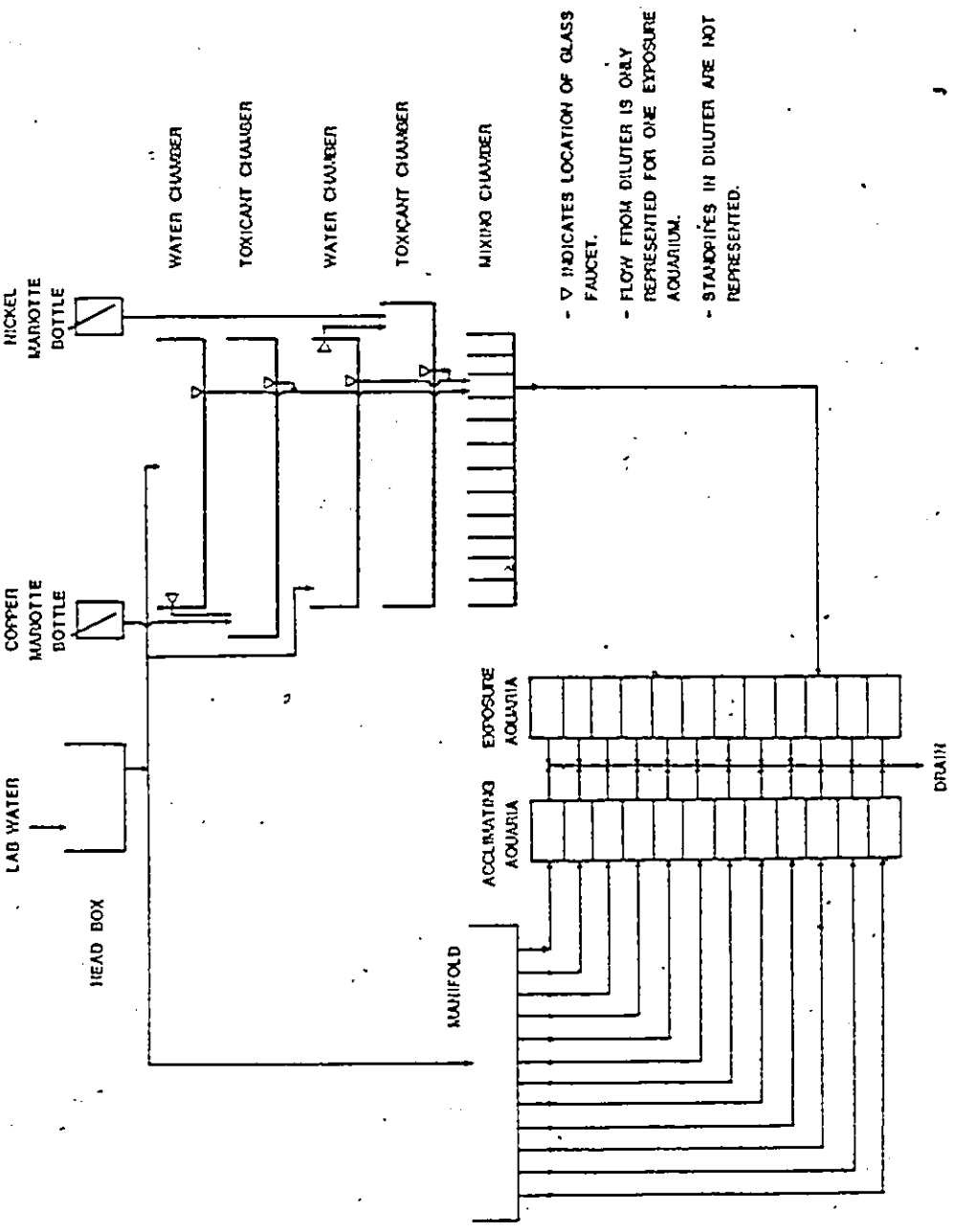

Figure 2. Schematic of exposure system

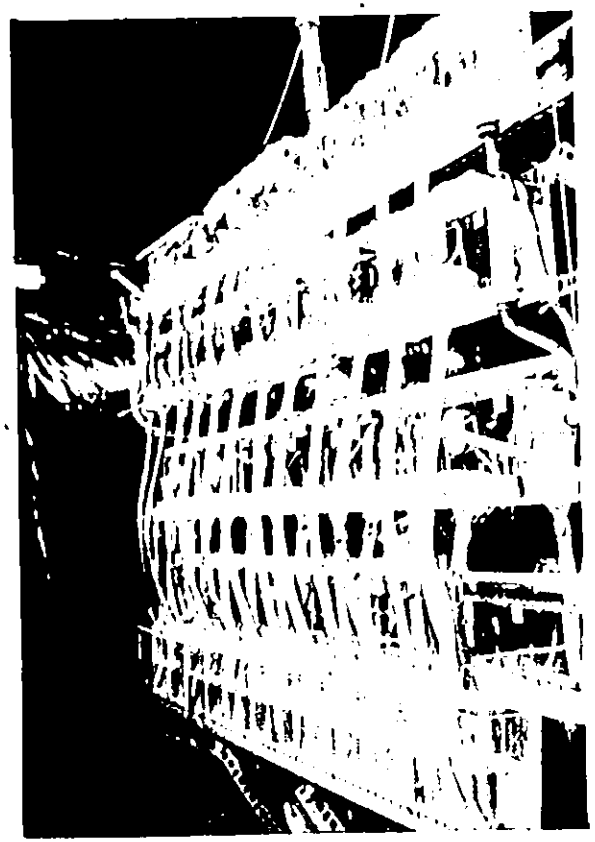

Fig. 3a.

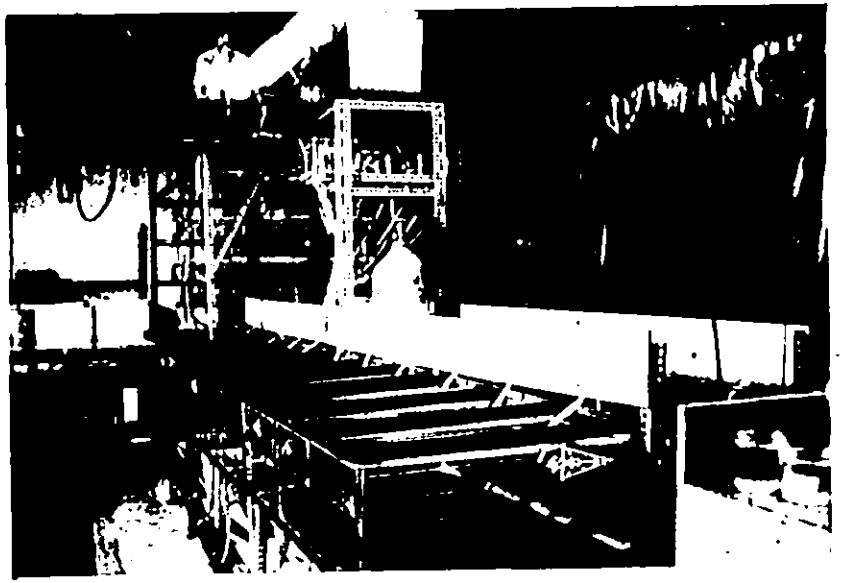

Fig. 3b.

Figure 3a. Photograph of diluter

Figure 3b. Experimental apparatus showing acclimating aquaria, head box, and Mariotte bottle; exposure aquaria on opposite long side of apparatus are not visible

water from a constant-output head box flowed by gravity through polyvinyl chloride pipes into the top and third chambers of the diluter. A rotatable glass faucet in the side of each of these "water chambers" was adjusted[1] to regulate a gravity-flow of diluent water into the second or fourth chamber below, each of which received a simultaneous input of its respective toxicant stock solution. These "toxicant chambers" were aerated in order to generate currents to mix the toxicant ions in solution, thus promoting a first phase of dilution. Water and toxicant solutions from the top four chambers were drained through adjustable faucets and collected in twelve independent channels of Tygon tubing, where second phase dilutions occurred. The resultant solutions flowed by gravity through these channels into the bottom chamber of the diluter. Here, final mixing took place within a separate compartment for each of the twelve diluted solutions. When the diluter was employed to prepare mixtures of copper and nickel, the compartments in the bottom chamber also served to combine the toxicant solutions, thus providing a third phase of dilution. After final mixing the test solutions were delivered through glass tubing and Tygon tubing to the respective exposure aquaria. To divert the flow of toxicant in the event of a "slow-down" or interruption of the supply of diluent water, a mechanical failsafe device was designed and employed (Figure 4).

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[1] At any instant the flow rate of water through a faucet was directly dependent on the distance between the faucet outlet and the surface of the headwater in the chamber. Thus, a flow rate could be adjusted by rotating the faucet such that this vertical distance would be appropriately increased or decreased.

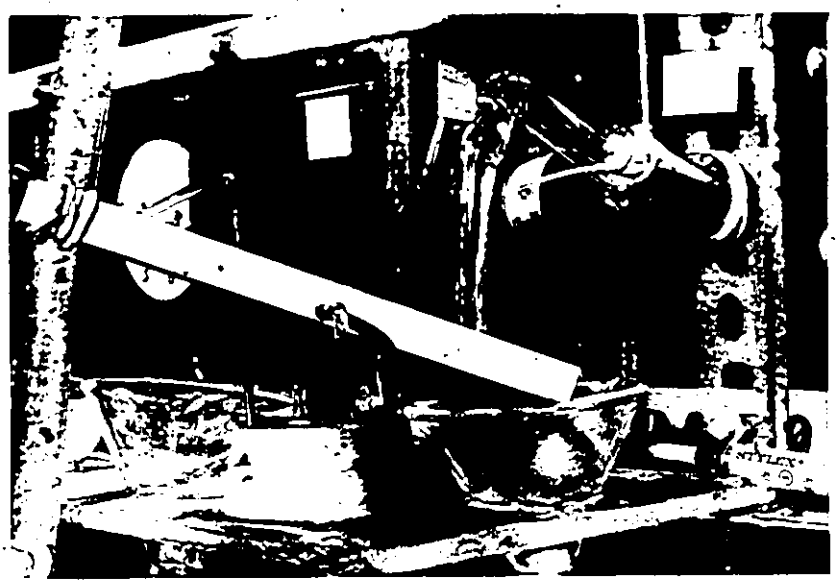

Figure 4. Failsafe device for diluter. The failsafe device consists of a pivoting plastic trough. In the position shown the trough serves to guide both toxicant stock solution (seen dripping from the polyethylene "bleeder tube" which is connected to a Mariotte bottle) and diluent water (seen pouring from the large faucet) into a funnel. This funnel empties its contents into the "toxicant chamber" below (not shown). The trough remains in this position only as long as the diluent water is pouring forcefully against it. In the event of a "slow-down" or interruption in the supply of diluent water, counterweights attached to the left end of the trough (see elastic bands) flip the trough so that the left end dips downward. The toxicant stock solution, which continues to drip independently, is thus diverted by the trough away from the funnel which empties into the diluter.

The exposure apparatus was designed to allow toxicant solutions to equilibrate in their respective test aquaria prior to the actual exposure of organisms. During this equilibration period fish were accommodated in the twelve acclimating aquaria which were served with an independent supply of diluent water from the head box.

The diluter was adjusted to deliver test solutions at a constant rate of 300 ml/min to each of the exposure aquaria, thereby providing for ninety percent replacement of solution in less than six hours (Sprague, 1973). This rate of replacement was considered to be adequate for maintaining fish.

Appendix I outlines sample calculations which demonstrate the protocol followed to compute nominal test concentrations and required flow rates for faucets and Mariotte bottles. Toxicant concentration is expressed as mg/l copper or nickel. All flow rates were checked daily using graduated cylinders and a stopwatch. Faucets were readjusted if necessary in order to ensure the dilution of toxicant solutions to precise nominal concentrations. The delivery of toxicant solutions was such that nominal test concentrations were randomized among the twelve exposure aquaria.

Water Analysis

It has been demonstrated that physical and chemical characteristics of water may modify the toxicities of aquatic pollutants (Brown, 1968; Sprague, 1970). During all experiments water temperature was regulated by a thermostatic heater which was installed in the head box. For the sublethal experiments additional thermostatic heaters in the aquaria were operated to regulate temperature more

precisely. Dissolved oxygen, pH, hardness, alkalinity, and temperature were measured periodically (temperature was recorded daily for sub-lethal experiments). Dissolved oxygen was determined by the azide modification of the iodometric method (APHA et al., 1965). Total alkalinity and total hardness were measured by the mixed brom-cresol green-methyl red and the titrimetric method respectively (APHA et al., 1965). According to these determinations the physical and chemical characteristics of the water remained fairly constant during each experiment. Table 2 reports the mean values of these parameters along with the results of water quality tests which were performed by the City of Montreal on the source water of the Water Pollution Research Laboratory.

Daily samples of all test solutions were routinely collected from exposure aquaria and stored in Pyrex test tubes for subsequent analysis. Two alternative techniques were employed in order to reduce the loss of detectable metal ions from sample solutions due to surface absorption on the glass walls of test tubes. According to one technique, which was devised by the author, test tubes were soaked in their respective exposure aquaria for approximately twenty-four hours prior to sample collection. The purpose of this procedure was to promote an equilibrium between toxicant ions in solution and toxicant ions absorbed on the walls of the sample tubes, thereby reducing the tendency for ions to become lost from solution after sample collection. According to the alternative technique samples were collected and acidified with two to four drops of concentrated nitric or hydrochloric acid.

All samples of test solutions were analyzed by a Perkin-Elmer 503 atomic absorption spectrophotometer. Samples containing low levels of copper were atomized in a graphite furnace; a flame atomizer was used for all other samples containing nickel or higher levels of copper.

Table 2. Water quality data

Analysis of laboratory water used in experiments		
dissolved oxygen	% saturation	87
temperature	°C	24 (lethal expts.), 27 (sublethal expts.)
pH		7.7
total alkalinity	mg/l as $CaCO_3$	81
total hardness	mg/l as $CaCO_3$	128
Analysis performed at the City of Montreal filtration plant		
colour	STD	5
turbidity	JTU (formazin)	0.4
total residue	103°C, mg/l	190
loss on ignition	550°C, mg/l	92
silica	mg/l SiO_2	37.4
calcium	mg/l Ca^{++}	37.4
magnesium	mg/l Mg^{++}	8.1
sulfates	mg/l SO_4^{--}	26
chlorides	mg/l Cl^-	27
sodium	mg/l Na^+	12.3
potassium	mg/l K^+	1.4
fluorides	mg/l F^-	0.15
iron	mg/l Fe^{+++}	0.012
carbon dioxide	mg/l CO_2	0.3
detergents	LAS	0.017

Design of Lethal Response Studies

Following the acclimation period lots of ten or fourteen fish were transferred in their discrete weight classes to the twelve exposure aquaria. During the ninety-six hours of exposure each experimental lot was observed at least every four hours for mortality; cessation of respiratory movements was adopted as the criterion of death. Observations were recorded and fish which had died were promptly removed.

Analysis of the lethal data was based on the probit model (Bliss, 1935). Finney's (1971) method of probit analysis was selected in preference to a number of alternative procedures which trade off precision in exchange for computational simplicity. In order to reduce the labour of processing large quantities of data a computer program was designed for Finney's method and applied (Appendix II).

Consideration of Body Weight as a Tolerance-Modifying Variable

Certain inherent biological factors such as sex, age, size, or nutritional state may influence the responses of organisms to chemical stimuli (Brown, in press). The fact that groups of organisms are generally non-homogeneous with respect to such factors provides an explanation for the apparent variability in the individual reactions to a toxic stressor. Theoretically, this variability could be eliminated if the experimental animals were selected to be completely homogeneous for all of the biological factors which modify their individual susceptibilities. Alternatively, the variability in reactions within a group of organisms may be

considerably reduced if very large samples are subjected to the toxic stressor so as to "average out" the effects of the inherent biological modifying factors. However, due to practical constraints these methods are often impossible to employ in research.

A more feasible and informative approach for dealing with biological variability is to determine the quantitative functions which represent the relationships between response and modifying factors. The information gained is then incorporated in the analysis of dose-response data. These data are standardized mathematically with respect to the biological influencing variables. In this way the responses of organisms are expressed as a quantitative function of dose or concentration as well as of the biological modifying factors studied.

The latter approach was adopted in this study in an attempt to adjust for variations in response which are hypothetically due to differences in the sizes of test organisms. Ordinarily, biases of size are unwittingly introduced when small samples of animals are selected. In order to incorporate the factor of size as a controlled variable, organisms were exposed in discrete arbitrarily defined weight classes to concentrations of toxicant (see "Preparations for Exposure of Test Organisms").

Analyses of the lethal dose-response data were based on Anderson's and Weber's (1975b) modification of the linear probit model (as well as on Finney's, 1971, method of probit analysis). Equation (15), which is based on this modified model, includes a term which expresses the functional relation of response to weight.

$$Y = a + b \log_{10}(C/W^h) \qquad (15)$$

where,

Y = response in probits

C = dose or concentration of toxicant

W = body weight

h = a proportionality constant

a = intercept

b = slope

The usefulness of this formula for studying the influence of body weight on the tolerances of fish to lethal levels of heavy metals has been demonstrated in previous studies (Anderson and Weber, 1975b; Spear and Anderson, 1975). Implicit in its application are two assumptions: (1) variability in the reactions of test organisms to a given concentration of toxicant are attributed primarily to differences in body weight; and (2) these variations are manifested as displacements from a linear dose-response curve. Based on these assumptions, a computer program was designed in the present study to select, for any particular set of data, the value of the proportionality constant h which yields the best correlation of Y and $\log_{10}(C/W^h)$ in Equation (15) (Appendix III).

Design of Sublethal Response Studies

Sublethal toxicity was measured in terms of the reduction in the total numbers of eggs produced by zebrafish and the potential viability of these eggs. Because zebrafish tend to eat their eggs (Gordon, 1960; Mertens, 1973), a breeding trap was

designed to confine spawning fish and to isolate demersal eggs (1 mm diameter). Each breeding trap (Figures 5a, 5b), which consisted of plexiglass sides and a polyethylene mesh (3 mm X 3 mm) bottom, was suspended in an aquarium by means of glass supporting struts. This arrangement allowed free-falling eggs to pass through the mesh and collect on the floor of the tank.

Each morning all tanks in which breeding traps were suspended were siphoned (Figure 6) in order to collect the eggs which had settled to the bottoms of the aquaria. Eggs collected from each tank were removed from the catchment device of the siphon apparatus and transferred to plastic petri dishes for enumeration and examination under a dissecting microscope. One hundred eggs were selected randomly from the collection of each tank (or all eggs from tanks from which less than one hundred were siphoned) and sorted into the following four categories (Figure 7):

1. unfertilized - transparent blastodisc with no evidence of cellular proliferation.
2. abnormal - showing evidence of any structural irregularities or fungal invasion.
3. collapsed - punctured or crushed chorion and evidence of fungal invasion (this criterion does not include eggs crushed due to handling because such eggs presumably could not become invaded by fungus within the brief interval between collection and examination).

Figure 5a. Exposure tanks containing breeding traps; zebrafish inside nearest breeding trap can be seen.

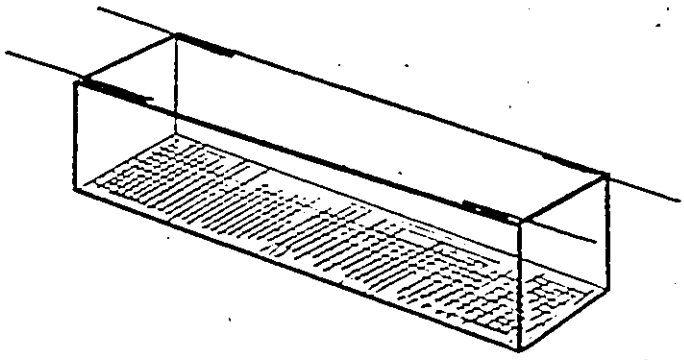

Figure 5b. Diagram of breeding trap

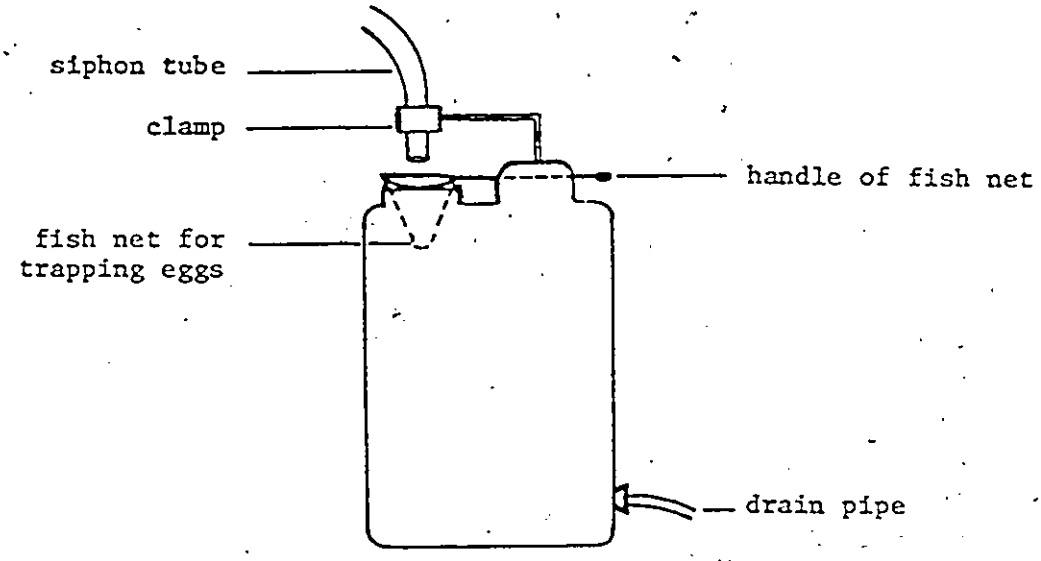

Figure 6. Siphon device for collecting zebrafish eggs from aquaria

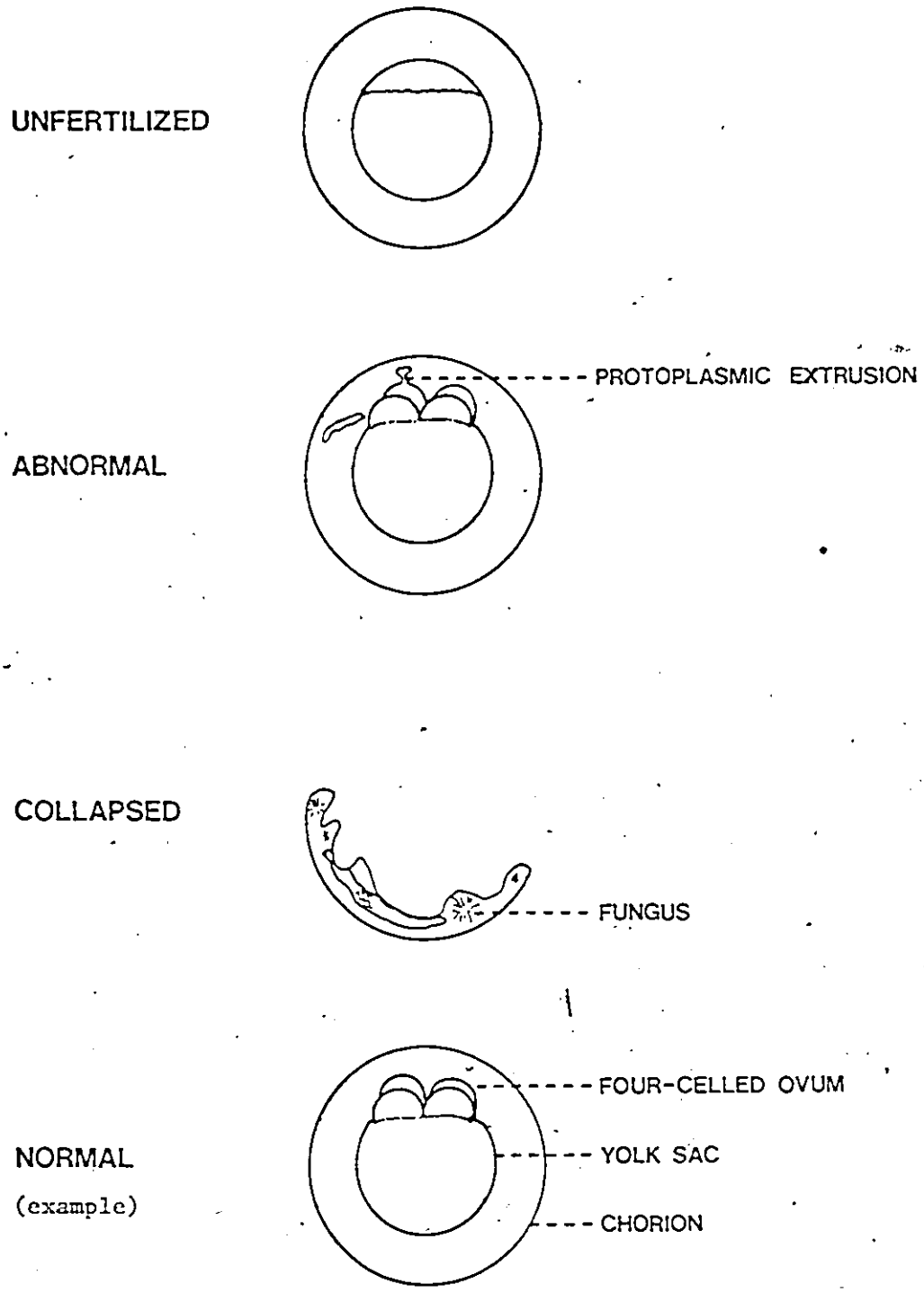

Figure 7. Categories of zebrafish eggs

4. normal – no apparent features of categories 1, 2, or 3; evidence of cellular proliferation.

Eggs described by categories 1, 2, or 3 were considered to be non-viable. All aquaria were siphoned each afternoon to remove settled debris which might interfere with the examination of the following morning's eggs.

Before subjecting zebrafish to toxic insult, lots of five males and five females were paired (in rank-correlated weight classes; see "Preparations for Exposure of Test Organisms") and confined to breeding traps in acclimating aquaria. For a period of ten days daily counts and classifications of eggs were performed. This information was recorded as control data. After the ten-day control period the spawning groups were transferred in their breeding traps from the acclimating tanks to their randomly-assigned test aquaria. Daily counts and classifications of eggs produced during the following ten days of exposure were recorded as experimental data.

As explained in the "Theory" section, probit analysis, which is based on the theory of quantal response, is not applicable for graded-response data. The sublethal data were analyzed using least squares linear regressions.

RESULTS

Zebrafish exposed to copper, nickel, or their mixtures demonstrated several common signs of distress. These manifestations included the following: hemorrhaging in the cardiac region and at the base of fins, extruded scales, darkening in body colouration, general sluggishness, slowness or failure to respond to visual or tactile stimuli, and loss of equilibrium. Rapid, erratic ventilation in fish insulted with copper, and gaping mouths and flared opercula in fish having died in nickel alone, or in mixtures with copper, were apparent. These observations suggest that the modes of action of copper and nickel evoke respiratory dysfunction in the zebrafish, possibly resulting in death due to hypoxia.

Lethal Studies - Quantal Response

Data accumulated from bioassays in which zebrafish were exposed to lethal levels of copper or nickel as discrete toxicants are pooled in Tables 3 and 4. The data for each toxicant were analyzed by a computer program to determine if the mean wet weight within each exposure lot of organisms could be incorporated as an additional variable in order to improve the fit of the customary linear model of dose-response. The following regression equations were computed for the data and compared in terms of their respective correlation coefficients.

Table 3. Lethal response data for zebrafish exposed to copper at various concentrations

Mean assayed level of copper (mg/l ± SD)	Number of fish	Mean wet weight of fish (g ± SD)	Observed % mortality in 96 hours
0 ± 0.01	14	0.69 ± 0.03	0
0	14	0.55 ± 0.04	0
0.01 ± 0.01	10	0.42 ± 0.04	0
0.05 ± 0.01	14	0.42 ± 0.05	0
0.05 ± 0.02	10	0.29 ± 0.03	0
0.09 ± 0.02	10	0.42 ± 0.04	0
0.13 ± 0.03	10	0.42 ± 0.04	10
0.15 ± 0.04	10	0.29 ± 0.03	20
0.17 ± 0.05	10	0.29 ± 0.03	30
0.22 ± 0.08	10	0.42 ± 0.04	30
0.22 ± 0.05	14	0.32 ± 0.03	43
0.24 ± 0.07	10	0.42 ± 0.04	30
0.27 ± 0.04	10	0.42 ± 0.04	50
0.31 ± 0.03	14	0.43 ± 0.03	100
0.31 ± 0.06	10	0.42 ± 0.04	60
0.35 ± 0.05	10	0.42 ± 0.04	80

Table 4. Lethal response data for zebrafish exposed to nickel at various concentrations

Mean assayed level of nickel (mg/l ± SD)	Mean wet weight of fish (g ± SD)	Observed % mortality in 96 hours
0 ± 0	0.90 ± 0.06	0
0 ± 0	0.69 ± 0.03	0
0 ± 0.1	0.69 ± 0.03	0
0 ± 0	0.55 ± 0.04	0
12.6 ± 1.3	0.67 ± 0.04	0
15.0 ± 0.2	0.71 ± 0.03	14
16.3 ± 0.6	0.79 ± 0.02	0
18.1 ± 0.3	0.57 ± 0.03	14
18.9 ± 0.6	0.86 ± 0.04	0
19.9 ± 1.5	0.45 ± 0.04	14
21.1 ± 0.6	0.47 ± 0.05	14
22.9 ± 0.7	0.55 ± 0.04	36
23.3 ± 0.4	0.90 ± 0.05	0
23.4 ± 3.1	0.69 ± 0.04	14
24.3 ± 0.7	0.44 ± 0.04	7
27.2 ± 0.8	0.82 ± 0.04	36
28.0 ± 0.5	0.56 ± 0.04	14
28.7 ± 1.2	0.43 ± 0.03	14
29.9 ± 0.6	0.58 ± 0.04	21
32.3 ± 1.5	0.55 ± 0.04	36
32.7 ± 1.0	0.69 ± 0.04	29
35.0 ± 1.7	0.69 ± 0.04	36
38.8 ± 1.2	0.44 ± 0.04	50
41.4 ± 1.2	0.55 ± 0.04	50
45.9 ± 2.4	0.94 ± 0.03	43
49.1 ± 1.4	0.54 ± 0.03	71
50.2 ± 4.3	0.54 ± 0.04	57
53.6 ± 2.3	0.43 ± 0.04	86
74.7 ± 5.8	0.44 ± 0.04	86
100.3 ± 6.9	0.31 ± 0.04	100

Fourteen fish were exposed at each concentration.

$$Y = a_1 + b_1 \log_{10} C \quad (16)$$

$$Y = a_2 + b_2 \log_{10} (C/W^h) \quad (17)$$

where,

Y = probit of % mortality

C = concentration of toxicant

W = mean wet weight of test organisms exposed to C

h = a proportionality constant

a = intercept

b = slope

The results of this computer analysis are given in Table 5. Although correlation coefficients associated with Equations (16) and (17) are highly significant ($P < 0.001$), the copper and nickel data are each more closely represented by Equation (17), which incorporates the mean wet weights of organisms. However, because the respective improvements in correlation using Equation (17) are not significant ($P > 0.05$), Equation (16), the simpler function, was selected as the dose-response formulation for these data.

Dose-response curves fitted to the lethal response data for copper (Table 3) and nickel (Table 4) are defined by the following linear regressions which were derived by a computer program based on Finney's (1971) method of probit analysis.

$$\text{Copper: } Y = 8.417 + 5.401 \log_{10} C_1 \quad (18)$$

Table 5. Computer analysis of the effects of wet body weight on the response of zebrafish to lethal levels of copper or nickel

Dose-response equation:	Equation (16) $Y = a_1 + b_1 \log_{10} C$	Equation (17) $Y = a_2 + b_2 \log_{10}(C/W^h)$
Copper:		
a	7.377	6.829
b	4.063	4.636
h	—	0.47
Correlation coeff.	0.935	0.962
Computed "t" value for correlation coeff. (N = 9)	7.290	9.071
Nickel:		
a	-0.868	-0.873
b	3.632	3.629
h	—	0.01
Correlation coeff.*	0.875	0.875
Computed "t" value for correlation coeff. (N = 21)	7.691	7.691

Y = probit of % mortality
C = concentration of toxicant (mg/l)
W = mean wet weight (g) of test organisms exposed to C
h = a proportionality constant
a = intercept
b = slope

* Correlation coefficient is relatively greater for Equation (17), but is identical at three decimal places to correlation coefficient for Equation (16).

$$\text{Nickel:}\quad Y = -1.924 + 4.299 \log_{10} C_2 \qquad (19)$$

where,

Y = probit of % mortality

C_1 = concentration of copper (mg/l)

C_2 = concentration of nickel (mg/l)

Chi-square tests comparing observed numbers killed with those predicted by Equations (18) and (19) suggest that both of these linear regressions adequately represent ($P < 0.05$) the respective empirical data.

The model of RESPONSE-ADDITION was tested in order to consider the possibility that lethal levels of copper and nickel were operating independently on different types of receptors in the zebrafish. Because it was not known to what extent tolerances to these toxicants were correlated for individual test organisms, Equations (1), (2), and (3) were applied to predict multiple toxicity.

Table 6 reveals the results of the bioassay in which zebrafish were exposed to copper and nickel in lethal mixtures. Percent mortalities predicted in accordance with the model of RESPONSE-ADDITION are listed. Because of the consistent and marked discrepancies between observed and predicted mortalities, the hypothesis of RESPONSE-ADDITION was rejected.

The possibility also existed that copper and nickel were operating similarly on the same type of receptor in the zebrafish. Therefore, the model of CONCENTRATION-ADDITION was tested.

Table 6. Lethal response data for zebrafish exposed to mixtures of copper and nickel and responses predicted in accordance with the model of RESPONSE-ADDITION

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of nickel (mg/l ± SD)	Observed % mortality in 96 hours	Predicted % mortality if r=-1	if r=0	if r=+1
0	0 ± 0	0	0	0	0
0.02 ± 0.01	3.8 ± 0.4	71	<1	<1	<1
0.02 ± 0.01	4.4 ± 0.4	86	<1	<1	<1
0.03 ± 0	3.3 ± 0.5	21	<1	<1	<1
0.03 ± 0.02	3.3 ± 0.7	50	<1	<1	<1
0.03 ± 0.01	3.6 ± 0.4	64	<1	<1	<1
0.01 ± 0.01	7.0 ± 0.6	21	<1	<1	<1
0.03 ± 0.01	4.4 ± 0.6	43	<1	<1	<1
0.01 ± 0.01	7.8 ± 0.7	29	<1	<1	<1
0.02 ± 0.01	6.6 ± 0.7	93	<1	<1	<1
0.01 ± 0.01	8.5 ± 0.6	36	<2	<2	<1
0.01 ± 0.01	9.0 ± 1.2	79	<2	<2	<1
0.01 ± 0.01	10.0 ± 0.8	71	<2	<2	<1
0.01 ± 0	11.8 ± 0.9	100	<3	<3	<2
0.01 ± 0.01	12.7 ± 1.1	100	<3	<3	<2
0.02 ± 0.01	11.1 ± 1.8	100	<2	<2	<1
0.01 ± 0.01	13.0 ± 1.3	93	<3	<3	<2

Fourteen fish were exposed to each mixture except 0.02 mg/l copper and 11.1 mg/l nickel, at which fifteen fish were exposed.

r = hypothetical correlation coefficient for tolerances to lethal levels of copper and nickel as discrete toxicants.

- if $r = -1$, Predicted % Mortality $= \begin{cases} 100\ (P_A + P_B), & \text{if } P_A + P_B < 1 \\ 100, & \text{if } P_A + P_B \geq 1 \end{cases}$

- if $r = 0$, Predicted % Mortality $= 100 \left[P_A + P_B(1-P_A) \right]$

- if $r = +1$, Predicted % Mortality $= \begin{cases} 100\ P_A, & \text{if } P_A > P_B \\ 100\ P_B, & \text{if } P_B > P_A \end{cases}$

where,

P_A = proportion of test organisms responding to copper alone

P_B = proportion of test organisms responding to nickel alone

P_A and P_B are determined from the probit regressions which were fitted independently to the lethal response data for copper and nickel as discrete toxicants (see Equations 18 and 19).

According to the Student's "t" test, there is no significant difference ($P > 0.05$) in the slopes of Equations (18) and (19). Thus, in order to facilitate the formulation of the CONCENTRATION-ADDITION hypothesis, a common slope was calculated, thereby constraining these probit regressions to parallelism. This procedure is recommended by Finney (1971). The resultant equations, constrained to parallelism as follows, are plotted against the lethal response data in Figure 8.

$$\text{Copper:} \quad Y = 7.779 + 4.476 \log_{10} C_1 \tag{20}$$

$$\text{Nickel:} \quad Y = -2.195 + 4.476 \log_{10} C_2 \tag{21}$$

where,

Y = probit of % mortality

C_1 = concentration of copper (mg/l)

C_2 = concentration of nickel (mg/l)

Chi-square tests comparing observed numbers killed with those predicted by Equations (20) and (21) attest to the close fit ($P < 0.005$) of these dose-response curves with the respective empirical data.

In the application of the model of CONCENTRATION-ADDITION to these data, the level of copper within each test mixture was expressed arbitrarily as an equipotent concentration of nickel. The relative potency factor, with which equipotent levels were determined, was computed by substituting the slope and intercepts of Equations (20)

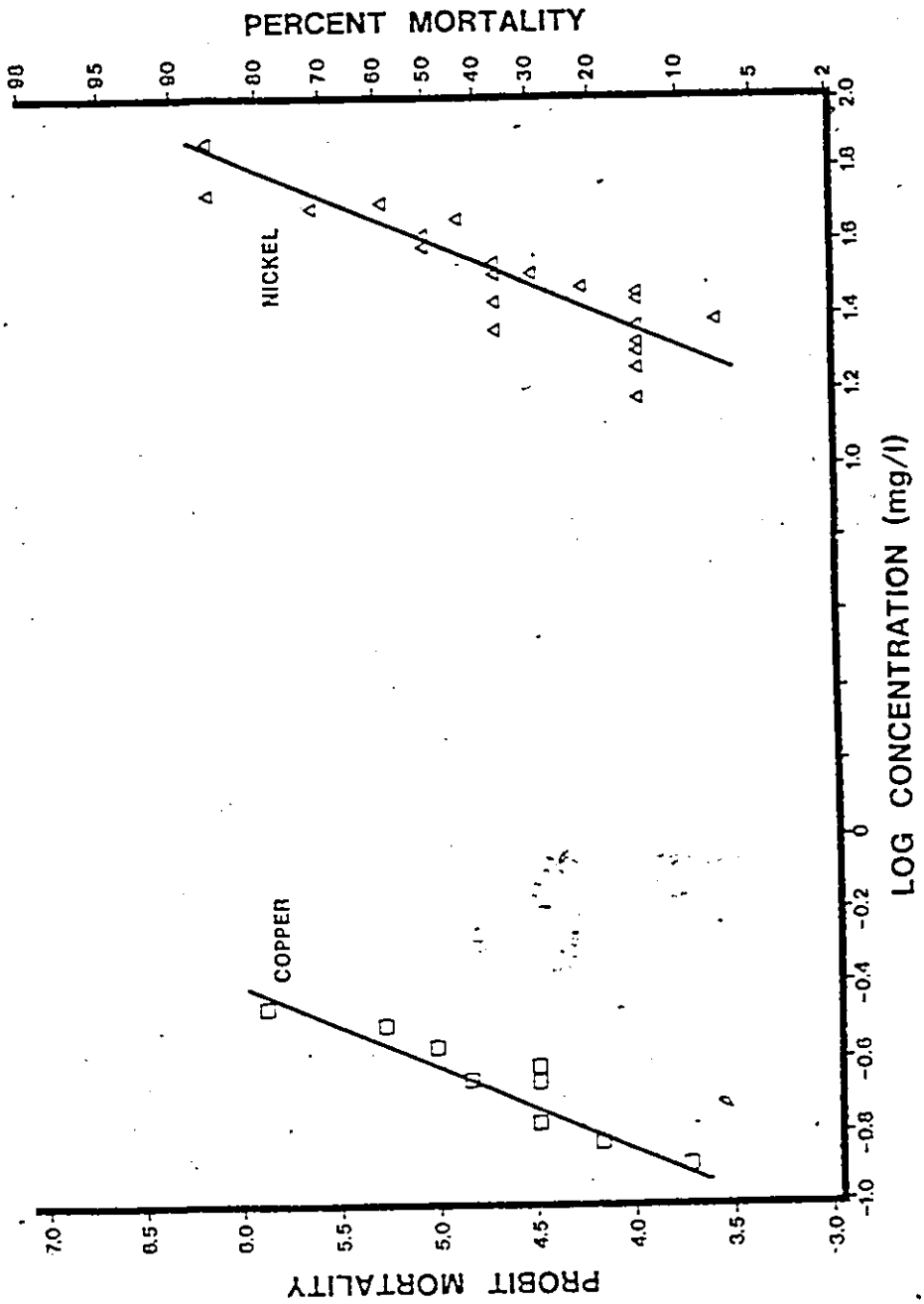

Figure 8. Lethal response curves for zebrafish exposed for ninety-six hours to various concentrations of copper or nickel

and (21) into Formula (7). According to this computation the relative potency factor is 169.17215. The total concentration of each mixture was calculated by algebraically adding the mean assayed level of nickel to the product of the mean assayed level of copper and the relative potency factor. In accordance with the model of CONCENTRATION-ADDITION, the hypothetical response for each mixture was predicted by substituting the total concentration for the term C_2 in Equation (21).

Figure 9 illustrates the results of bioassays in which zebrafish were exposed to lethal mixtures of copper and nickel. Observed responses are plotted against total concentrations which are expressed in terms of nickel. The parallel dose-response curves for copper and nickel (Equations 20 and 21) are depicted on the same graph for reference.

Table 7 compares observed mortalities with those predicted in accordance with the model of CONCENTRATION-ADDITION. Included in this table is a variable which expresses the mean assayed level of nickel as a proportion of the total concentration of the corresponding mixture. Because of the lack of agreement between observed and predicted mortalities, the hypothesis of CONCENTRATION-ADDITION was rejected.

According to the results of the lethal studies, observed mortalities for mixtures exceeded predictions based on models of RESPONSE-ADDITION and CONCENTRATION-ADDITION. Thus, copper and nickel were classified as SUPRA-ADDITIVE in terms of their lethal multiple toxicity to zebrafish.

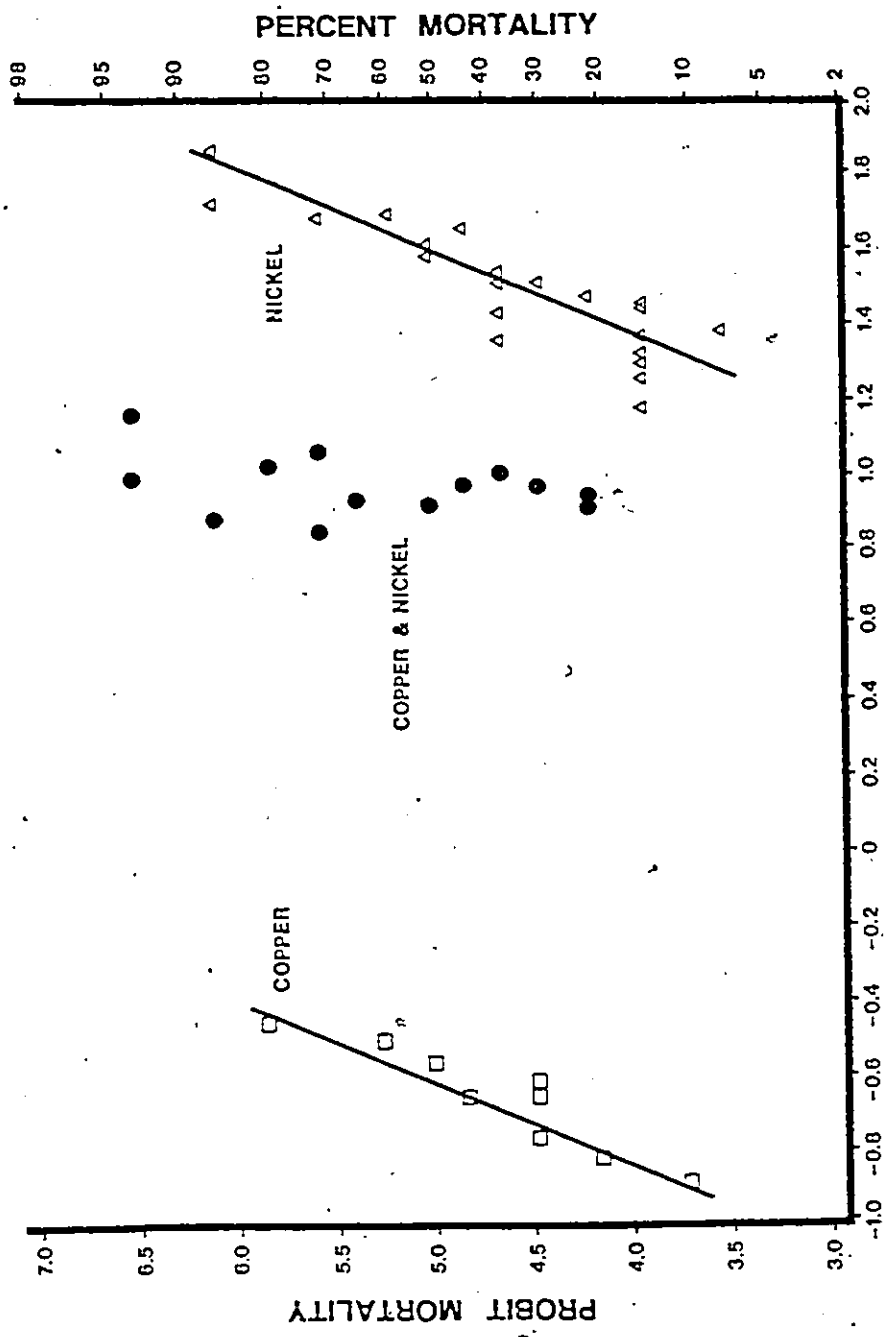

Figure 9. Lethal response data for zebrafish exposed for ninety-six hours to mixtures of copper and nickel; (the parallel lethal response curves for copper and nickel as discrete toxicants are depicted for reference; dose-response coordinates which are predicted for these mixtures by the model of CONCENTRATION-ADDITION are found at the intersections of vertical lines dropped from observed coordinates (black dots) and the extrapolated lethal response curve for nickel)

Table 7. Lethal response data for zebrafish exposed to mixtures of copper and nickel and responses predicted in accordance with the model of CONCENTRATION-ADDITION

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of nickel, (C_2) (mg/l ± SD)	Copper as equipotent level of nickel, (C_2') (mg/l)	Total mixture level as nickel (mg/l)	Nickel as proportion of total mixture level	Observed % Mortality in 96 hours	Predicted % Mortality
0	0	0	0	--	0	0
0.02 ± 0.01	3.8 ± 0.4	3.38	7.18	0.53	71	<1
0.02 ± 0.01	4.4 ± 0.4	3.38	7.78	0.57	86	<1
0.03 ± 0	3.3 ± 0.5	5.08	8.38	0.39	21	<1
0.03 ± 0.02	3.3 ± 0.7	5.08	8.38	0.39	50	<1
0.03 ± 0.01	3.6 ± 0.4	5.08	8.68	0.42	64	<1
0.01 ± 0.01	7.0 ± 0.6	1.69	8.69	0.81	21	<1
0.03 ± 0.01	4.4 ± 0.6	5.08	9.48	0.46	43	<1
0.01 ± 0.01	7.8 ± 0.7	1.69	9.49	0.82	29	<1
0.02 ± 0.01	6.6 ± 0.7	3.38	9.98	0.66	93	<1
0.01 ± 0.01	8.5 ± 0.6	1.69	10.19	0.83	36	<1
0.01 ± 0.01	9.0 ± 1.2	1.69	10.69	0.84	79	<1
0.01 ± 0.01	10.0 ± 0.8	1.69	11.69	0.86	71	1
0.01 ± 0	11.8 ± 0.9	1.69	13.49	0.87	100	2
0.01 ± 0.01	12.7 ± 1.1	3.38	14.39	0.88	100	2
0.02 ± 0.01	11.1 ± 1.8	1.69	14.48	0.77	100	2
0.01 ± 0.01	13.0 ± 1.3	1.69	14.69	0.89	93	3

Fourteen fish were exposed to each mixture except 0.02 mg/l copper and 11.1 mg/l nickel, at which fifteen fish were exposed.

Total mixture level as nickel = $C_2' + C_2$

Theoretically, mortality in a mixture of SUPRA- or INFRA-ADDITIVE toxicants must be a function of the constituents' concentrations as well as of the ratio of these concentrations. Consequently, a linear dose-response model could not be applied to the present study's multiple toxicity data because the constituent ratios for the test mixtures were not mutually equivalent (see chemical assay data in Table 7). A model of SUPRA-ADDITIVE SYNERGISM which compensates for these discrepancies is derived in the "Discussion".

Sublethal Studies - Graded Response

As discussed in "Materials and Methods", the sublethal toxicities of copper, nickel, and their mixtures were studied in terms of the graded effects on reproduction. Eggs laid by groups of zebrafish during control and subsequent exposure periods were counted and examined. The percentage reduction in the number of eggs produced was calculated for each group and related to the common logarithm of toxicant concentration by a least squares linear regression.

The results of the heavy metal analyses of water samples taken from the sublethal, discrete-toxicant experiments were atypically erratic. For this reason the response data from these particular bioassays were studied with reference to the nominal test concentrations of copper and nickel.

Tables 8 and 9 present the results of the sublethal, discrete-toxicant bioassays. Total numbers of eggs enumerated for each lot of

Table 8. Sublethal response data for zebrafish exposed to copper at various concentrations

Nominal level of copper (mg/l)	Total number of eggs		
	Ten-day control period	Ten-day exposure period	% reduction
0	3358	3527	-5.0
0	2178	2657	-22.0
0	2274	2377	-4.5
0.01	2458	3059	-24.5
0.02	2617	1318	+49.6
0.03	3148	2323	+26.2
0.03	2750	659	+76.0
0.04	3764	560	+85.1
0.07	3367	494	+85.3

Five males and five females were exposed at each concentration.

Negative % reduction denotes a relative increase in number of eggs.

Table 9. Sublethal response data for zebrafish exposed to nickel at various concentrations

Nominal level of nickel (mg/l)	Total number of eggs		
	Ten-day control period	Ten-day exposure period	% reduction
0	3358	3527	-5.0
0	2178	2657	-22.0
0	2274	2377	-4.5
1.5	2671	2467	+7.6
2.1	2316	2638	-13.9
2.9	2685	2695	-0.4
4.1	2592	325	+87.5
4.4	2194	1022	+53.4
5.7	3091	1093	+67.7
8.0	2716	963	+64.5

Five males and five females were exposed at each concentration.

Negative % reduction denotes a relative increase in number of eggs.

five males and five females during the ten-day control period and subsequent ten-day exposure to copper or nickel are reported along with the corresponding percentage reductions in numbers of eggs. The following linear regressions represent the dose-response curves which were fitted to these data:

$$\text{Copper:}\quad Y = 250.3 + 129.6 \log_{10} C_1 \tag{22}$$

$$\text{Nickel:}\quad Y = -29.8 + 121.9 \log_{10} C_2 \tag{23}$$

where,

Y = % reduction in total number of eggs

C_1 = concentration of copper (mg/l)

C_2 = concentration of nickel (mg/l)

"F" tests based on analyses of variance suggest that Equations (22) and (23) adequately represent ($P < 0.05$) the respective empirical data.

The model of RESPONSE-ADDITION was tested in order to consider the possibility that the decreases in egg production may have been due to the independent actions of copper and nickel on different types of receptors in the zebrafish. Equation (9) was applied to predict multiple toxicity.

Table 10 discloses the results of bioassays in which zebrafish were exposed to copper and nickel in "sublethal" mixtures. Predictions of decreased egg production based on the model of RESPONSE-ADDITION are listed. Within the first ninety-six hours of

Table 10. Sublethal response data for zebrafish exposed to mixtures of copper and nickel, and responses predicted in accordance with the model of RESPONSE-ADDITION

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of nickel (mg/l ± SD)	Observed % reduction in number of eggs	Predicted % reduction in number of eggs	Observed % mortality in 96 hours
0 ± 0	0 ± 0	- 4.5	0	0
0 ± 0	0 ± 0	- 22.0	0	0
0.01 ± 0.01	0.7 ± 0.5	+ 93.1	<1	0
0.01 ± 0	1.5 ± 0.9	+ 98.8	<1	0
0.02 ± 0.01	1.4 ± 0.9	+ 86.5	<1	0
0.02 ± 0	2.1 ± 1.6	+100	<1	10
0.02 ± 0.01	2.7 ± 1.7	+100	<1	0
0.02 ± 0	4.2 ± 2.6	+ 97.5	<1	30
0.03 ± 0	3.6 ± 2.2	+100	<1	80
0.04 ± 0	4.4 ± 2.3	+ 99.9	<1	90

Five males and five females were exposed to each mixture.

Negative % reduction denotes a relative increase in number of eggs.

exposure, mortalities ranging from ten to ninety percent were observed in four of the test mixtures. Because of the consistent and marked discrepancies between observed and predicted percentage reductions in numbers of eggs, the hypothesis of RESPONSE-ADDITION was rejected.

The possibility also existed that the decreases in egg production were due to the toxic actions of copper and nickel on the same type of receptor in the zebrafish. Therefore, the model of CONCENTRATION-ADDITION was tested. According to the Student's "t" test, the slopes of Equations (22) and (23) are not significantly different ($P > 0.05$). Thus, in order to facilitate the formulation of the CONCENTRATION-ADDITION hypothesis, and to be consistent with the protocol followed to analyze the lethal multiple toxicity data, a common slope was calculated for these linear regressions. The resultant equations, constrained to parallelism as follows, are plotted against the sublethal response data in Figure 10.

$$\text{Copper: } Y = 244.8 + 125.9 \log_{10} C_1 \qquad (24)$$

$$\text{Nickel: } Y = -31.7 + 125.9 \log_{10} C_2 \qquad (25)$$

where,

Y = % reduction in total number of eggs

C_1 = concentration of copper (mg/l)

C_2 = concentration of nickel (mg/l)

"F" tests based on analyses of variance suggest that Equations (24) and (25) adequately fit ($P < 0.05$) the respective empirical data.

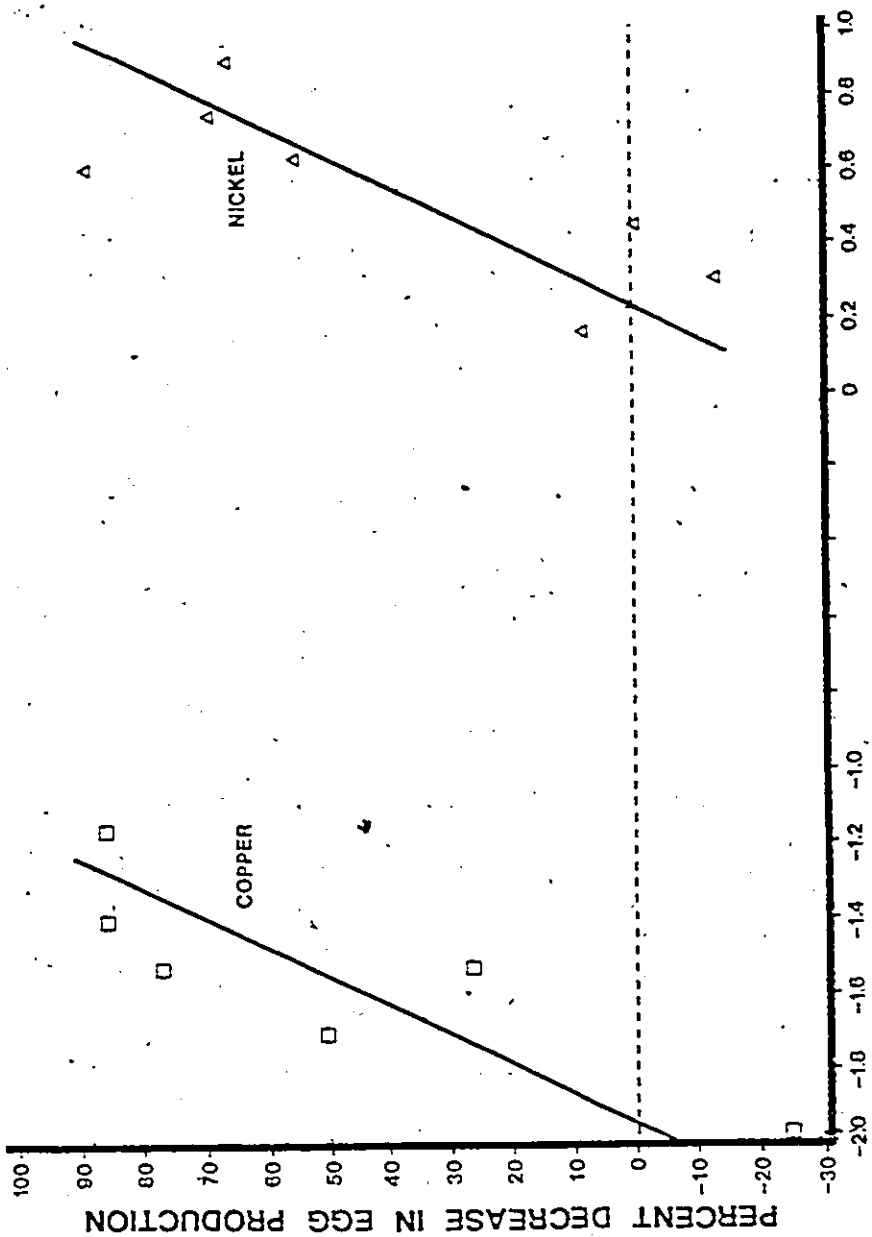

Figure 10. Percentage decreases in egg production by zebrafish exposed for ten days to various concentrations of copper or nickel

The steps taken to formulate the CONCENTRATION-ADDITION hypothesis for the sublethal data are equivalent to the procedure followed for the lethal data. In order to express for each mixture, the sum of the constituents' concentrations in terms of nickel, a relative potency factor was determined with which copper concentrations could be mathematically converted to equipotent nickel concentrations. When the slope and intercepts of Equations (24) and (25) are substituted appropriately into Formula (13), the relative potency factor is 157.10407. The total concentration of each mixture was calculated by algebraically adding the mean assayed nickel concentration to the product of the mean assayed level of copper and the derived relative potency factor. In accordance with the model of CONCENTRATION-ADDITION, the predicted decrease in egg production for each mixture was computed by substituting the total concentration for the term C_2 in Equation (25).

The results of bioassays in which zebrafish were exposed to "sublethal" mixtures of copper and nickel are illustrated in Figure 11. Observed responses are plotted against total concentrations which are expressed in terms of nickel. For reference, the parallel dose-response curves for copper and nickel (Equations 24 and 25) are depicted on the same graph.

Table 11 compares the observed decreases in egg production with those predicted in accordance with the model of CONCENTRATION-ADDITION. Included in this table is a variable which expresses the mean assayed level of nickel as a proportion of the total concentration of the corresponding mixture. Because of the apparent lack of

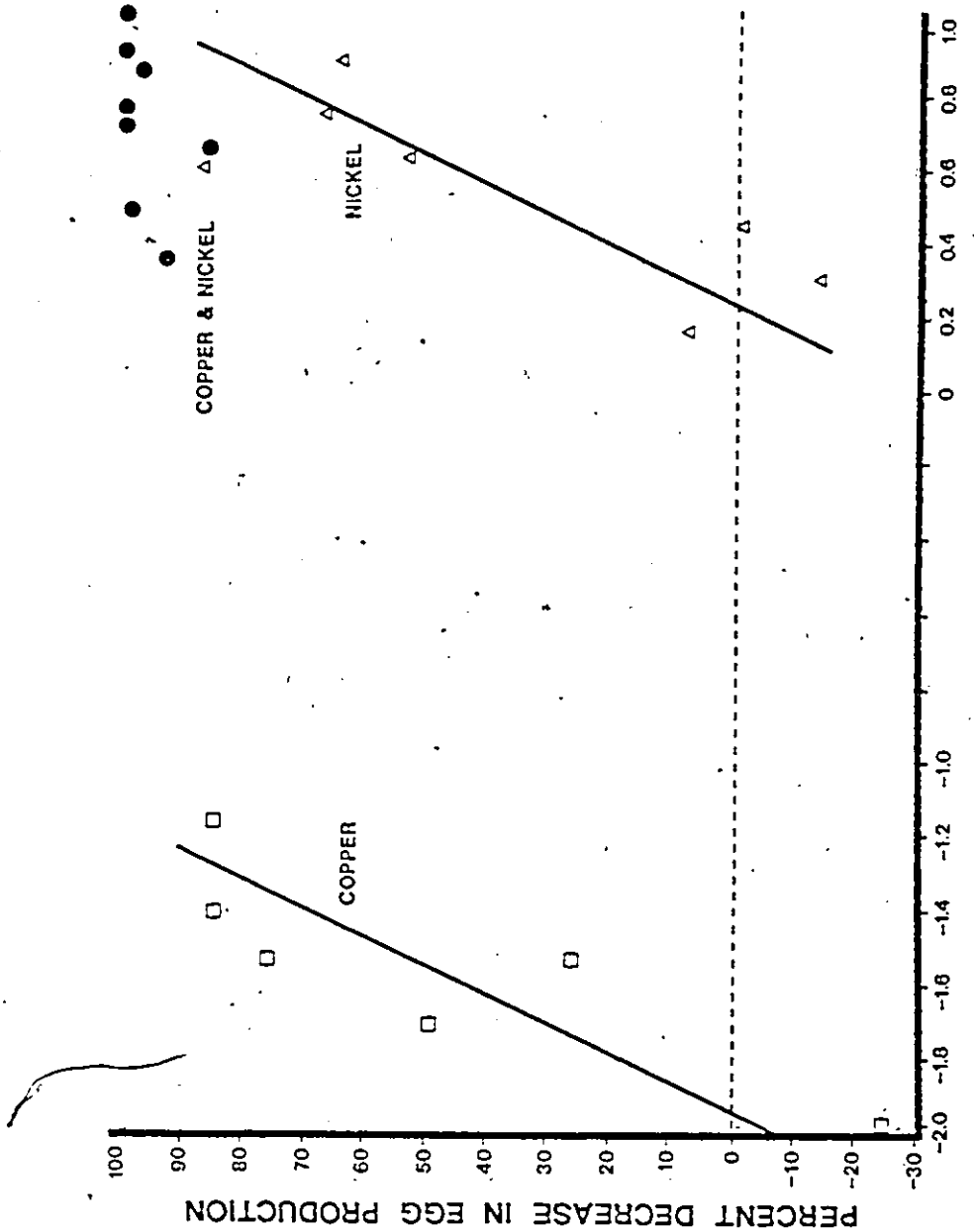

Figure 11. Percentage decreases in egg production by zebrafish exposed for ten days to mixtures of copper and nickel; (the parallel sublethal response curves for copper and nickel as discrete toxicants are depicted for reference; dose-response coordinates which are predicted for these mixtures by the model of CONCENTRATION-ADDITION are found at the intersections of vertical lines dropped from observed coordinates (black dots) and the extrapolated sublethal response curve for nickel)

Table 11. Sublethal response data for zebrafish exposed to mixtures of copper and nickel, and responses predicted in accordance with the model of CONCENTRATION-ADDITION

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of nickel, (C_2) (mg/l ± SD)	Copper as equipotent level of nickel, (C_2') (mg/l)	Total mixture level as nickel (mg/l)	Nickel as proportion of total mixture level	Observed % reduction in number of eggs	Predicted % reduction in number of eggs	Observed % mortality in 96 hours
0 ± 0	0 ± 0	0	0	--	- 4.5	0	0
0 ± 0	0 ± 0	0	0	--	- 22.0	0	0
0.01 ± 0.01	0.7 ± 0.5	1.57	2.27	0.31	+ 93.1	+13.6	0
0.01 ± 0	1.5 ± 0.9	1.57	3.07	0.49	+ 98.8	+30.0	0
0.02 ± 0.01	1.4 ± 0.9	3.14	4.54	0.31	+ 86.5	+51.4	0
0.02 ± 0	2.1 ± 1.6	3.14	5.24	0.40	+100.0	+58.9	10
0.02 ± 0.01	2.7 ± 1.7	3.14	5.84	0.46	+100.0	+65.2	0
0.02 ± 0	4.2 ± 2.6	3.14	7.34	0.57	+ 97.5	+77.8	30
0.03 ± 0	3.6 ± 2.2	4.71	8.31	0.43	+100.0	+84.1	80
0.04 ± 0	4.4 ± 2.3	6.28	10.68	0.41	+ 99.9	+98.0	90

Five males and five females were exposed to each mixture.

Total mixture level as nickel = $C_2' + C_2$

Negative % reduction denotes a relative increase in number of eggs.

agreement between observed and predicted responses, the hypothesis of CONCENTRATION-ADDITION was rejected.

According to the results of the sublethal bioassays, observed responses for mixtures exceeded predictions based on models of RESPONSE-ADDITION and CONCENTRATION-ADDITION. Thus, copper and nickel were classified as SUPRA-ADDITIVE with respect to their combined effects on egg production in the zebrafish. Because observed responses are within the narrow range of 86.5% to 100%, the model of SUPRA-ADDITIVE SYNERGISM which is derived in the "Discussion" was not applied to these data.

Tables 12 and 13 contain the classification data for zebrafish eggs which were collected during a ten-day control period and the ten-day exposure to various sublethal concentrations of copper or nickel. These data suggest that the changes in the percentages of "unfertilized", "abnormal", "collapsed", and "total non-viable" eggs (see "Materials and Methods") are not significantly correlated ($P > 0.05$) with the log concentration of copper or nickel at which spawning zebrafish were exposed (Table 14). Thus, linear dose-response curves could not be adequately fitted to these data and consequently, the models of RESPONSE-ADDITION and CONCENTRATION-ADDITION could not be applied to predict multiple toxicity.

The classification data for eggs produced by zebrafish during a ten-day control period and the ten-day exposure to mixtures of copper and nickel are reported in Table 15. Although these mixtures dramatically reduced the production of eggs (Table 11), the changes in the percentages of "unfertilized", "abnormal", "collapsed", and

Table 12. Classification of zebrafish eggs collected during control period and during exposure to various sublethal concentrations of copper

Nominal level of copper (mg/l)	Total number of eggs C	Total number of eggs E	% Unfertilized C	% Unfertilized E	% Unfertilized INC	% Abnormal C	% Abnormal E	% Abnormal INC	% Collapsed C	% Collapsed E	% Collapsed INC	Total % non-viable C	Total % non-viable E	Total % non-viable INC
0	3358	3527	14.3	18.5	+ 4.2	9.7	6.8	- 2.9	9.3	0.3	- 9.0	33.3	25.6	- 7.7
0	2178	2657	8.2	3.5	- 4.7	4.2	4.5	+ 0.3	0.6	1.2	+ 0.6	13.0	9.1	- 3.9
0	2274	2377	9.5	7.9	- 1.6	3.2	6.9	+ 3.7	0.8	2.6	+ 1.8	13.5	17.4	+ 3.9
0.01	2458	3059	25.7	38.3	+12.6	7.2	5.8	- 1.4	17.7	0		50.6	44.1	- 6.5
0.02	2617	1318	16.5	23.1	+ 6.6	11.5	14.7	+ 3.2	21.1	0		49.0	37.9	-11.1
0.03	3148	2324	24.4	39.7	+15.3	0.8	15.5	+14.7	0	0		25.2	55.2	+30.0
0.03	2750	659	2.1	7.0	+ 4.9	4.5	3.8	- 0.7	0.3	0		6.9	10.8	+ 3.9
0.04	3764	560	18.7	26.1	+ 7.4	5.9	16.4	+10.5	6.8	0		31.5	42.5	+11.0
0.07	3367	494	13.8	24.3	+10.5	7.5	21.7	+14.2	23.7	1.6	-22.1	45.0	47.6	+ 2.6

Five males and five females were exposed to each mixture.

C = control period
E = exposure period
INC = increase in % classified during exposure period = E - C

Negative INC denotes a relative decrease in % classified during exposure period

Total # Non-viable = # Unfertilized + # Abnormal + # Collapsed

Table 13. Classification of zebrafish eggs collected during control period and during exposure to various sublethal concentrations of nickel

Nominal level of nickel (mg/l)	Total number of eggs		% Unfertilized			% Abnormal			% Collapsed			Total % non-viable		
	C	E	C	E	INC	C	E	INC	C	E	INC	C	E	INC
0	3358	3527	14.3	18.5	+ 4.2	9.7	6.8	- 2.9	9.3	0.3	- 9.0	33.3	25.6	- 7.7
0	2178	2657	8.2	3.5	- 4.7	4.2	4.5	+ 0.3	0.6	1.2	+ 0.6	13.0	9.1	- 3.9
0	2274	2377	9.5	7.9	- 1.6	3.2	6.9	+ 3.7	0.8	2.6	+ 1.8	13.5	17.4	+ 3.9
1.5	2671	2467	26.7	41.5	+14.8	4.8	8.3	+ 3.5	2.7	0.2	- 2.5	34.1	49.9	+15.8
2.1	2316	2638	26.8	51.0	+24.2	8.3	6.7	- 1.6	11.0	0.7	-10.3	46.0	58.5	+12.5
2.9	2685	2695	11.2	36.5	+25.3	7.6	5.8	- 1.8	12.5	0	-12.5	31.2	42.3	+11.1
4.1	2592	325	12.3	28.6	+16.3	7.0	15.4	+ 8.4	3.3	0	- 3.3	22.6	44.0	+21.4
4.4	2194	1022	4.0	17.4	+13.4	5.7	5.6	- 0.1	3.4	2.4	- 1.0	13.0	25.4	+12.4
5.7	3091	1093	9.2	9.1	- 0.1	15.5	42.0	+26.5	15.5	1.0	-14.5	40.2	52.1	+11.9
8.0	2716	963	20.3	50.6	+30.3	5.0	27.1	+22.1	4.3	0.9	- 3.4	29.6	78.7	+49.1

Five males and five females were exposed at each concentration.

C = control period
E = exposure period
INC = increase in % classified during exposure period = E - C

Negative INC denotes a relative decrease in % classified during exposure period

Total # Non-viable = # Unfertilized + # Abnormal + # Collapsed

Table 14. Statistical analysis for linear relationships between increases in percentages of classified eggs and the common logarithm of sublethal concentrations of copper or nickel

Category of eggs	Unfertilized	Abnormal	Collapsed	Total non-viable
Copper:				
Correlation coeff.	-0.148	0.742	0.081	0.396
Computed "t" value of correlation coeff. (N = 6)	-0.300	2.211	0.140	0.863
Nickel:				
Correlation coeff.	-0.060	0.735	0.004	0.584
Computed "t" value of correlation coeff. (N = 7)	-0.135	2.423	0.009	1.607

These computed "t" values indicate that none of the correlation coefficients are significant ($P > 0.05$).

Table 15. Classification of zebrafish eggs collected during control period and during exposure to mixtures of copper and nickel

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of nickel (mg/l ± SD)	Copper as equipotent level of nickel (mg/l)	Total mixture level as nickel (mg/l)	% Unfertilized			% Abnormal			% Collapsed			Total % non-viable		
				C	E	INC	C	E	INC	C	E	INC	C	E	INC
0 ± 0	0 ± 0	0	0	9.5	7.9	- 1.6	3.2	6.9	+ 3.7	0.8	2.6	+ 1.8	13.5	17.4	+ 3.9
0 ± 0	0 ± 0	0	0	8.2	3.5	- 4.7	4.2	4.5	+ 0.3	0.6	1.2	+ 0.6	13.0	9.1	- 3.9
0.01 ± 0.01	0.7 ± 0.5	1.57	2.27	2.1	7.0	+ 4.9	3.1	3.0	- 0.1	0.5	0	- 0.5	5.6	10.0	+ 4.4
0.01 ± 0	1.5 ± 0.9	1.57	3.07	2.1	96.8	+94.7	8.7	0	- 8.7	3.5	3.2	- 0.3	14.3	100.0	+85.7
0.02 ± 0.01	1.4 ± 0.9	3.14	4.54	2.8	8.1	+ 5.3	16.4	28.0	+11.6	0.8	3.8	+ 3.0	20.0	39.9	+19.9
0.02 ± 0	2.1 ± 1.6	3.14	5.24 *	3.4			4.7			0.8			8.8		
0.02 ± 0.01	2.7 ± 1.7	3.14	5.84 *	5.7			2.6			2.3			10.6		
0.02 ± 0	4.2 ± 2.6	3.14	7.34	4.4	8.2	+ 3.8	4.2	5.5	+ 1.3	1.6	9.1	+ 7.5	10.1	21.8	+11.7
0.03 ± 0	3.6 ± 2.2	4.71	8.31	15.0	0	-15.0	12.6	0	-12.6	0.3	0	- 0.3	27.8	0	-27.8
0.04 ± 0	4.4 ± 2.3	6.28	10.68	1.7	0	- 1.7	7.7	66.7	+59.0	1.8	33.3	+31.5	11.1	100.0	+88.9

Five males and five females were exposed to each mixture.

Total mixture level as nickel = $C_2' + C_2$

C = control period
E = exposure period
INC = increase in % classified during exposure period = E - C.

Negative INC denotes a relative decrease in % classified during exposure period

Total # Non-viable = # Unfertilized + # Abnormal + # Collapsed

* = no eggs were produced during exposure to this mixture

"total non-viable" eggs are not significantly correlated ($P > 0.05$) with the logarithm of mixture concentration (Table 16).

Table 16. Statistical analysis for linear relationships between increases in percentages of classified eggs and the common logarithm of mixture concentration (expressed in terms of nickel)

Category of eggs	Unfertilized	Abnormal	Collapsed	Total non-viable
Correlation coefficient	-0.518	0.501	0.661	0.016
Computed "t" value of correlation coefficient (N = 6)	-1.213	1.158	1.761	0.031

These computed "t" values indicate that none of the correlation coefficients are significant ($P > 0.05$).

DISCUSSION

Lethal Potencies of Copper and Nickel

Based on the ninety-six hour LC50's of copper and nickel in these studies (0.24 mg/l and 40.5 mg/l respectively), copper may be considered highly toxic and nickel may be regarded as moderately toxic to the zebrafish. This apparent difference in the relative potencies of copper and nickel is supported by the findings of several other workers (too numerous to mention here) who have exposed a variety of aquatic organisms to lethal concentrations of these heavy metals. Thus, with reference to their lethal toxicities, copper is generally considered to be more hazardous than nickel to aquatic biota.

A comparison of the lethal response data for copper and nickel indicates that copper was 169 times more potent than nickel to the zebrafish (Figure 8). This means that approximately the same % mortality is evoked in a given solution of copper as in a solution of nickel that is 169 times the concentration of the copper solution. A similar pattern was observed by Anderson and Weber (1975a), who reported a relative potency factor of 152 for the lethal toxicities of copper and nickel to the guppy, *Poecilia reticulata*.

Body Weight as a Tolerance-Modifying Variable

According to computer analysis of the lethal response data, there appears to be some suggestion that tolerance of zebrafish to

copper is dependent on body weight. Maximum linearity of the dose-response curve for copper is attained when 0.47 is substituted for the proportionality factor, h, in Equation (17) (Table 5). However, the correlation coefficient associated with the "weight-adjusted" curve is not significantly greater ($P > 0.05$) than that corresponding to the "unadjusted" curve. Furthermore, in terms of the computed linear regression parameters, these dose-response curves are similar. Therefore, the simpler and more customary form of the dose-response equation was used in subsequent analyses of the data for copper.

Other researchers have reported a relationship between lethal copper toxicity and body weight in the guppy, *Poecilia reticulata*, (Anderson and Weber, 1975b) and in the sunfish, *Lepomis gibbosus*, (Spear and Anderson, 1975). In each of these previous studies the calculated value of the proportionality factor is between 0 and 1. This suggests that the tolerances within some species to lethal levels of copper may vary disproportionately with body weight such that smaller individuals are more susceptible than larger specimens. Anderson and Weber (1975b) noted that their empirically determined proportionality factor, 0.72, approximates the value of the weight exponent which expresses the disproportionality between oxygen consumption rate and body weight in guppies, and suggested a possible inference to copper's mode of action.

Analysis of the lethal response data for nickel suggests that body weight does not modify the tolerance of zebrafish to this heavy metal. Linearity of the dose-response curve is maximum when "h" in Equation (17) is equal to 0.01 (Table 5). However, this value of

h is approximately zero, which would cancel out the weight factor, W^h.

As for the copper data, the correlation coefficient associated with the "weight-adjusted" curve is not significantly greater ($P > 0.05$) than that corresponding to the "unadjusted" curve. Thus, the simpler and more customary form of the dose-response equation was employed in subsequent analyses of the data for nickel. In contrast to these results Anderson and Weber (1975b) found that the tolerance of guppies to nickel is modified by body weight according to a proportionality factor of 0.67.

Reproduction in Sublethal Response Studies

The results of the sublethal response studies suggest that 0.01 mg/l copper and 1.8 mg/l nickel approximate the threshold concentrations for the effects of these heavy metals on egg production in the zebrafish (Figure 10). Concentrations only slightly greater than these levels inhibited reproduction almost completely.

During exposure to some of the lower concentrations of copper or nickel zebrafish produced more eggs than they had laid during the previous control period (Tables 8 and 9); these increases however, were not profound and are presumably within the normal range of variation for this species. Nevertheless, it may be that because copper is generally an essential element for metabolism, trace levels in the environment are beneficial for reproduction in the zebrafish. In contrast, nickel is not generally considered to be an essential element. However, it has been shown that certain animals, if fed on deficient diets, require nickel in order to

perform essential functions (WHO, 1973). Thus, it may be that the increased egg production at low levels of nickel reflected a deficiency in the feeding regime which was employed in this study.

Although the effects of copper and nickel on the numbers of eggs laid increased with concentration, no such dose-response trends could be demonstrated for changes in the percentages of "unfertilized", "abnormal", "collapsed", or "total non-viable" eggs (see "Materials and Methods") (Tables 12, 13, 14). Thus, according to these studies, the hazards of copper and nickel with respect to impairment of reproduction in the zebrafish, seem to be related to the numbers of eggs produced.

This pattern of response may provide insights concerning the nature of the underlying toxic actions of copper and nickel. Because the percentage of unfertilized eggs did not increase with concentration it is unlikely that the production, viability, or virility of sperm cells were affected. Reductions in the numbers of eggs produced may have been due to impairment of spawning behaviour in either sex, however this possibility was not examined during the course of the experiment. Other possible causes for the decreased egg production may be related to the effects of copper and nickel on gametogenesis, ovulation, or on the "scope for activity" (Fry, 1947) in the female.

"Safe" Concentrations of Single Toxicants

In an attempt to safeguard aquatic organisms against the hazards of sublethal levels of chemical pollutants government

regulatory agencies have employed "application factors" to estimate "safe" concentrations from LC50's. Initially these application factors were established on the basis of intuition. As an alternative to this method Mount and Stephan (1967) proposed that application factors be determined on the basis of toxicity tests; the empirically derived application factor for a toxicant is found by dividing the highest concentration that does not inhibit growth or reproduction during a life-cycle test by the 48-hour or 96-hour LC50.

Because parameters of reproduction are particularly sensitive as indicators of sublethal toxicity (Brungs, 1969; Sprague, 1971), it might be expected that concentrations which do not affect egg production or egg viability would not impair other vital systems in the organism. The results of the present study suggest that 0.01 mg/l copper and 1.8 mg/l nickel are the highest concentrations which do not inhibit egg production or egg viability in the zebrafish (Figure 10). Thus, if the adult is the most sensitive stage of the zebrafish life cycle, then it may be that these particular concentrations are, under the conditions of these experiments, "safe" levels of copper and nickel for this species. Based on these supposed safe levels, application factors for copper and nickel are 0.04 and 0.05 of the respective 96-hour LC50's.

These results are similar to those of other researchers who have studied impairment of reproduction as a sublethal response of various aquatic organisms to copper (Mount, 1968; Mount and Stephan, 1969; Arthur and Leonard, 1970; McKim and Benoit, 1971; Biesinger and Christensen, 1972; Benoit, 1975; Brungs et al., 1976; Pickering

et al., 1977) or nickel (Biesinger and Christensen, 1972; Pickering, 1974). A concensus of their empirical data indicates that 0.03 to 0.04 of the LC50's for copper and 0.01 to 0.05 of the LC50's for nickel include the safe levels for the various species tested.

Multiple Toxicity Studies

The results of the multiple toxicity studies suggest that if nickel, generally considered to be relatively innocuous as a toxic agent, is combined with copper, the resultant mixtures are extremely potent. According to theoretical models of RESPONSE-ADDITION and CONCENTRATION-ADDITION the mixtures to which zebrafish were exposed in the lethal bioassay should not have caused more than 3% mortality at any of the concentrations tested. However, mortalities ranging from 21 to 100% were observed (Tables 6 and 7). Greater than predicted responses were also recorded during the sublethal response studies (Tables 10 and 11). Egg production was almost completely blocked (86.5 to 100%) in all of the mixtures tested. According to the models of RESPONSE-ADDITION and CONCENTRATION-ADDITION no zebrafish were expected to die within the first 96 hours of the ten-day "sublethal" exposure. Yet, mortalities ranging from 10 to 90% were observed during this period. By the end of the ten-day exposure 100% mortality had occurred in two mixtures. Thus, the multiple toxicity observed in lethal as well as in sublethal mixtures of copper and nickel was considered to be a form of SUPRA-ADDITIVE SYNERGISM.

The results of the lethal study are in contrast with those of

Anderson and Weber (1975a). These researchers employed a similar approach and reported that copper and nickel were CONCENTRATION-ADDITIVE in mixtures which were lethal to the guppy within 96 hours of exposure. Because the toxicity-modifying characteristics of the water used in this previous experiment were similar to those of the water used in the present study, the difference in the observed forms of multiple toxicity for copper and nickel were presumably due to interspecies variations in the pharmacokinetic or pharmacodynamic actions of these poisons.

The observation that copper and nickel were SUPRA-ADDITIVE in sublethal as well as in lethal mixtures for the zebrafish should not be interpreted as a suggestion that the same form of multiple toxicity will generally prevail at low and high concentrations of these or other toxic mixtures, and for different response parameters. Heavy metals which have gained access to the circulation of an organism may, amongst other effects, derange electrolyte homeostasis, interfere with enzymatic processes, cause organ lesions, or affect the central nervous system (Shulman and Dwyer, 1965). The metabolic pathways which underlie these systems can react with various sensitivities to a given metal (Passow et al., 1961). Stated otherwise, different metabolic pathways exhibit distinct reaction thresholds for a particular toxicant. Consequently, high concentrations of a given metal may stimulate different metabolic pathways and therefore, different responses, than do low levels of the same metal. Thus, it is not unreasonable to assume that the form of multiple toxicity which is observed for a particular response

parameter at lethal concentrations of a mixture can be different from the form of multiple toxicity observed at sublethal mixture concentrations.

This hypothesis is supported by the results of other researchers who have studied the multiple toxicity of copper and nickel. Anderson and Weber (1975a) found that lethal mixtures of copper and nickel were CONCENTRATION-ADDITIVE for the guppy. However, while sublethal mixtures of these heavy metals were apparently CONCENTRATION-ADDITIVE with respect to the guppy's gross growth efficiency, they were SUPRA-ADDITIVE with regard to food consumption (Muska and Weber, 1977).

Theory of Toxic Interactions

Based on the pharmacological concepts of Ariens (1972), Anderson and d'Apollonia (in press) presumed that toxic interactions may occur at any of the phases which punctuate the course of environmental toxicants from the ambient medium to the target site in exposed organisms (Figure 12).

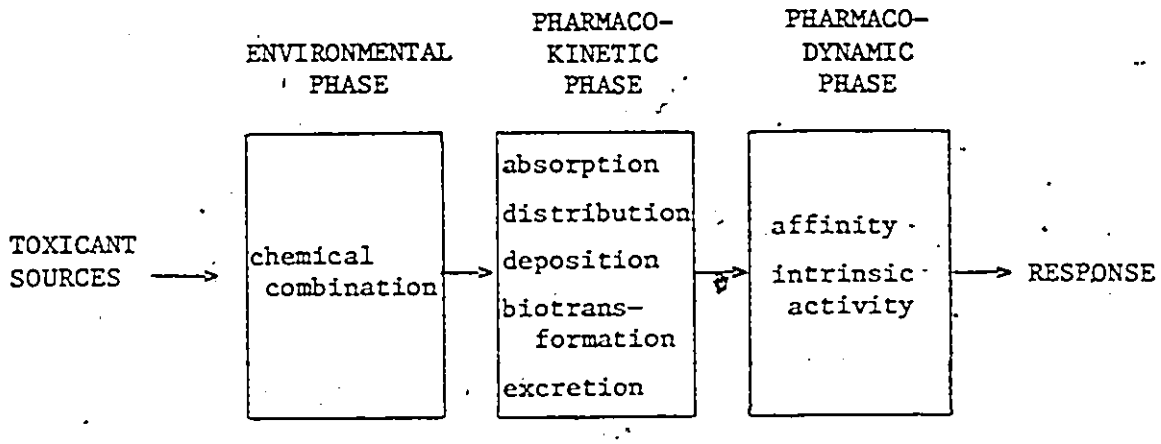

Figure 12. Phases in which environmental toxicants may interact (after Ariens, 1972; Anderson and d'Apollonia, in press)

In the "environmental phase" discrete toxicants may, in some cases, combine chemically and produce a single compound whose toxicity is greater or less than that predicted for the mixture, based on a knowledge of the individual toxicities of the original reactants. Interactions in the "pharmacokinetic phase" presumably alter the concentrations of toxicants that ultimately reach the target sites in exposed organisms. In the "pharmacodynamic phase" interactions may change the affinities of receptors for available toxicants or alter the intrinsic activities which are initiated by the binding of receptors with toxicant.

Based on a knowledge of the chemical forms of copper and nickel in water it is unlikely that these metallic cations would combine chemically in the environmental phase and produce a highly toxic ionic or molecular entity (J. Dick[1], personal communication). Therefore, it is probable that the apparent SUPRA-ADDITIVE SYNERGISM of these heavy metals in the present study resulted from interactions in the pharmacokinetic and/or pharmacodynamic phases.

For example, in the pharmacokinetic phase, processes such as storage, elimination, or biotransformation may result in a proportion of a drug or toxicant becoming lost before it ever reaches its target site (Veldstra, 1956). Veldstra referred to the loci at which such pharmacokinetic processes occur as "sites of loss". He suggested that the synergistic interaction of two compounds may be due to the less active compound competing successfully with the more active compound for a common site of loss. Consequently, a greater proportion of the more active entity is free to reach its specific

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target site. This mechanism of interaction may account for the apparent SUPRA-ADDITIVE SYNERGISM of copper and nickel in the present study.

A hypothesis for interaction in the pharmacodynamic phase may be proposed based on the following considerations. It is suspected that some drugs may conformationally alter and deform their receptors (Albert, 1973). If this suspicion is correct, it is conceivable that the steric changes which are elicited by one drug or toxicant may bring about the induced fit of a second drug or toxicant to receptors which are normally non-specific for the latter entity. This increase in the affinity of a receptor for a toxicant which it generally does not bind may account for the apparent SUPRA-ADDITIVE effects of copper and nickel in mixtures tested in the present study.

The Use of Models as an Approach for Studying Multiple Toxicity

The approach which was adopted to study multiple toxicity in these experiments is based on the premise that, in many cases, the potencies of mixtures may be predicted from a knowledge of the potencies of the discrete constituents. However, the theoretical models underlying this premise only apply to mixtures in which the constituents are RESPONSE-ADDITIVE or CONCENTRATION-ADDITIVE.

Initiative in the conception and development of predictive models in the field of environmental toxicology can be gained by considering aspects which pertain to fundamental principles of statistics and pharmacology. For example, it can often be assumed

that tolerances to a particular toxicant are normally distributed within a population. Another aspect which may be worth considering is that the tolerances of individual organisms to two or more poisons may or may not be correlated. The models of RESPONSE-ADDITION and CONCENTRATION-ADDITION which are previously outlined in the "Theory" section of this thesis, distinguish conceptually between toxicants that stimulate different types of receptors in organisms and toxicants that operate on the same type of receptor. These particular models assume implicitly that within an individual organism, the pharmacokinetic and pharmacodynamic processes which are initiated by one constituent are not influenced by the presence of a second constituent.

This latter pharmacological assumption does not apply to the multiple toxicities of SUPRA-ADDITIVE or INFRA-ADDITIVE toxicants. In contrast, these forms of multiple toxicity are presumed to result from toxicant interactions with pharmacokinetic and pharmacodynamic processes (Anderson and d'Apollonia, in press). However, the principles governing the quantitative aspects of these interactions have not been elucidated. Consequently, there are at this time, no theoretical models for predicting the potencies of mixtures of SUPRA-ADDITIVE or INFRA-ADDITIVE toxicants from a knowledge of the potencies of the discrete constituents.

The following discussion describes a quantitative approach developed by the author for assessing the potential hazards of toxic mixtures whose constituents have been identified as SUPRA-ADDITIVE. A quantitative model of SUPRA-ADDITIVE SYNERGISM is derived and

applied to the multiple toxicity data which were generated in the present study.

Derivation of a Quantitative Model of SUPRA-ADDITIVE SYNERGISM

In bioassays which are designed to estimate the dose-response relationships for mixtures of toxicants, the relative proportions of the discrete constituents in the test combinations are usually held constant. Yet, implicit in most theoretical approaches to the study of toxic mixtures is the assumption that forms of multiple toxicity other than CONCENTRATION-ADDITION, depend on the relative proportions as well as the absolute concentrations of a mixture's constituents. Thus, comprehensive evaluations of the toxicities of poisons applied jointly require the testing of several series of mixtures, with each series representing a unique ratio of constituents (Bliss, 1939). However, such tests tend to be very time-consuming and costly and are therefore avoided.

The incentive to develop a more pragmatic approach to evaluating multiple toxicity arose serendipitously upon examination of the empirical data which were recorded in the present study. These data suggest that copper and nickel in combination are SUPRA-ADDITIVE as lethal agents for the zebrafish (Tables 6, 7; Figure 9). However, chemical analyses revealed that the relative proportions of these heavy metals in test mixtures were not constant (see chemical assay data in Table 7). Consequently, the multiple toxicity data could not be readily analyzed and expressed quantitatively without first being adjusted to compensate for the apparent discrepancies in the

constituent ratios representing the various mixtures. The rationale for this "adjustment" is the basis of the proposed model.

As a point of departure in the derivation of the model, it will be assumed that if SUPRA-ADDITIVE poisons are similarly acting, i.e., they stimulate the same type of receptor in exposed organisms, then mixtures which combine these poisons in constant relative proportions may be treated as different concentrations of a single discrete toxicant. If this assumption is correct, the multiple toxicity data for similarly acting toxicants which are SUPRA-ADDITIVE should theoretically generate a linear dose-response curve, provided that the constituents of mixtures are combined according to a fixed ratio. Conversely, the multiple toxicity of SUPRA-ADDITIVE poisons in mixtures which do not represent a common ratio would be represented by dose-response points that deviate from linearity. In the latter case, the extent of lateral displacement between a point and a theoretical dose-response curve for constituents combined in constant proportions, is hypothetically related to the discrepancy between the corresponding constituent ratios.

The approach of the proposed model is to quantify this relationship and define a mathematical operator which, as a function of the differences between constituents ratios, shifts all displaced dose-response coordinates so as to describe a straight line.

Figure 13 illustrates hypothetical relationships between the relative potencies of mixtures of two similarly acting toxicants and the constituent ratios, for various forms of multiple toxicity.

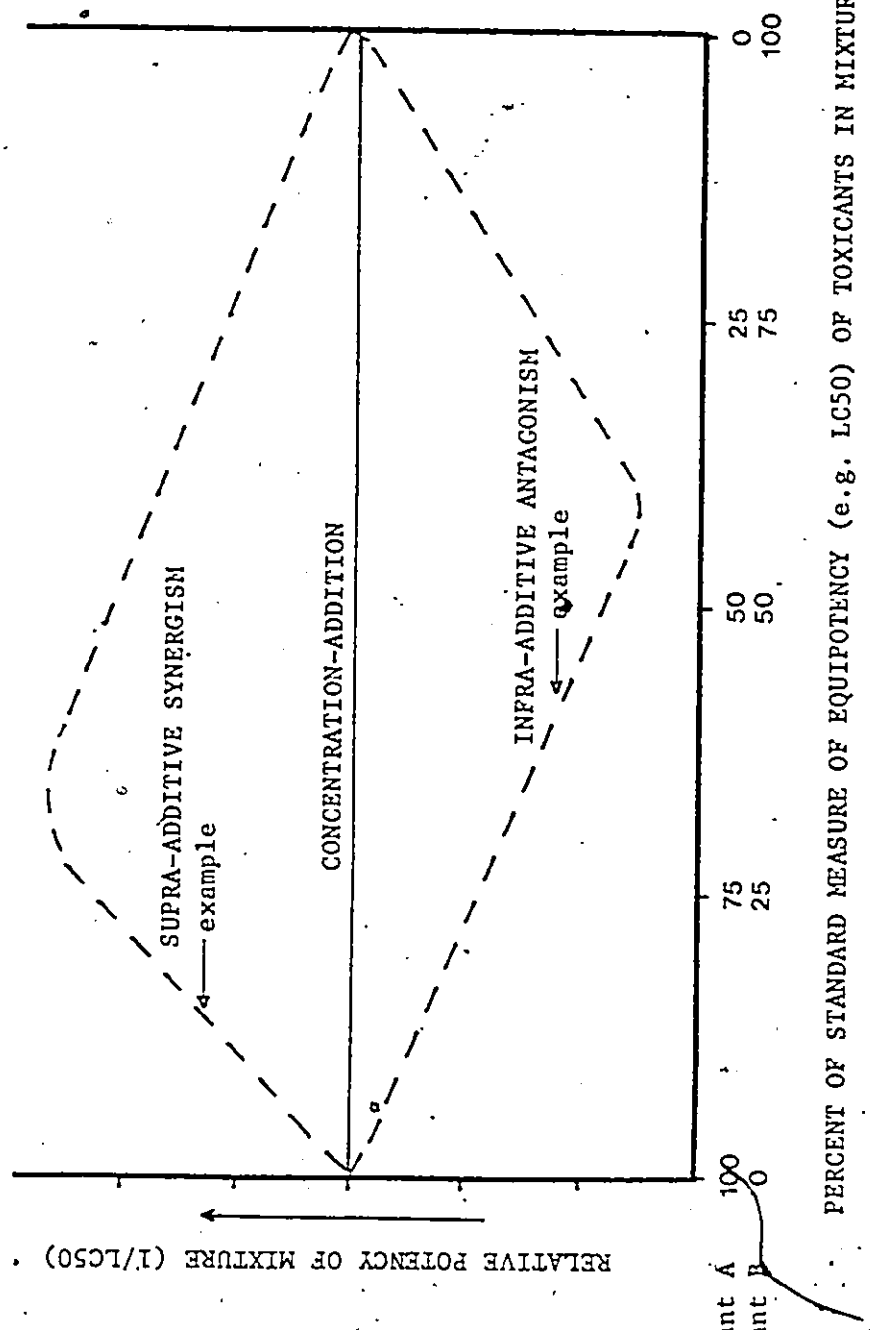


Figure 13. Relative potencies of mixtures in which two "similarly acting" toxicants are combined in various proportions (adapted from Warren, 1971)

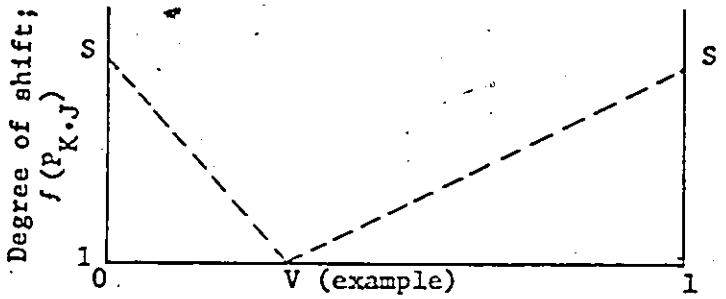
Although this aspect of toxicology has received little attention, there is some empirical evidence to support the shape of the INFRA-ADDITION curve (Osterhout, 1914; Jones, 1939; Roales and Perlmutter, 1974 a,b). Examination of the isoboles depicted in Figure 13 reveals that the curves for SUPRA-ADDITION and INFRA-ADDITION each tend toward a single maximum and minimum respectively. These trends suggest that at a given level of response, a single constituent ratio represents the most potent SUPRA-ADDITIVE, or conversely, the least potent INFRA-ADDITIVE combination of two toxicants. By definition, a relatively low concentration of a highly potent mixture is required to produce the same response as a relatively high concentration of a less potent mixture. Therefore, the more potent a mixture is, the lower its total concentration must be in order to evoke a particular response. Thus, if the constituent ratio representing the most potent SUPRA-ADDITIVE combination at a given level of response is common for all response levels, then the corresponding dose-response curve must fall farthest to the left on a graph which depicts the dose-response curves corresponding to other constant constituent ratios.

The application of this rationale in the present study is based on the following assumptions: (1) copper and nickel are similarly acting at the lethal level; and (2) departures from linearity in the multiple toxicity data which are plotted in Figure 9 are attributable primarily to discrepancies in the ratios of copper and nickel in the different test mixtures. According to the concentration scale in Figure 9, dose-response coordinates

which are farthest to the left at each response level denote highly potent mixtures. Conversely, coordinates which are farthest to the right at each response level represent mixtures of comparitively low potency. Presumably, the constituent ratios associated with those multiple toxicity coordinates which are farthest to the left approach the hypothetical constituent ratio which is associated with the most potent combination of copper and nickel. On the other hand, the constituent ratios for those multiple toxicity coordinates which are farthest to the right in Figure 9 should deviate markedly from this "hypothetical ratio". Theoretically, if these data were to be standardized with respect to a common constituent ratio and adjusted accordingly, the resultant dose-response coordinates would describe a straight line. On this premise, the following procedure is applied. Those empirical coordinates (Figure 9) whose constituent ratios approximate an arbitrarily chosen estimate of the "hypothetical ratio" are virtually "unadjusted", while all other coordinates are mathematically shifted to the left. The degree of shift for each coordinate is quantitatively related to the discrepancy between its empirical constituent ratio and the arbitrarily chosen estimate of the "hypothetical ratio". This relationship is depicted in Figure 14.

Once the multiple toxicity data are standardized in this way, a linear regression is fitted to the adjusted coordinates. Based on the derived model the regression is represented by the following equation. This expression modifies the equation predicted by the model of CONCENTRATION-ADDITION (Equation 8) with the "shift

Figure 14. Shift function for derived model of SUPRA-ADDITIVE SYNERGISM for mixture of similarly acting toxicants J and K



$f(P_{K\cdot J})$ = shift function

$P_{K\cdot J}$ = proportion of K in mixture (J and K) whose total concentration is expressed in terms of K

$$= \frac{C_K}{R_{K\cdot J}C_J + C_K}$$

C_J = concentration of J

C_K = concentration of K

$R_{K\cdot J}$ = relative potency factor used to express C_J as equipotent concentration of C_K; see Equation (6)

V = a constant denoting the arbitrarily chosen estimate of $P_{K\cdot J}$ which hypothetically represents the most potent SUPRA-ADDITIVE combination of J and K

S = a constant denoting a relative measure of the degree of SUPRA-ADDITIVE SYNERGISM for the most potent combination of J and K; "S" is the value of $(P_{K\cdot J})$ for single pure solutions of J $(P_{K\cdot J} = 0)$ or K $(P_{K\cdot J} = 1)$

Constraints on shift function, $f(P_{K\cdot J})$:

1. $f(P_{K\cdot J})$ is continuous from $P_{K\cdot J} = 0$ to 1
2. $f(V) = 0$
3. $f(0) = f(1) =$ a constant, S

Shift function:

$$f(P) = \begin{cases} 1 + (S-1)(V-P_{K\cdot J})/V & \text{,if } P_{K\cdot J} \leq V \\ 1 + (S-1)(V-P_{K\cdot J})/(V-1) & \text{,if } P_{K\cdot J} > V \end{cases}$$

function" formulated in Figure 14.

$$Y = a + b \log_{10} \left[\frac{R_{K \cdot J} C_J + C_K}{f(P_{K \cdot J}) = \begin{cases} 1+(S-1)(V-P_{K \cdot J})/V & , \text{ if } P_{K \cdot J} \leq V \\ 1+(S-1)(V-P_{K \cdot J})/(V-1), & \text{if } P_{K \cdot J} > V \end{cases}} \right] \quad (27)$$

Equation (27) expresses mortality as a function of the independent variables C_J, C_K, $R_{K \cdot J}$, and $P_{K \cdot J}$. In the present study C_J and C_K represent the concentrations (mg/l) of copper and nickel respectively in each mixture; $R_{K \cdot J}$ is equal to 169.17215, which is the relative potency factor that expresses copper in terms of equipotent nickel at lethal levels (see "Results"); and $P_{K \cdot J}$ is equal to the proportion of nickel in each mixture (see Table 7). The terms a, b, V, and S are constants which are theoretically related to the particular toxicants, organisms, and any toxicity-modifying factors associated with the empirical data.

The solution of Equation (27) for the multiple toxicity data of the present study was determined by computer analysis. This analysis was based on a program which was designed to arbitrarily select values of V and S which, when substituted in Equation (27), maximize the linearity of the adjusted dose-response data (Appendix IV). The resultant linear regression computed for these adjusted data is compared with the linear regression computed for the original unadjusted data. Tables 17a and 17b present the results of this analysis.

Table 17a. Computer analysis of lethal multiple toxicity data according to derived model of SUPRA-ADDITIVE SYNERGISM.

Linear regression for unadjusted data:

$$Y = 1.615 + 3.749 \log_{10}(169.17215\ C_1 + C_2) \qquad (28)$$

where,

Y = probit of % mortality

C_1 = concentration of copper (mg/l)

C_2 = concentration of nickel (mg/l)

computed correlation coefficient = 0.3799

Linear regression for adjusted data:

$$Y = -4.084 + 11.348 \log_{10} \left[\frac{(169.17215\ C_1 + C_2)}{\begin{cases} 1 + (2.10-1)(0.60-P_{2\cdot1})/0.60 & \text{, if } P_{2\cdot1} \leq 0.60 \\ 1 + (2.10-1)(0.60-P_{2\cdot1})/(0.60-1) & \text{, if } P_{2\cdot1} > 0.60 \end{cases}} \right] \qquad (29)$$

where,

$V = 0.60$ = the arbitrarily estimated value of $P_{2\cdot1}$ which represents the most potent SUPRA-ADDITIVE combination of copper and nickel

$S = 2.10$ = the value of $f(P_{2\cdot1})$ for single pure solutions of copper or nickel. "S" denotes a relative measure of the degree of SUPRA-ADDITIVE SYNERGISM for the most potent SUPRA-ADDITIVE mixtures

$P_{2\cdot1}$ = proportion of nickel in mixture

$$= \frac{C_2}{169.17215\ C_1 + C_2}$$

$f(P_{2\cdot1})$ = shift function (see Figure 14)

computed correlation coefficient = 0.8091

Table 17b. Unadjusted and adjusted coordinates for lethal multiple toxicity data

Mean assayed level of copper, (C_1) (mg/l)	Mean assayed level of nickel, (C_2) (mg/l)	"Unadjusted" total mixture level as nickel (mg/l)	Nickel as proportion of "unadjusted" total mixture level, ($P_{2 \cdot 1}$)	"Adjusted" total mixture level as nickel (mg/l)	Observed % mortality in 96 hours
0	0	0	--	--	0
0.02	3.8	7.18	0.53	6.36	71
0.02	4.4	7.78	0.57	7.37	86
0.03	3.3	8.38	0.39	6.05	21
0.03	3.3	8.38	0.39	6.05	50
0.03	3.6	8.68	0.42	6.53	64
0.01	7.0	8.69	0.81	5.51	21
0.03	4.4	9.48	0.46	7.54	43
0.01	7.8	9.49	0.82	5.91	29
0.02	6.6	9.98	0.66	8.57	93
0.01	8.5	10.19	0.83	6.24	36
0.01	9.0	10.69	0.84	6.44	79
0.01	10.0	11.69	0.86	6.82	71
0.01	13.0	14.69	0.89	8.17	93

"Unadjusted" total mixture level as nickel = $169.17215\ C_1 + C_2$

"Adjusted" total mixture level as nickel = $\left[\frac{169.17215\ C_1 + C_2}{f(P_{2 \cdot 1})}\right]$

These results suggest that the most potent combination of copper and nickel contains 60% nickel, i.e. $V = 0.60$, and 40% copper with respect to the total concentration of the mixture. Such mixtures are 2.1 times more potent, i.e. $S = 2.10$, than predicted according to the model of CONCENTRATION-ADDITION.

Statistical analysis of these results indicates that the correlation coefficient (0.8091) based on the adjusted dose-response data is highly significant ($P < 0.001$); conversely, the correlation coefficient (0.3799) associated with the unadjusted data is not significant ($P > 0.05$). Thus the derived model, as represented by Equation (27), increases the linearity of these data (Figure 15).

The results of the present study suggest that the derived model may serve as an effective tool for quantifying SUPRA-ADDITIVE SYNERGISM by means of fitting a linear regression to adjusted dose-response data. The general application of the model may provide a practical approach to predicting, on the basis of the observed responses of aquatic organisms to a few test mixtures, the following phenomena: (1) the responses of aquatic organisms to other combinations of these toxicants representing other constituent ratios; and (2) the particular combinations of the toxicants that are likely to be most potent and consequently, most hazardous to aquatic organisms. The feasibility of these and other possible applications of the proposed model should be determined by future investigations.

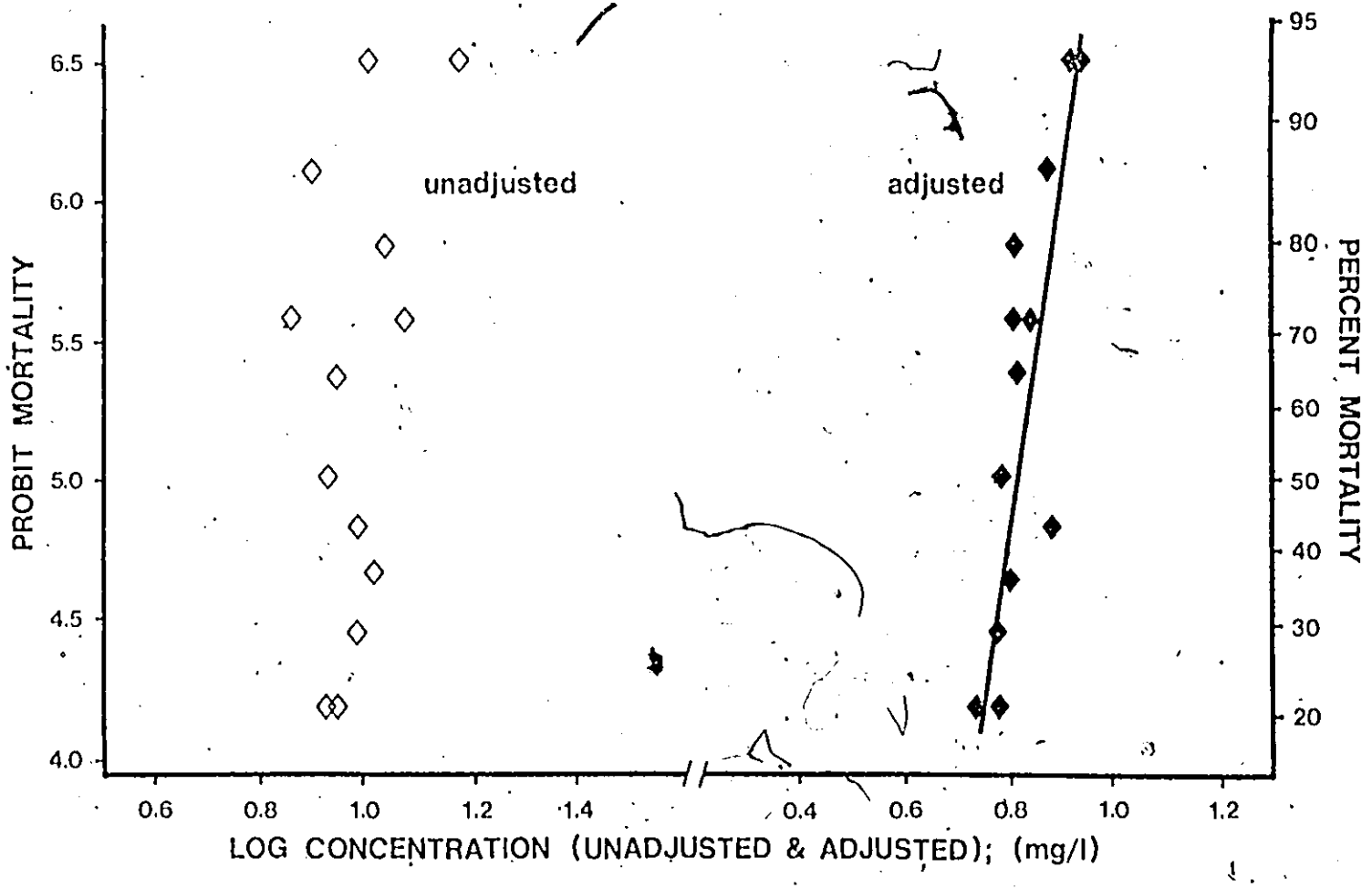


Figure 15. Dose-response curves for unadjusted and adjusted lethal toxicity data for mixtures of copper and nickel

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APPENDIX I

SAMPLE CALCULATIONS DEMONSTRATING THE PROTOCOL FOLLOWED TO COMPUTE NOMINAL CONCENTRATIONS OF A SINGLE TOXICANT AND THE REQUIRED FLOW RATES FOR THE CONTINUOUS-FLOW DILUTION APPARATUS

Protocol (refer to Figure 2)

1. Decide on the range of concentrations to be tested, based on previous knowledge of the toxicant's potency if the information is available.

2. Using the following derived formula, interpolate the range of concentrations in order to determine "n" concentrations spaced at equal intervals along a logarithmic scale.

$$C_x = \text{antilog}\left[\log C_1 + (x-1)(\log C_n - \log C_1)/(n-1)\right] \qquad (30)$$

where,

n = the number of concentrations to be determined

C_x = the concentration which is at position "x" in rank when all concentrations to be determined are listed as variables in order of increasing magnitude, i.e. C_1 = lowest concentration, C_2 = second lowest concentration, ..., C_n = highest concentration.

3. Decide on a concentration to be established in the toxicant chamber of the diluter. This concentration should be greater or equal to the highest concentration (C_n) which is to be delivered to an exposure aquarium, but low enough so that metal salts do not precipitate in the chamber.

4. Calculate a flow rate for each faucet in the toxicant chamber (see Equation 31).

$$R_p C_p = R_{p+1} C_{p+1} \tag{31}$$

where,

R_p = flow rate of toxicant solution at any phase "p" of dilution

C_p = concentration of toxicant solution at phase "p" of dilution.

R_{p+1} = flow rate of toxicant solution at phase "p+1" of dilution

C_{p+1} = concentration of toxicant solution at phase "p+1" of dilution.

5. Select a flow rate for the faucet located in the side of the water chamber which serves the toxicant chamber below with diluent water. This flow rate may be arbitrarily selected, however it must exceed the sum of the previously calculated flow rates for faucets in the toxicant chamber. The purpose of this constraint is to maintain a constant head level in the

toxicant chamber by feeding in a surplus flow of diluent water which can only escape through the standpipe in the chamber.

6. Calculate a flow rate for each of the other faucets in the water chamber to provide a final flow rate of 300 ml/min of diluted toxicant solution into each exposure aquarium.

7. Decide on a drip rate for Mariotte bottle and calculate concentration of stock solution required (see Equation 31). Based on this drip rate, determine the volume of stock solution which is required for the duration of the experiment.

Note: The same approach is used to compute flow rates when mixtures of two toxicants are to be studied.

Sample Calculations (refer to Table 18)

For test concentrations ranging from 15.0 mg/l to 35.0 mg/l, a nominal concentration is determined for each exposure aquarium according to Equation (30). Based on a final flow rate of 300 ml/min through each exposure aquarium and a selected concentration of 200 mg/l in the toxicant chamber,

$$R_1 C_1 = R_2 C_2 \qquad (32)$$

$$R_1(200) = (300) C_2$$

$$\therefore R_1 = 1.5\ C_2$$

where,

R_1 = flow rate for faucet in toxicant chamber

C_2 = nominal concentration to be tested.

According to Table 18, the sum of the flow rates for faucets in the toxicant chamber is 392.0 ml/min. A flow rate of 500.0 ml/min for the faucet in the side of the water chamber should provide a sufficient surplus flow to maintain a constant head level in the toxicant chamber below. To provide a total flow rate of 300 ml/min into each exposure aquarium, the flow rate for each of the other faucets in the water chamber must be 300 minus R_1.

The concentration of toxicant stock solution in the Mariotte bottle is calculated based on the following information: (1) drip rate of stock solution from Mariotte bottle is 2.0 ml/min; (2) total flux of diluted solution through toxicant chamber is 2.0 + 500.0 = 502 ml/min; and (3) concentration in toxicant chamber is 200 mg/l. Thus,

$$\begin{aligned} R_0C_0 &= R_1C_1 \\ 2C_0 &= (502)(200) \\ C_0 &= 50{,}200.0 \end{aligned}$$

where,

C_0 = concentration (mg/l) of toxicant stock solution in Mariotte bottle.

Table 18. Results of sample calculations to compute nominal concentrations of a single toxicant and the required flow rates for the diluter's faucets

Nominal test concentration of toxicant (mg/l)	Faucet flow rate for toxicant chamber (ml/min)	Faucet flow rate for water chamber (ml/min)
15.0	22.5	277.5
16.3	24.5	275.3
17.8	26.7	273.3
19.3	29.0	271.0
21.1	31.7	268.3
22.9	34.4	265.6
24.9	37.4	262.6
27.1	40.7	259.3
29.5	44.3	255.7
32.2	48.3	251.7
35.0	52.5	247.5
	Sum = 392.0	

APPENDIX II

COMPUTER PROGRAM DESIGNED AS COMPUTATIONAL AID FOR FINNEY'S (1971) METHOD OF PROBIT ANALYSIS; COMPUTER OUTPUT IS GIVEN FOR SAMPLE DATA

This program performs a single iteration of analysis in the "maximum likelihood" estimation of a probit regression line. When necessary, subsequent iterations may be performed by re-executing the program using information derived from previous iterations.

```
C     NORMAN WEINSTEIN
C
C     COMPUTATIONAL AID FOR TABLE 4.1, P.62 IN FINNEY,1971.
C     PROGRAM CALCULATES REGRESSION LINE, PREDICTED Y'S AND THEIR
C     95% FIDUCIAL LIMITS.
C
C     SUBSTITUTE N=#ROWS, TC=CRITICAL T VALUE FOR (N-2)DF AT P=.05
C     ENTER DATA ACCORDING TO "2 FORMAT", WITH X=LOG CONC,
C     NW=WEIGHTING COEFFICIENT * #TEST ORGANISMS, WPY=WORKING PROBIT.
C
      PROGRAM MAIN(INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
      REAL X(100),NW(100),WPY(100),NWX(100),NWY(100)
      REAL NWXS(100),NWXY(100),NWYS(100),Y(100),YNEW(100),VY(100)
      REAL SY(100),FLL(100),FLU(100),FL(100),FU(100)
      N=26
      TC=2.064
      SNW=0
      SNWX=0
      SNWY=0
      SNWXSA=0
      SNWXYA=0
      SNWYSA=0
      WRITE(6,1)
    1 FORMAT(*1*,6X,*X*,13X,*NW*,12X,*WPY*,11X,*NWX*,11X,*NWY*,11X,
     1*NWXSQ*,9X,*NWXY*,10X,*NWYSQ*)
      DO 8 I=1,N
      READ(5,2)X(I),NW(I),WPY(I)
    2 FORMAT(3F10.2)
C
C     CALCULATE COLUMNS AND THEIR SUMS
C
      NWX(I)=NW(I)*X(I)
      NWY(I)=NW(I)*WPY(I)
      NWXS(I)=NW(I)*(X(I)**2)
      NWXY(I)=NW(I)*X(I)*WPY(I)
      NWYS(I)=NW(I)*(WPY(I)**2)
      WRITE(6,3)X(I),NW(I),WPY(I),NWX(I),NWY(I),NWXS(I),NWXY(I),
     1NWYS(I)
    3 FORMAT(* *,8F14.7)
      SNW=SNW+NW(I)
      SNWX=SNWX+NWX(I)
```

```
      SNWY=SNWY+NWY(I)
      SNWXSA=SNWXSA+NWXS(I)
      SNWXYA=SNWXYA+NWXY(I)
      SNWYSA=SNWYSA+NWYS(I)
    8 CONTINUE
      CALL ROUND(SNWXSA,5,SNWXS)
      CALL ROUND(SNWXYA,4,SNWXY)
      CALL ROUND(SNWYSA,3,SNWYS)
C
C     CALCULATE DATA MATRIX STATISTICS
C
      AA=(SNWX**2)/SNW
      BA=(SNWX*SNWY)/SNW
      CA=(SNWY**2)/SNW
      CALL ROUND(AA,5,A)
      CALL ROUND(BA,4,B)
      CALL ROUND(CA,3,C)
      SXX=SNWXS-A
      SXY=SNWXY-B
      SYY=SNWYS-C
      XBAR1=SNWX/SNW
      YBAR1=SNWY/SNW
      CALL ROUND(XBAR1,4,XBAR)
      CALL ROUND(YBAR1,4,YBAR)
      WRITE(6,4)
    4 FORMAT(*0*,*SUMS OF COLUMNS ARE AS FOLLOWS*)
      WRITE(6,5)SNW,SNWX
    5 FORMAT(*0*,*SNW=*,F15.2,12X,*SNWX=*,F15.4)
      WRITE(6,6)SNWY,SNWXS
    6 FORMAT(* *,*SNWY=*,F16.4,10X,*SNWXSQ=*,F14.5)
      WRITE(6,7)SNWXY,SNWYS
    7 FORMAT(* *,*SNWXY=*,F15.4,10X,*SNWYSQ=*,F12.3)
      WRITE(6,9)
    9 FORMAT(*0*,*STATISTICS OF DATA MATRIX ARE AS FOLLOWS*)
      WRITE(6,10)XBAR,YBAR
   10 FORMAT(*0*,*XBAR=*,F10.4,8X,*YBAR=*,F10.4)
      WRITE(6,11)SXX,SXY,SYY
   11 FORMAT(* *,*SXX=*,F12.5,7X,*SXY=*,F11.4,7X,*SYY=*,F12.3)
C
C     CALCULATE REGRESSION EQUATION PARAMETERS
C
      SLOPEA=SXY/SXX
      YINTA=YBAR-SLOPEA*XBAR
      CALL ROUND(SLOPEA,3,SLOPE)
      CALL ROUND(YINTA,3,YINTCP)
      WRITE(6,12)
   12 FORMAT(*0*,*REGRESSION EQUATION PARAMETERS ARE AS FOLLOWS*)
      WRITE(6,13)YINTCP,SLOPE
   13 FORMAT(*0*,*INTERCEPT=*,F10.3,8X,*SLOPE=*,F10.3)
C
C     CALCULATE PREDICTED Y'S
C
      WRITE(6,14)
   14 FORMAT(*0*,*PREDICTED Y'S AND 95% FIDUCIAL LIMITS ARE AS FOLLOWS
     1*)
```

```
      WRITE(6,15)
   15 FORMAT(*0*,7X,*X*,8X,*YNEW*,5X,*LOWER*,5X,*UPPER*)
      DO 17 J=1,N
      Y(J)=YINTCP+X(J)*SLOPE
      CALL ROUND(Y(J),3,YNEW(J))
C
C     CALCULATE 95% FIDUCIAL LIMITS IN TERMS OF Y AT EACH X
C     SEE EQUATION 4.31, P.76 IN FINNEY,1971.
C
      VY(J)=1/SNW+((X(J)-XBAR)**2)/SXX
      SY(J)=SQRT(VY(J))
      FLL(J)=YNEW(J)-TC*SY(J)
      FLU(J)=YNEW(J)+TC*SY(J)
      CALL ROUND(FLL(J),3,FL(J))
      CALL ROUND(FLU(J),3,FU(J))
      WRITE(6,16)X(J),YNEW(J),FL(J),FU(J)
   16 FORMAT(* *,F10.2,3F10.3)
   17 CONTINUE
C
C     FIND LC50 AND ITS 95% FIDUCIAL LIMITS
C     SEE EQUATION 4.37, P.79 IN FINNEY,1971.
C
      V=(5.0-YINTCP)/SLOPE
      G=(1.96**2)/((SLOPE**2)*SXX)
      P=SQRT((1-G)/SNW+((V-XBAR)**2)/SXX)
      Q=(1.96/(SLOPE*(1-G)))*P
      VL=V+(G/(1-G))*(V-XBAR)-Q
      VU=V+(G/(1-G))*(V-XBAR)+Q
      WL=10.0**VL
      WU=10.0**VU
      VA=10.0**V
      CALL ROUND(VA,2,VLC50)
      CALL ROUND(WL,2,AL)
      CALL ROUND(WU,2,AU)
      WRITE(6,18)V
   18 FORMAT(*0*,*ESTIMATED LOG LC50 IS*,F14.9)
      WRITE(6,19)VLC50
   19 FORMAT(* *,*ESTIMATED LC50 IS*,F11.2)
      WRITE(6,20)AL
   20 FORMAT(* *,*LOWER 95% FIDUCIAL LIMIT OF ESTIMATED LC50 IS*,F7.2)
      WRITE(6,21)AU
   21 FORMAT(* *,*UPPER 95% FIDUCIAL LIMIT OF ESTIMATED LC50 IS*,F7.2)
      STOP
      END
      SUBROUTINE ROUND(F,L,D)
C
C     SUBROUTINE TO ROUND OFF NUMBERS TO "L" DECIMAL PLACES
      Z=ABS(F)
      H=Z+5.0/(10.0**(L+1))
      IR=H*(10.0**L)
      D=IR/(10.0**L)
      IF(F.LT.0)D=-1.0*D
      RETURN
      END
```

X	NW	WPY	NWX	NWY	NWXSQ	NWXY	NWYSQ
1.1000000	1.2900000	2.4100000	1.4190000	3.1089000	1.5609000	3.4197900	7.4924490
1.1800000	2.5200000	4.5200000	2.9736000	11.3904000	3.5088480	13.4406720	51.4846080
1.2100000	2.9100000	2.8300000	3.5211000	8.2353000	4.2605310	9.9647130	23.3058990
1.2600000	3.7700000	4.0700000	4.7502000	15.3439000	5.9852520	19.3333140	62.4496730
1.3000000	4.7000000	3.9500000	6.1100000	18.5650000	7.9430000	24.1345000	73.3317500
1.3200000	5.1800000	3.9300000	6.8376000	20.3574000	9.0256320	26.8717680	80.0045820
1.3300000	5.1800000	3.2100000	6.8894000	16.6278000	9.1629020	22.1149740	53.3752380
1.3600000	5.6700000	4.9300000	7.7112000	27.9531000	10.4872320	38.0162160	137.8087830
1.3700000	6.1400000	3.3400000	8.4118000	20.5076000	11.5241660	28.0954120	68.4953840
1.3700000	6.1400000	3.9200000	8.4118000	24.0688000	11.5241660	32.9742560	94.3496960
1.3900000	6.6000000	3.6700000	9.1740000	24.2220000	12.7518600	33.6685800	88.8947400
1.4300000	7.0400000	4.7100000	10.0672000	33.1584000	14.3960960	47.4165120	156.1760640
1.4500000	7.4400000	3.9700000	10.7880000	29.5368000	15.6426000	42.8283600	117.2610960
1.4600000	7.8100000	4.0000000	11.4026000	31.2400000	16.6477960	45.6104000	124.9600000
1.4800000	7.8100000	4.2100000	11.5588000	32.8801000	17.1070240	48.6625480	138.4252210
1.5100000	8.4100000	4.6400000	12.6991000	39.0224000	19.1756410	58.9238240	181.0639360
1.5100000	8.4100000	4.4500000	12.6991000	37.4245000	19.1756410	56.5109950	166.5390250
1.5400000	8.6300000	4.6400000	13.2902000	40.0432000	20.4669080	61.6665280	185.8004480
1.5900000	8.8800000	5.0000000	14.1192000	44.4000000	22.4495280	70.5960000	222.0000000
1.6200000	8.9100000	5.0000000	14.4342000	44.5500000	23.3834040	72.1710000	222.7500000
1.6600000	8.7800000	4.8200000	14.5748000	42.3196000	24.1941680	70.2505360	203.9804720
1.6900000	8.6300000	5.5400000	14.5847000	47.8102000	24.6481430	80.7992380	264.8685080
1.7000000	8.4100000	5.1700000	14.2970000	43.4797000	24.3049000	73.9154900	224.7900490
1.7300000	8.1300000	5.9800000	14.0649000	48.6174000	24.3322770	84.1081020	290.7320520
1.8700000	5.6700000	6.0800000	10.6029000	34.4736000	19.8274230	64.4656320	209.2004880
2.0000000	3.3300000	7.0900000	6.6600000	23.6097000	13.3200000	47.2194000	167.3937730

SUMS OF COLUMNS ARE AS FOLLOWS

SNW=	166.39	SNWX=	252.0524
SNWY=	762.9458	SNWXSQ=	386.80604
SNWXY=	1177.1788	SNWYSQ=	3617.332

STATISTICS OF DATA MATRIX ARE AS FOLLOWS

XBAR= 1.5148 YBAR= 4.5853
SXX= 4.98975 SXY= 21.4464 SYY= 119.007

REGRESSION EQUATION PARAMETERS ARE AS FOLLOWS

INTERCEPT= -1.925 SLOPE= 4.298

PREDICTED Y'S AND 95% FIDUCIAL LIMITS ARE AS FOLLOWS

X	YNEW	LOWER	UPPER
1.10	2.803	2.388	3.218
1.18	3.147	2.799	3.495
1.21	3.276	2.952	3.600
1.26	3.490	3.205	3.775
1.30	3.662	3.407	3.917
1.32	3.748	3.507	3.989
1.33	3.791	3.557	4.025
1.36	3.920	3.705	4.135
1.37	3.963	3.754	4.172
1.37	3.963	3.754	4.172
1.39	4.049	3.852	4.246
1.43	4.221	4.043	4.399
1.45	4.307	4.136	4.478
1.46	4.350	4.182	4.518
1.48	4.436	4.273	4.599
1.51	4.565	4.405	4.725
1.51	4.565	4.405	4.725
1.54	4.694	4.532	4.856
1.59	4.909	4.735	5.083
1.62	5.038	4.851	5.225
1.66	5.210	5.001	5.419
1.69	5.339	5.111	5.567
1.70	5.382	5.148	5.616
1.73	5.511	5.256	5.766
1.87	6.112	5.747	6.477
2.00	6.671	6.195	7.147

ESTIMATED LOG LC50 IS 1.611214518
ESTIMATED LC50 IS 40.85
LOWER 95% FIDUCIAL LIMIT OF ESTIMATED LC50 IS 37.49
UPPER 95% FIDUCIAL LIMIT OF ESTIMATED LC50 IS 45.39

APPENDIX III

COMPUTER PROGRAM DESIGNED TO QUANTIFY TOLERANCE AS A FUNCTION OF CONCENTRATION OF TOXICANT AND BODY WEIGHT OF ORGANISMS; COMPUTER OUTPUT IS GIVEN FOR SAMPLE DATA

```
C     NORMAN WEINSTEIN
C
C     PROGRAM TO DETERMINE BEST PROPORTIONALITY FACTOR FROM -2.00 TO
C     +2.00
C
C     TAILOR PROGRAM TO SPECIFIC DATA BY SUBSTITUTING N=#ROWS FOR
C     CARD AFTER "DIMENSION" CARD.
C     ENTER DATA IN 3 COLUMNS ACCORDING TO "1 FORMAT" WITH
C     W=WEIGHT, C=CONCENTRATION, Y=RESPONSE IN PROBITS.
C
      PROGRAM MAIN(INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
      DIMENSION W(100),C(100),Y(100),WP(401),R(401)
      N=9
      DO 2 I=1,N
      READ(5,1)W(I),C(I),Y(I)
    1 FORMAT(3F10.2)
    2 CONTINUE
C
C     COMPUTE PROPORTIONALITY FACTORS
C
      DO 7 J=1,401
      WP(J)=-2.01+J*0.01
C
C     COMPUTE CORRELATIONS
C
      SD=0.0
      SY=0.0
      SDY=0.0
      SSD=0.0
      SSY=0.0
      DO 3 K=1,N
      CW=C(K)/W(K)**WP(J)
      D=ALOG10(CW)
      SD=SD+D
      SY=SY+Y(K)
      SDY=SDY+D*Y(K)
      SSD=SSD+D**2
      SSY=SSY+(Y(K))**2
    3 CONTINUE
      SD2=SD**2
```

```
      SY2=SY**2
      RNUM=N*SDY-SD*SY
      RDENOM=SQRT((N*SSD-SD2)*(N*SSY-SY2))
      R(J)=RNUM/RDENOM
C
C     FIND BEST CORRELATION
C
      IF(J-1)4,4,5
    4 RMAX=ABS(R(J))
      KA=J
      GO TO 7
    5 IF(ABS(R(J)).LT.RMAX)GO TO 6
      RMAX=ABS(R(J))
      KA=J
      GO TO 7
    6 IF(J.EQ.0)GO TO 8
    7 CONTINUE
    8 CONTINUE
      BESTPF=-2.01+KA*0.01
      WRITE(6,9)BESTPF
    9 FORMAT(*1*,*PROPORTIONALITY FACTOR (P.F.) GIVING BEST CORRELATION
     1IS*,F7.2)
      WRITE(6,10)R(KA)
   10 FORMAT(*0*,*CORRELATION USING BEST P.F. IS*,F9.4)
      WRITE(6,11)R(201)
   11 FORMAT(*0*,*CORRELATION USING NO P.F. IS*,F9.4)
      WRITE(6,12)
   12 FORMAT(*0*,*C.EFF = EFFECTIVE CONC = CONC/(W RAISED TO BEST P.F.)*
     1)
      WRITE(6,13)
   13 FORMAT(*0*,6X,*WEIGHT*,7X,*CONC*,6X,*LOG CONC*,6X,*C.EFF*,
     14X,*LOG C.EFF*,4X,*RESPONSE*)
      DO 15 L=1,N
      E=ALOG10(C(L))
      CW=C(L)/W(L)**BESTPF
      GINDP=ALOG10(CW)
      WRITE(6,14)W(L),C(L),E,CW,GINDP,Y(L)
   14 FORMAT(*0*,6F12.4)
   15 CONTINUE
C
C     COMPUTE LINEAR REGRESSIONS
C
      WRITE(6,16)
   16 FORMAT(*0*,*LINEAR REGRESSIONS AS FOLLOWS*)
      DO 25 IJ=1,2
      SUMD=0.0
      SUMY=0.0
      SUMDY=0.0
      SUMSD=0.0
      IF(IJ-2)17,18,18
   17 Z=BESTPF
      GO TO 19
   18 Z=0.0
   19 DO 20 M=1,N
```

```
      CW=C(M)/W(M)**Z
      D=ALOG10(CW)
      SUMD=SUMD+D
      SUMY=SUMY+Y(M)
      SUMDY=SUMDY+D*Y(M)
      SUMSD=SUMSD+D**2
   20 CONTINUE
      SUMD2=SUMD**2
      BNUM=SUMDY-SUMD*SUMY/N
      BDENOM=SUMSD-SUMD2/N
      SLOPE=BNUM/BDENOM
      SINTCP=(SUMY-SLOPE*SUMD)/N
      IF(IJ-2)21,23,23
   21 WRITE(6,22)SINTCP,SLOPE
   22 FORMAT(*0*,*RESPONSE=*,F10.3,*  +*,F10.3,*  LOG C.EFF*)
      GO TO 25
   23 WRITE(6,24)SINTCP,SLOPE
   24 FORMAT(*0*,*RESPONSE=*,F10.3,*  +*,F10.3,*  LOG CONC*)
   25 CONTINUE
      STOP
      END
```

PROPORTIONALITY FACTOR (P.F.) GIVING BEST CORRELATION IS .47

CORRELATION USING BEST P.F. IS .9618

CORRELATION USING NO P.F. IS .9354

C.EFF = EFFECTIVE CONC = CONC/(W RAISED TO BEST P.F.)

WEIGHT	CONC	LOG CONC	C.EFF.	LOG C.EFF	RESPONSE
.4200	.1300	-.8861	.1954	-.7090	3.7200
.2900	.1500	-.8239	.2684	-.5712	4.1600
.2900	.1700	-.7696	.3042	-.5169	4.4800
.3200	.2200	-.6576	.3758	-.4250	4.8200
.4200	.2200	-.6576	.3307	-.4805	4.4800
.4200	.2400	-.6198	.3608	-.4427	4.4800
.4200	.2700	-.5686	.4059	-.3916	5.0000
.4200	.3100	-.5086	.4661	-.3316	5.2500
.4200	.3500	-.4559	.5262	-.2789	5.8400

LINEAR REGRESSIONS AS FOLLOWS

RESPONSE= 6.829 + 4.636 LOG C.EFF

RESPONSE= 7.377 + 4.063 LOG CONC

APPENDIX IV

COMPUTER PROGRAM DESIGNED FOR QUANTITATIVE MODEL OF SUPRA-ADDITIVE SYNERGISM; COMPUTER OUTPUT IS GIVEN FOR SAMPLE DATA

```
C     NORMAN WEINSTEIN
C
C     MULTIPLE TOXICITY MODEL FOR SUPRA-ADDITIVE TOXICANTS IN
C     BINARY MIXTURES
C
C     PROGRAM SELECTS BEST "V" FROM 0 TO 1.00 AND BEST
C     "S" FROM 1.00 TO 5.00
C
C     TAILOR PROGRAM TO SPECIFIC DATA BY SUBSTITUTING N=#ROWS FOR 2ND
C     CARD AFTER "DIMENSION" CARD. ENTER DATA IN 3 COLUMNS
C     ACCORDING TO "1 FORMAT" WITH P=RELATIVE PROPORTION OF ONE TOXICANT
C     IN MIXTURE, C=TOTAL CONCENTRATION OF MIXTURE EXPRESSED IN TERMS
C     OF ONE TOXICANT, AND Y=RESPONSE IN PROBITS.
C
      PROGRAM MAIN(INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
      DIMENSION P(100),C(100),Y(100),V(101),S(401),R(401),
     1 RM(101),KB(101)
      N=13
      DO 2 ID=1,N
      READ(5,1)P(ID),C(ID),Y(ID)
    1 FORMAT(3F10.2)
    2 CONTINUE
C
C     COMPUTE V'S
C
      DO 13 I=1,101
      V(I)=0.01*(I-1)
C
C     COMPUTE S'S
C
      DO 9 J=1,401
      S(J)=0.01*(J-1)+1.00
C
C     COMPUTE CORRELATIONS
C
      SD=0.0
      SY=0.0
      SDY=0.0
      SSD=0.0
      SSY=0.0
```

```
      DO 6 K=1,N
      IF(P(K)-V(I))3,3,4
    3 F=1.00+(S(J)-1.00)*(V(I)-P(K))/V(I)
      GO TO 5
    4 F=1.00+(S(J)-1.00)*(V(I)-P(K))/(V(I)-1.00)
    5 CE=C(K)/F
      D=ALOG10(CE)
      SD=SD+D
      SY=SY+Y(K)
      SDY=SDY+D*Y(K)
      SSD=SSD+D**2
      SSY=SSY+Y(K)**2
    6 CONTINUE
      SD2=SD**2
      SY2=SY**2
      RNUM=N*SDY-SD*SY
      RDENOM=SQRT((N*SSD-SD2)*(N*SSY-SY2))
      R(J)=RNUM/RDENOM
C
C     FIND BEST CORRELATION
C
      IF(J-1)7,7,8
    7 RMAX=ABS(R(J))
      KS=J
      GO TO 9
    8 IF(ABS(R(J)).LT.RMAX)GO TO 10
      RMAX=ABS(R(J))
      KS=J
    9 CONTINUE
   10 CONTINUE
      RM(I)=R(KS)
      KB(I)=KS
      IF(I-1)11,11,12
   11 RM=ABS(RM(I))
      KV=I
      GO TO 13
   12 IF(ABS(RM(I)).LT.RM)GO TO 13
      RM=ABS(RM(I))
      KV=I
   13 CONTINUE
      RMX=RM(KV)
      JJ=KB(KV)
      BESTS=0.01*(JJ-1)+1.00
      WRITE(6,14)R(1)
   14 FORMAT(*1*,*CORRELATION FOR UNADJUSTED DATA IS*,F8.4)
      WRITE(6,15)RM(KV)
   15 FORMAT(*0*,*CORRELATION FOR ADJUSTED DATA IS*,F8.4)
      WRITE(6,16)V(KV)
   16 FORMAT(*0*,*     V=*,F5.2,*  = ESTIMATE OF PROP.(P) OF 1 TOX. IN *
     1*MOST POTENT COMBINATION OF TOX'S*)
      WRITE(6,17)BESTS
   17 FORMAT(*0*,*     S=*,F5.2,*  = REL. DEGREE SUPRA-ADD. FOR MOST *
     1*POTENT COMBINATION OF TOX'S*)
      WRITE(6,18)
```

```
   18 FORMAT(*0*,*C.ADJ=ADJUSTED  CONC.=CONC./F(P)*)
      WRITE(6,19)
   19 FORMAT(*0*,*WHERE F(P)=1+(S-1)(V-P)/V, IF P < OR = V*)
      WRITE(6,20)
   20 FORMAT(*0*,*WHERE F(P)=1+(S-1)(V-P)/(V-1), IF P > V*)
      WRITE(6,21)
   21 FORMAT(*0*,9X,*P*,9X,*CONC*,6X,*LOG CONC*,6X,*C.ADJ*,4X,
     1*LOG C.ADJ*,4X,*RESPONSE*)
      DO 26 L=1,N
      E=ALOG10(C(L))
      IF(P(L)-V(KV))22,22,23
   22 F=1.00+(BESTS-1.00)*(V(KV)-P(L))/V(KV)
      GO TO 24
   23 F=1.00+(BESTS-1.00)*(V(KV)-P(L))/(V(KV)-1.00)
   24 CE=C(L)/F
      GINDP=ALOG10(CE)
      WRITE(6,25)P(L),C(L),E,CE,GINDP,Y(L)
   25 FORMAT(*0*,6F12.4)
   26 CONTINUE
C
C     COMPUTE LINEAR REGRESSIONS
C
      WRITE(6,27)
   27 FORMAT(*0*,*LINEAR REGRESSIONS AS FOLLOWS*)
      DO 39 IJ=1,2
      SUMD=0.0
      SUMY=0.0
      SUMDY=0.0
      SUMSD=0.0
      IF(IJ-2)28,29,29
   28 Z=BESTS
      GO TO 30
   29 Z=1.00
   30 DO 34 M=1,N
      IF(P(M)-V(KV))31,31,32
   31 F=1.00+(Z-1.00)*(V(KV)-P(M))/V(KV)
      GO TO 33
   32 F=1.00+(Z-1.00)*(V(KV)-P(M))/(V(KV)-1.00)
   33 CE=C(M)/F
      D=ALOG10(CE)
      SUMD=SUMD+D
      SUMY=SUMY+Y(M)
      SUMDY=SUMDY+D*Y(M)
      SUMSD=SUMSD+D**2
   34 CONTINUE
      SUMD2=SUMD**2
      BNUM=SUMDY-SUMD*SUMY/N
      BDENOM=SUMSD-SUMD2/N
      SLOPE=BNUM/BDENOM
      SINTCP=(SUMY-SLOPE*SUMD)/N
      IF(IJ-2)35,37,37
   35 WRITE(6,36)SINTCP,SLOPE
   36 FORMAT(*0*,*RESPONSE=*,F10.3,*  +*,F10.3,*  LOG C.ADJ    (FOR *
     1*ADJUSTED DATA)*)
```

```
      GO TO 39
   37 WRITE(6,38)SINTCP,SLOPE
   38 FORMAT(*0*,*RESPONSE=*,F10.3,*  +*,F10.3,*  LOG CONC      (FOR *
     1*UNADJUSTED DATA)*)
   39 CONTINUE
      STOP
      END
```

CORRELATION FOR UNADJUSTED DATA IS .3799

CORRELATION FOR ADJUSTED DATA IS .8091

V= .60 = ESTIMATE OF PROP.(P) OF 1 TOX. IN MOST POTENT COMBINATION OF TOX'S

S= 2.10 = REL. DEGREE SUPRA-ADD. FOR MOST POTENT COMBINATION OF TOX'S.

C.ADJ=ADJUSTED = CONC.=CONC./F(P)

WHERE F(P)=1+(S-1)(V-P)/V, IF P < OR = V

WHERE F(P)=1+(S-1)(V-P)/(V-1), IF P > V

P	CONC	LOG CONC	C.ADJ	LOG C.ADJ	RESPONSE
.5300	7.1800	.8561	6.3634	.8037	5.5500
.5700	7.7800	.8910	7.3744	.8677	6.0800
.3900	8.3800	.9232	6.0505	.7818	4.1900
.3900	8.3800	.9232	6.0505	.7818	5.0000
.4200	8.6800	.9385	6.5263	.8147	5.3600
.8100	8.6900	.9390	5.5087	.7411	4.1900
.4600	9.4800	.9768	7.5438	.8776	4.8200
.8200	9.4900	.9773	5.9128	.7718	4.4500
.6600	9.9800	.9991	8.5665	.9328	6.4800
.8300	10.1900	1.0082	6.2420	.7953	4.6400
.8400	10.6900	1.0290	6.4398	.8089	5.8100
.8600	11.6900	1.0678	6.8163	.8336	5.5500
.8900	14.6900	1.1670	8.1725	.9124	6.4800

LINEAR REGRESSIONS AS FOLLOWS

RESPONSE= -4.084 + 11.348 LOG C.ADJ (FOR ADJUSTED DATA)

RESPONSE= 1.615 + 3.749 LOG CONC (FOR UNADJUSTED DATA)