

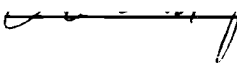
AN ABSTRACT OF THE THESIS OF

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Title: EVALUATION OF AN APPROACH FOR STUDYING THE
QUANTITATIVE RESPONSES OF WHOLE ORGANISMS
TO MIXTURES OF ENVIRONMENTAL TOXICANTS

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An approach previously proposed for studying the effects of mixtures of environmental toxicants on the quantal (all or none) responses of whole organisms was evaluated in terms of its applicability to quantitative (graded) response studies. Four types of multiple toxicity were defined respectively as concentration, response, supra- and infra-addition. Hypothetical dose response curves and their respective isobole diagrams were used to illustrate the relationships between the types of toxicant interaction discussed.

Growth was selected as the quantitative response for study in order to empirically evaluate the proposed approach. The effects of copper, nickel, and dieldrin and selected mixtures of these compounds on the relative growth and food consumption rates of juvenile guppies (Poecilia reticulata) fed a restricted and an unrestricted ration of tubificid worms were expressed as a function of the natural logarithm of toxicant concentration. The resulting dose response

curves for binary mixtures of copper-nickel and dieldrin-nickel were compared to curves predicted on the basis of the previously mentioned types of toxicant interaction. Mixtures of copper and nickel appeared to be either concentration additive or slightly supra-additive depending upon the defined response under consideration. The results of the dieldrin-nickel study were inconclusive regarding the determination of the nature of the interaction for mixtures of these toxicants. Possible reasons for the difficulties encountered in interpreting the results of the dieldrin-nickel studies are discussed. In summary, the proposed approach appears to provide a useful frame of reference for empirically describing the combined effects of multiple toxicants on the performances of whole organisms; however, to offer explanations as to why mixtures of toxicants interact in a particular manner requires further studies on the effects of combined toxicants on underlying biochemical processes and physiological functions.

Evaluation of an Approach for Studying the Quantitative
Responses of Whole Organisms to Mixtures
of Environmental Toxicants

by

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EVALUATION OF AN APPROACH FOR STUDYING THE QUANTITATIVE RESPONSES OF WHOLE ORGANISMS TO MIXTURES OF ENVIRONMENTAL TOXICANTS

INTRODUCTION

An extensive methodology has been developed for evaluating the effects of discrete environmental toxicants on a variety of test organisms. Where environmental pollution does occur, however, several toxicants are usually present simultaneously. The recognition of this situation by environmental toxicologists and those responsible for assessing the potential hazards of man-made pollutants has generated considerable interest in developing approaches for evaluating the effects of mixtures of environmental toxicants. Sprague (1970) in his series of papers on the measurement of pollutant toxicity to fish reviewed the approaches and some of the results of previous studies assessing the joint toxicity of aquatic pollutants.

Although of relatively recent interest in the field of environmental toxicology, the exposure of animals to two or more drugs has for years been the concern of many pharmacologists for both practical and theoretical reasons. In studying the biological responses of organisms to drugs, pharmacologists make a useful distinction between the action of a drug and the effects it produces. The action of a drug or toxicant, although sometimes considered synonymously with effect, is considered to be the underlying processes by which

a compound initiates alterations in some pre-existing physiological or biochemical process. The sequence of biochemical and physiological events which are initiated by the action of a compound are regarded as drug effects. It is commonly recognized that only pharmacological studies on the modes of action of toxicants applied separately or jointly can definitively determine the type of interaction between them (Plackett and Hewlett 1948). However, the primary actions of toxicants have been elucidated in only a few cases. Even in these cases it can probably be expected that the more a presumed action is studied the more likely it will be found to be an effect (Fingl and Woodbury 1965). Consequently, the classical pharmacological method for evaluating the toxicity of compounds involves studying the relationship between the concentration of a toxicant and the effects it produces.

Historically, pharmacologists have studied the interaction of two or more drugs or toxicants in basically two distinct ways (Schild 1961), differentiated primarily on the basis of the level of biological organization under consideration. One approach has been to deduce theoretical concentration effect relationships based on assumed mechanisms of interaction and then to relate empirical results to these curves. This approach has received considerable attention by Clark (1937) and Ariens (1964) and is based on concepts found in molecular pharmacology and receptor theory such as affinity and

intrinsic activity. Investigators using this approach have been primarily interested in the effects of multiple toxicants or drugs on biochemical and physiological processes. The second approach describes general biological models for toxicant interaction and provides them with a mathematical foundation based upon statistical considerations. Since the early contributions of Trevan (1927) and Gaddum (1953), this approach, particularly for quantal (all or none) response studies, has been largely developed by Bliss (1939) and more recently by Hewlett and Plackett (1959). This approach has been used to study the effects of mixtures of toxicants on the survival of whole organisms.

The latter approach was adopted for the present study because it provides a potentially useful frame of reference for evaluating the effects of mixtures of environmental toxicants on whole organism performances such as survival, growth and reproduction. The selection of an appropriate response for evaluating the toxicity of an environmental pollutant depends on the objectives of the toxicologist. Lethality is often used as a starting point for studying the toxic properties of a compound. Thus, it is not surprising that most studies in the literature on the joint toxicity of environmental toxicants have utilized death as the index of toxicity. However, to insure the success of organisms in nature, it is also necessary to study the effects of toxic substances on such whole organism performances

as growth, reproduction, and behavioral activities.

Plackett and Hewlett (1948) proposed that the mathematical examination of the concentration mortality curves for individual toxicants may indicate the types of combined effects that occur when toxicants are present simultaneously. Using their approach, Anderson and Weber (1977) were able to predict in most cases the effects of mixtures of selected environmental toxicants on the survival of guppies (Poecilia reticulata). Based on these results, a series of experiments were designed for the present study to evaluate the applicability of the approach to graded (sublethal) responses.

The objectives of this study are: (1) to examine the rationale of the proposed approach for studying both the quantal and graded responses of whole organisms to mixtures of environmental toxicants and (2) to empirically evaluate the approach by studying the effects of selected environmental toxicants and their mixtures on a whole organism performance using an aquatic organism as the test animal. Hypothetical dose response curves with their associated isobole diagrams are presented to illustrate the different types of toxicant interaction discussed.

RATIONALE

Using Bliss's paper (1939) as their point of departure, Plackett and Hewlett (1952) described general biological models for toxicant interactions and deduced mathematical models for each based largely upon statistical considerations. They proposed general types of toxicant interaction from the following two-way classification scheme:

	Similar	Dissimilar
Non-interactive	Simple similar (concentration addition)	Independent (response addition)
Interactive	Complex similar	Dependent

Toxicant mixtures were defined as "similar" or "dissimilar" according to whether the toxicants acted upon the same or different biological systems (i. e. , biochemical, physiological) and as "interactive" or "non-interactive" according to whether one toxicant influenced the "biological action" of the other toxicants. "Simple similar" and "independent action" were regarded as special cases in a range of biological possibilities and the mathematical models proposed for "complex similar and dependent" were generalizations of the models proposed for "simple similar and independent action" respectively.

Their mathematical models, particularly for the quantal responses to mixtures of "interactive" toxicants, are very complex and

require the knowledge of certain parameters which are normally unattainable when evaluating the effects of toxicant mixtures on whole organism performances. However, Hewlett and Plackett's models for "joint action" are useful for elucidating the limitations of and the assumptions required for the special cases of "simple similar and independent joint action." As a first approach to evaluating the effects of toxicant mixtures on the whole organism performances such as survival and growth, the present discussion only considers the special cases of "non-interactive" toxicant mixtures.

A multitude of terms have been suggested to describe the various types of combined toxicant effects. Ariens (1972) and Fedeli et al. (1972) review the various terminologies that have been used. As Sprague (1970) and Warren (1971) point out, the nomenclature is confusing particularly since certain terms have been defined in more than one way by different authors. Furthermore, terminology describing mechanisms of toxicant action is not appropriate for studies evaluating the effects of toxicant mixtures on whole organism performances without knowledge of the action of the individual toxicants. To avoid both ambiguities in terminology and assumptions implying knowledge of sites and mechanisms of toxicant action, Anderson and Weber (1977) introduced the terms concentration and response addition which are mathematically analogous to the "simple similar" and "independent action" defined by Plackett and Hewlett

(1952).

Concentration addition is mathematically defined as the summation of the concentrations of the individual constituents in a mixture after adjusting for differences in their respective potencies. The primary assumption governing this type of addition is that the toxicants in a mixture act upon similar biological systems and contribute to a common response in proportion to their respective potencies. Bliss (1939) and others have assumed that if two toxicants act similarly the variations in susceptibility of individual organisms to the toxicants are completely correlated. As a consequence the dose response curves for the components and the mixture are parallel. This has been observed for some toxicant mixtures; however, Plackett and Hewlett (1952) presented examples of chemically related insecticides which gave non-parallel lines. They and other toxicologists (Ariens and Simonis 1961; Casarett 1975) have quite correctly pointed out that parallelism and hence complete correlation of individual susceptibilities is not a necessary prerequisite for this type of addition.

In cases where the dose response curves for the individual toxicants are parallel, a dose response curve for the mixture can be calculated based upon the assumption of concentration addition. With the regression equations for the individual toxicants in the form of $y = a + b \ln x$ (where y is the % response to each toxicant and x is

its concentration), the regression equation for a binary mixture can be represented by (Finney 1971):

$$Y_m = a_1 + b \ln (\pi_1 + p\pi_2) + b \ln X \quad (1)$$

where,

Y_m = % response to the mixture

a_1 = y intercept of the first toxicant

b = common slope

π_1 = proportion of the first toxicant in the mixture

π_2 = proportion of the second toxicant in the mixture

p = potency of the second toxicant relative to the first

X = concentration of the mixture

This equation can be readily adapted to represent mixtures containing more than two toxicants. It should be noted that equation (1) for concentration addition is similar in principle to the toxic unit method used by Lloyd (1961), Brown (1968) and others. Whereas the toxic unit method measures the toxicity of mixtures only at particular levels of response (LC10, LC50, etc.), equation (1) incorporates the entire dose response curve.

Response addition is methodologically defined as the summation of the responses of the organism to each toxicant in a mixture. This form of addition is based on the assumption that the toxic constituents of a mixture act upon different biological systems within the organism.

Each organism in a population is assumed to have a tolerance for each of the toxicants in a mixture and will only show a response to a toxicant if the concentration exceeds its tolerance. Thus, the responses to a binary mixture are additive only if the concentrations of both toxicants are above their respective tolerance thresholds. For quantal responses the tolerances to the toxicants in a mixture may vary from one individual to another in a population; therefore, the response of the test animals depends also upon the correlation between the susceptibilities of the individual organisms to the discrete toxicants. For example, in order to predict the proportion of organisms killed by a binary mixture, it is necessary to know not only the proportion that would be killed by each toxicant alone but also to what degree the susceptibility of organisms to one toxicant is correlated with their susceptibility to the other toxicant.

Plackett and Hewlett (1948) recognized this statistical concept and developed mathematical models that account for the correlation of individual tolerances ranging from total negative to total positive correlation. If the correlation is completely negative ($r=-1$) so that the organisms most susceptible to one toxicant (A) are least susceptible to the other (B), then the proportion of individuals responding to the mixture (P_m) can be represented by:

$$P_m = P_A + P_B \quad \text{if } (P_A + P_B \leq 1) \quad (2a)$$

where P_A and P_B are the respective proportion of organisms responding to the individual toxicants A and B. With no correlation ($r=0$) in susceptibility the relationship is expressed by:

$$P_m = P_A + P_B (1 - P_A) \quad (2b)$$

In the limiting case of complete and positive correlation ($r=1$), individuals very susceptible to toxicant A in comparison with the population will be correspondingly very susceptible to toxicant B. In this situation the proportion of animals responding to the mixture is equal to the response to the most toxic constituent in the mixture. Mathematically this is represented by:

$$\begin{aligned} P_M &= P_A \quad \text{if } P_A \geq P_B \\ P_M &= P_B \quad \text{if } P_B \geq P_A \end{aligned} \quad (2c)$$

For response addition no significance can be placed on the slope of the dose response curves because the toxicants in a mixture are acting primarily upon different biological systems with varying degrees of susceptibility between organisms. Even if the regression equations for the constituents in a mixture are parallel for toxicants acting in this manner, the dose response curve for the mixture will not be linear (Finney 1971) except in the special case where $r = 1$. This will be illustrated later for two hypothetical toxicants whose dose response curves are parallel. Although the

mathematical equations (2 a, b, c) representing response addition are relatively simple, the statistical consequences of this type of addition are more complicated than those of concentration addition (Finney 1971).

Terms such as supra- and infra-addition are used to describe toxicant interactions which are greater or less than those predicted on the basis of either concentration or response addition.

Quantal Response Studies

Hypothetical Dose Response Curves

To illustrate the relationship between concentration and response addition, hypothetical dose response curves for two toxicants (A and B) are plotted in Figure 1 expressing percent response in probits as a function of the logarithm of total concentration. In this example the dose response curves for the discrete toxicants are parallel with A being 100 times more toxic than B. Non-parallel curves could have also been used; however, for these cases equation (1) for concentration addition is not appropriate. Hewlett and Plackett (1959) developed a more generalized model (from which equation (1) can be deduced) which does not depend on the assumption of parallel dose response curves.

Dose response curves for mixtures of toxicants A and B are obtained when the total concentration is varied and the ratio of the

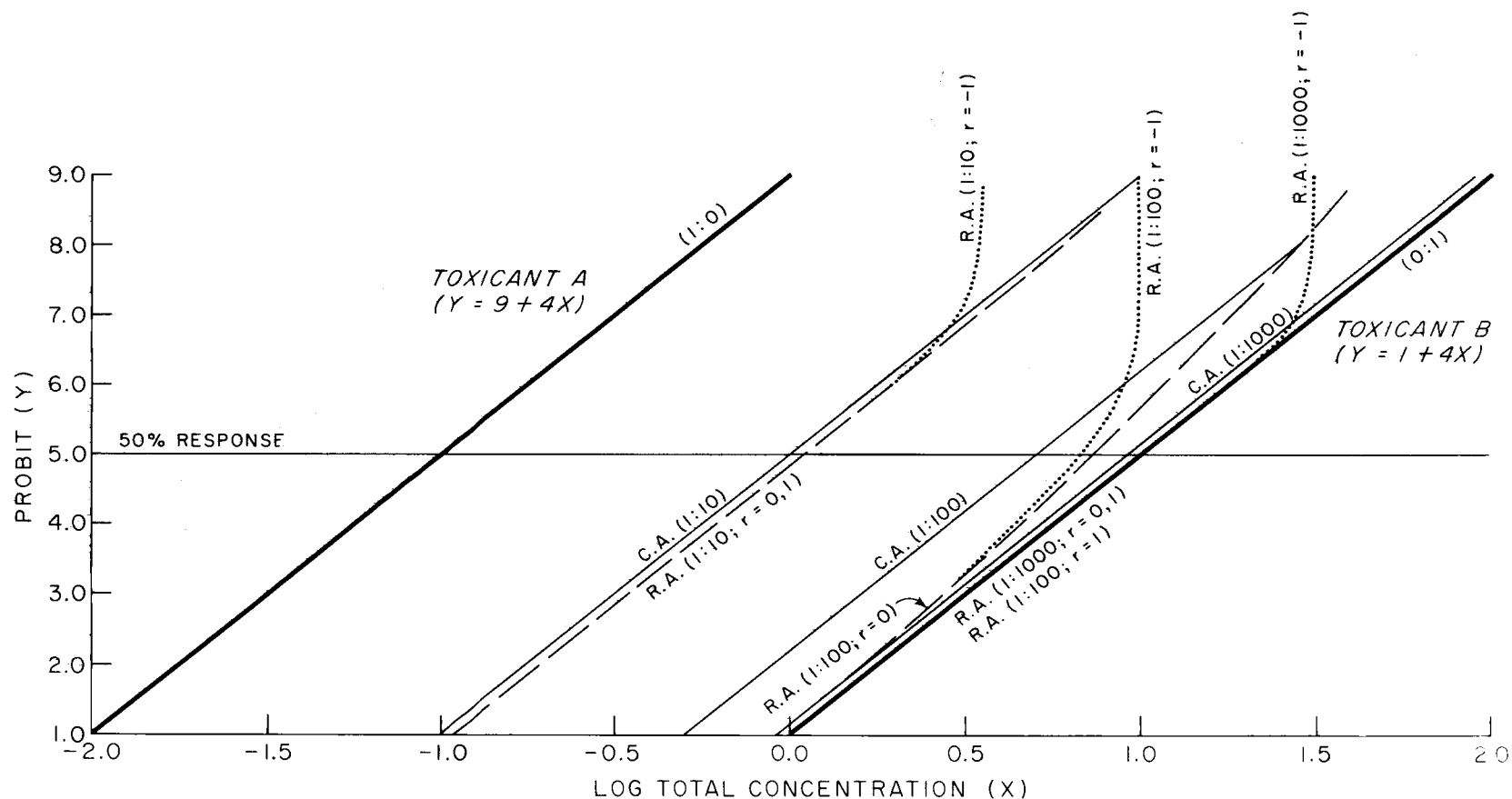


Figure 1. Hypothetical dose response curves for toxicant A (1:0), toxicant B (0:1) and their mixture containing the fixed proportions (1:10, 1:100, 1:1000). See text for explanation.

concentrations for the individual toxicants is kept constant. Using the equations (1 and 2 a, b, c) for concentration (C. A.) and response addition (R. A.), dose response curves were calculated for mixtures containing different fixed proportions of toxicants A:B (1:10, 1:100, 1:1000). In Figure 1, the curves for the mixtures are shown graphically in relation to the dose response curves of toxicants A and B.

Several observations can be made from the relationships between the dose response curves in Figure 1. As should be expected, the relative toxicity of the mixture depends on the ratio of its constituents. In Figure 1, a 1:10 mixture is more toxic than the other ratios depicted because of the greater proportion of the more toxic component, toxicant A. At certain fixed proportions the relative toxicity of mixtures acting in either a concentration or a responsive additive manner are very similar except at very high levels of response where $r = -1$. This is observed in Figure 1 for fixed proportions of 1:10 and 1:1000. For intermediate ratios the relationship between the dose response curves for concentration and response addition ($r=1, 0, -1$) is very dependent on the level of response. For example, at low levels of response (i. e., at the probit of 2 which corresponds to approximately a 0% response) mixtures in a 1:100 ratio acting in a concentration additive manner are considerably more toxic than those acting by response addition regardless of the degree of correlation (r). This is due to a fundamental difference in the two types of addition. At

threshold or below threshold concentrations of toxicants A and B, a mixture acting in a concentration additive manner can elicit a measurable effect because both toxicants are acting upon similar biological systems; their concentrations sum to produce a concentration for the mixture which is above the threshold level. However, the responses to toxicants acting upon different biological systems (response addition) are only additive if each toxicant in a binary mixture is present in concentrations above its respective threshold level. For similar reasons, as the concentrations for the toxicants in a 1:100 mixture increase, the toxicants acting in a response additive manner (except in the special limiting case where $r = 1$) become progressively more toxic relative to the dose response curve for concentration addition. It is even possible that at high levels of response (in this example, for responses greater than 84%; probit of 6.0), mixtures acting in a response additive manner ($r = -1$) can be more toxic than those acting on the basis of concentration addition.

These factors--the type of interaction, the ratio of the toxicants in a mixture, and the level of response--must also be considered along with the toxic properties of the individual compounds in assessing the relative toxicity of a mixture. The failure to recognize these factors can potentially lead to erroneous conclusions concerning the nature of the interaction.

Isobole Diagram

It is difficult to visualize the relationships between the dose response curves in Figure 1, primarily due to the number of curves presented. However, the relationships between the hypothetical curves in Figure 1 can be readily conceptualized with isobole diagrams, a technique introduced by Loewe (1928, 1953). Isoboles are lines of equivalent response. They are constructed by plotting on a two-dimensional diagram the concentrations of a binary mixture of toxicants that produce a quantitatively defined response, i. e. a 10%, 50% or 90% lethal response. It should be noted that an isobole diagram can be constructed for any level of response and that the relationship between the isoboles may vary depending upon the response level selected.

The isobole diagram for the 50% level of response of the hypothetical dose response curves in Figure 1 is presented in Figure 2. The x and y axes in this diagram represent the concentrations of toxicant B and A respectively. The radiating dashed lines or mixing rays correspond to a series of mixtures (A:B) of fixed proportions. If the 50% response, produced by combinations of the two toxicants, is represented by points inside the square area, the toxicants are additive. Antagonistic interactions are indicated by combinations of concentrations falling outside the square.

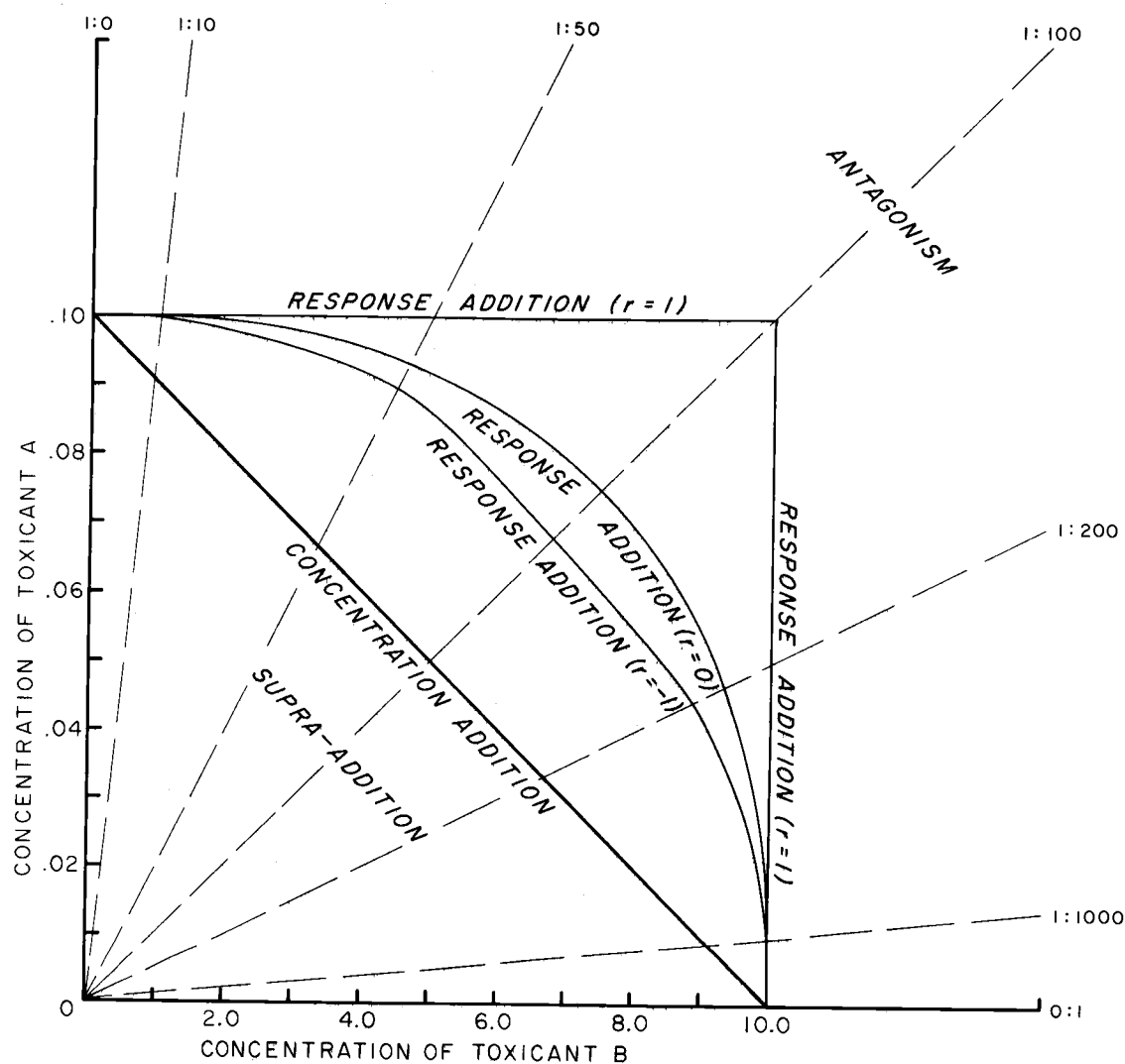


Figure 2. Isobole diagram for quantal response data. Isoboles for concentration and response addition were determined from hypothetical dose response curves in Figure 1.

The isoboles for concentration and response addition are determined by the concentrations of the two toxicants corresponding to the points of intersection between the 50% response line (Figure 1) and the respective hypothetical dose response curves. These concentrations are plotted in Figure 2 on the appropriate mixing ray. The lines connecting these points define the course of the isobole. Concentration addition is shown by the diagonal isobole. For quantal data, response addition is defined by the curved isoboles for complete negative ($r=-1$) and for no correlation ($r=0$) in susceptibility. The upper and right boundaries of the square correspond to the limiting case of response addition with complete positive correlation ($r=1$).

The term "no interaction" has been used by other authors (Sprague 1970; Warren 1971) to describe the response additive isobole in Figure 2 corresponding to complete positive correlation of susceptibilities. It is recognized that the equation (2c) used to determine this isobole is not additive in a strictly mathematical sense. For example, in lethality studies, organisms whose tolerances to the individual toxicants are positively correlated ($r=1$) die in response to the most toxic constituent in the mixture; there is no addition of responses. In experimental situations, it is unlikely that complete positive or for that matter complete negative correlation will often be observed. However, complete positive correlation is represented as a limiting case of response addition to be consistent in the

terminology and more importantly to emphasize that the isobole for response addition will for most toxicant mixtures fall between the extreme cases of $r = -1$ to $r = 1$ depending upon the degree of correlation.

For reasons similar to the ones presented by Warren (1971), the terms supra- and infra-addition are used to describe interactions which are greater or less than expected on the basis of either concentration or response addition. It is important that these terms be used in reference to a particular type of addition. For example, an isobole falling between the ones for concentration and response addition ($r = -1$) could be designated as both infra- and supra-additive depending on the nature of the interaction. This potentially confusing situation is avoided by using the terms in the manner suggested.

The term antagonism in Figure 2 refers to a physiological or functional antagonism. In the present discussion, toxicants that chemically or physically react in the external medium of an organism to form an inactive or less toxic product (chemical antagonism) are not considered. Some investigators have used the term antagonism to describe interactions that are less toxic than strict additivity (concentration addition) but whose mixture still has a combined effect greater than either constituent applied alone. The term infra-addition is preferred to describe these cases and antagonism is reserved for those situations where the presence of one toxicant necessitates that

a higher concentration of another toxicant be present to obtain the defined level of response.

Graded Response Studies

A consideration of the nature of the dose response curves for quantal and graded responses reveals that the effects they express are quite different. Quantal dose response curves express the incidence of an all-or-none effect (usually death) when varying concentrations are applied to a group of organisms. The curve is derived by observing the number of organisms which respond or fail to respond at various concentrations. Consequently, the slopes of these curves primarily express the individual variation of the population to a particular toxicant. Graded dose response curves characterize the relationship between the concentration of a toxicant and the magnitude of the effect under consideration. The dose response curve can be derived by measuring on a continuous scale the average response of a group of organisms at each concentration.

As Clark (1937) and others have pointed out, it is possible to represent any graded response quantally provided that the response of each individual organism can be measured. However, the adoption of this procedure is at the expense of some "loss of information" (Gaddum 1953). Quantal response data disclose only the number of organisms that respond or fail to respond at some particular

concentration. On the other hand, graded response data not only reveal whether or not a group of organisms respond but also how much they respond.

The mathematical equations (2 a, b, c) for the response addition are not appropriate for graded effects for two reasons. First, there is a difference in the way the two types of data are measured. For quantal responses the proportion of organisms responding to any concentration is determined by the ratio of number of organisms showing the response to the total number subjected to the concentration. For graded responses the mean response to each dose is measured, but, in general, the entire range of possible responses is not known. In these cases, no proportional response can be calculated. This is particularly true for growth experiments where an organism's response can potentially range from growth enhancement to negative growth depending on the concentration of a particular toxicant. Secondly, the statistical concept of correlation between the susceptibilities of the organisms to the discrete toxicants in a mixture is not appropriate for graded responses measured in the manner described earlier. Graded response data represent the average response of a group of organisms. Therefore, the response of each individual organism to the toxicants is not known. To be sure the tolerances of the individuals in the group will vary for the different toxicants in a mixture; however, this factor will not alter the relative

toxicity of the mixture because the range of tolerances of the population is theoretically represented in the sample of organisms from this population.

For graded response data, the combined response to a mixture of toxicants acting in a response additive manner is represented simply as the sum of the intensities of response which each component toxicant produces when administered alone. A similar relationship was defined by Loewe (1953). Concentration addition can be predicted using equation (1) if the component toxicants exhibit parallel dose response curves. Figure 3 represents an isobole diagram for a graded response. The isoboles for concentration and response addition were determined with the appropriate mathematical equations discussed above.

The simple types of isoboles represented in Figures 2 and 3 should only be expected for relatively simple in vitro systems or in situations where there is a clearcut relationship between dose and effect. Given the complexity and interdependency of physiological systems, it is reasonable to suppose a priori that the special types of additivity as represented by strict concentration and response addition will be approximated only occasionally in the responses of whole organisms to mixtures of environmental toxicants. Furthermore, as mentioned earlier, the relative toxicity of a mixture depends on several factors which include the level of response (i. e. , 10%, 50%,

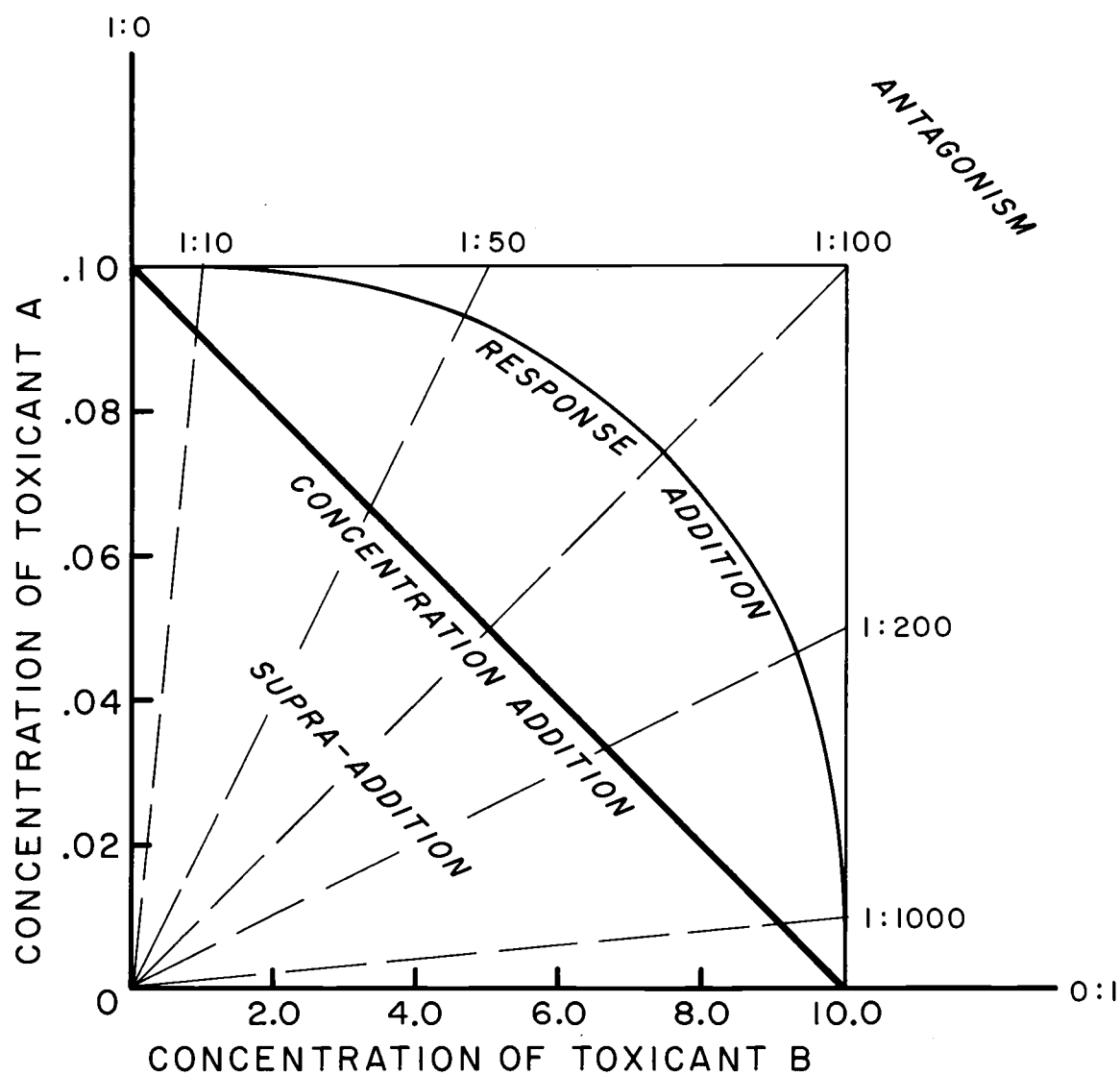


Figure 3. Isobole diagram for graded response data.

90% response), the ratio of the toxicants in a mixture (i. e. 1:10, 1:100, 1:1000) and the nature of the response itself. It should be noted that the type of addition can only be described in relation to the response under consideration. With the same mixture of toxicants, different types of interaction might be expected for different responses (i. e. , survival, growth, reproduction). However, these special types of toxicant interaction do provide a frame of reference for evaluating the effects of toxicant mixtures on whole organism performances.

Isobole diagrams are useful for visualizing the relationship between different types of toxicant interactions and for delineating the various factors which can influence the relative toxicity of multiple toxicants. However, in practice, isoboles are difficult to derive requiring a series of dose response curves for the mixture at different ratios of the component toxicants. Furthermore, there are no statistical criteria which might be used to distinguish between one form of interaction and another (Plackett and Hewlett, 1952). Following the procedures of Anderson and Weber (1977), the interaction of selected environmental toxicants was empirically studied by deriving a dose response curve for mixtures at fixed proportions. The dose response curve determined for the mixture was statistically compared to curves predicted on either the basis of concentration or response addition. This approach, utilized by Anderson and Weber (1977) for

lethality studies, was adopted in the present study to test its applicability to graded response data.

EXPERIMENTAL PROCEDURES

Growth was selected as the quantitative response for this study because it represents a performance of the integrated activities of the whole organism and as such is often a sensitive indicator of the suitability of the environment (Warren 1971). To grow, an organism must first meet the other energy requiring processes necessary to the sustenance of life. The growth of an animal is dependent not only on the quantity and quality of food consumed but also on its existing metabolic state and the energy required for maintenance and behavioral activities. Environmental toxicants can affect the growth of an organism by (1) changing its rate of food consumption, and/or (2) altering the distribution of food energy among its other energy requiring processes. Changes in the efficiency with which an animal converts food material into body tissue might be expected to reflect the effects of toxicants on the distribution of food energy among its other possible fates in the body.

The effects of toxicants on the growth of an organism as reflected in the efficiency of food utilization and in the rate of food consumption can be studied by subjecting experimental animals to two feeding regimes, a restricted and an unrestricted ration. By relegating organisms to a fixed restricted ration, the effect of toxicant concentration on the food conversion or growth efficiency can be

studied. A fixed ration was used because, as Warren (1971) points out, the amount of food consumed as well as environmental toxicants can affect the efficiency with which food is utilized for growth. The effects of toxicants on the food consumption can be studied by feeding test organisms an unrestricted ration and measuring the amount of food ingested at varying toxicant concentrations.

Experimental Fish

Juvenile guppies (Poecilia reticulata) were selected as the experimental organism for this study. Newborn guppies were collected daily from a laboratory population. They were transferred in lots containing 30-35 fish to individual ten liter acclimation tanks supplied by well water identical to the water feeding the exposure tanks except that no toxicants were present. During the acclimation period the test fish were fed daily an excess ration of tubificid worms. After approximately two weeks in the acclimation tanks, the fish were selected for uniformity of size and placed into groups containing 15 fish. Each group was weighed and transferred to the appropriate exposure tank. The initial wet body weight of the groups of fish ranged from .325 to .525 g. A representative sample of these fish was immediately killed and placed in an oven at 70° C. After five days, these fish were removed and weighed so that the initial wet weight to dry body weight relationship could be determined.

Toxicants

The toxicants studied were dieldrin and the chlorides of copper, and nickel. Stock solutions of copper and nickel were made with distilled well water and stored in Mariotte bottles for delivery to the diluter apparatus. Technical grade dieldrin, a sparingly soluble insecticide, was introduced to the system according to the technique of Chadwick and Kiigemagi (1968). On five of the seven day growth and food consumption studies, samples of the toxic solutions were taken from each exposure tank. These samples were assayed on the day after their collection as follows: dieldrin by gas chromatography, and nickel and copper by flame atomic absorption spectrophotometry.

Because copper concentrations greater than 12-13 $\mu\text{g}/\text{l}$ (incipient lethal level) resulted in fish mortality, very low levels of this toxicant were used in the experiments. Special procedures were developed to concentrate the copper in the samples prior to atomic absorption analysis. This assay involved a modification of a technique (Baetz and Kenner 1975) that utilized a chelating ion exchange resin (Chelex-100) to concentrate heavy metals.

The average concentration for each toxicant during the bioassays was used in the derivation of the dose response curves. Relatively low standard deviations for the toxicant concentrations reflected the precision of the analytical techniques and adequate performance of the diluter apparatus.

Bioassay Apparatus

The dosing apparatus consists of a series of plexiglass chambers designed to continuously dilute stock solutions of the individual toxicants to the desired concentration. It is arranged such that any one of six toxicants or any combination of these toxicants can be delivered to 24 individual ten liter exposure tanks. A schematic diagram of the experimental system and a description of its operation has been presented elsewhere (Anderson and Weber 1977). The total flow rate of the toxicant mixture and of the diluting well water was maintained at 100 ml/min throughout the experiments. These flow-rates were monitored daily to insure that the fish in each exposure tank received the proper dosage.

Environmental Conditions

Because chemical and physical conditions have been shown to influence the response of fish to toxicants, many of these factors were controlled or regularly monitored during both the acclimation and exposure portions of the experiment. Temperature was thermostatically maintained at $25 \pm 0.5^{\circ} \text{C}$ and the photoperiod set for 18 hours of light. The pH was checked daily in the acclimation and exposure tanks and adjusted to $7.0 \pm .15$ by controlled bubbling of CO_2 . The levels of certain water quality characteristics such as alkalinity

(144 mg/l as CaCO_3), hardness (124 mg/l as CaCO_3) and dissolved oxygen (8.3 mg/l) were similar to the ones reported by Anderson and Weber (1977).

Growth Studies

Groups of fish were fed once daily a restricted ration to determine the effect of toxicant concentration on food conversion efficiency. For the purposes of these experiments, a feeding level between the maintenance (ration required to just maintain body weight) and maximum food ration was selected to insure that over the range of toxicant concentrations studied the restricted ration would be entirely consumed. Preliminary experiments indicated that a ration of tubificid worms equivalent to 20% of the initial wet weight of each group of 15 fish would be satisfactory. After seven days on the predetermined ration, the groups of experimental fish were weighed to determine their final wet body weight. The fish were subsequently killed and placed in a drying oven at 70° C. Five days later they were removed and weighed to measure the final dry body weight.

The efficiency with which an animal converts food energy into body tissue is often called the total or gross growth efficiency (E_G) and can be represented by the equation (Warren 1971):

$$E_G = \frac{G}{I} \times 100 \quad (3)$$

where G is the growth measured as the change in body weight and I is the food intake. This parameter was used to determine the dose response curves relating toxicant concentration to growth efficiency.

To investigate the effect of the individual toxicants and their mixtures on the food consumption of guppies, each lot of 15 fish was fed daily an unrestricted ration. The test fish in each exposure tank were supplied with a preweighed quantity of tubificid worms in excess of their maximum food consumption rate. Twenty-four hours after each feeding the unconsumed worms were siphoned from the exposure tanks and reweighed to determine the amount of food consumed. This procedure was continued throughout the seven day food consumption experiments. At the termination of the study, the fish were weighed according to the procedures already described.

An appropriate measurement of growth for both the restricted and unrestricted ration studies is the relative growth rate (RGR), a growth rate relative to body weight. This can be calculated by the following equation (Warren 1971):

$$RGR = \frac{\ln W_f - \ln W_i}{t_f - t_o} \quad (4)$$

where W_i is the initial weight of each fish lot at the beginning of an experiment (t_o) and W_f is the final weight at the conclusion of the growth study (t_f).

The effects of the individual toxicants and selected mixtures on the gross growth efficiency, food consumption and relative growth rate of the guppies were studied by exposing the fish to concentrations ranging from levels causing small changes in these responses to concentrations approaching the incipient lethal level.

Derivation of Dose Response Curves

To adjust for experimental variables that might have fluctuated during the course of the study (i. e., caloric content of the tubificid worms, seasonal changes in water quality characteristics), approximately one half of the experimental tanks in each test were designated as controls. Experimental conditions were identical to the exposure tanks except no toxicant was introduced. The various responses studied (gross growth efficiency, food consumption rate, and relative growth rate) were normalized relative to the control or unexposed fish and expressed as percentages of control responses on the dose response curves. This procedure was adopted to facilitate the comparison of the results of the individual toxicant studies to the multiple toxicant tests performed at a later date.

Dose response curves for the individual toxicants and selected mixtures were derived by plotting the defined response after normalization against the natural logarithm of toxicant concentration. Standard linear regression techniques were used to quantify this

relationship. The regression equation for each experiment was calculated in the following form:

$$y = a + b \ln x \quad (5)$$

where y equals the defined response expressed as a percentage of the response of control fish, and x is the mean daily toxicant concentration.

Toxicant Interaction Studies

After studying the effects of copper, nickel, and dieldrin on the gross growth efficiency, food consumption, and relative growth rate of the guppies, fish were exposed to selected binary mixtures of these toxicants at fixed proportions. Based on the model under investigation, the slopes of the individual toxicants were compared statistically (t-test) for parallelism. All statistical tests were applied at the .05 level of significance. If parallelism between the regression lines for the individual toxicants could not be disproven, concentration addition was predicted as the type of interaction for the mixture. This prediction was tested by performing a bioassay with the toxicant mixture. The observed dose response curve for the mixture was then statistically compared to the predicted regression equation determined by equation (1) for concentration addition. The common slope (b) used in the predicted equation was calculated from the data for the individual toxicants by an analysis of

covariance. By determining the best fit of the data for the individual toxicants with the common regression coefficient, the relative potency (p) of the toxicants was calculated. Since the slopes of the regression equations for the individual toxicants are similar, the relative potency is constant at all levels of effect. The proportionality factor ($\pi_1:\pi_2$; $\pi_1 + \pi_2 = 1$) in equation (1) was determined on the basis of the ratio between the actual assayed concentrations of the toxicants in the mixture.

The slopes and intercepts of the predicted and observed regression equations were statistically compared using Student's t-test. The standard error terms used in this comparison were determined from the error terms associated with the slopes and intercepts of the regression equations of the individual toxicants.

If the slopes of the dose response curves were significantly different or if the individual toxicants were thought to primarily affect different biological systems, response addition was predicted for the mixture. For graded response data, the combined response to a mixture of toxicants acting in a response additive manner can be expressed as the sum of the intensities of response which each component toxicant produces when administered alone. The experimental procedure for determining the observed dose response curve for the mixture was similar to the one described for concentration addition; however, the curvilinear nature of the dose response curves for

toxicants interacting in this manner complicates the comparison of the observed and predicted curves.

RESULTS

Preliminary and Control Studies

The results of a preliminary experiment studying the relationship between the relative growth rate, gross growth efficiency and food consumption rate of juvenile guppies are shown in Figure 4. To determine if body weight over the range of weights studied (.325-.525 g) influenced relative growth and food consumption rates, three groups of 15 fish with low, medium and high body weights were fed either a restricted (10%, 20%, 30%, or 50% of the initial body weight per day) or an unrestricted ration (approximately 60-80% of the initial body weight per day). At each of the restricted rations, the relative growth rates of groups of fish representing the three weight classes were similar. These results indicated that the size or body weight did not affect the relative growth rate of guppies on the restricted rations. However, the relative growth and food consumption rates of fish fed an unrestricted ration was weight dependent over the range of body weights studied. The heavier fish demonstrated a lower relative food consumption rate (approximately 60% of their initial body weight per day) and hence a lower relative growth rate (100 mg/g/day) than the groups of lighter fish whose relative food consumption and growth rates were approximately 80% and 130 mg/g/day, respectively. In other words, the relative food consumption and growth rate

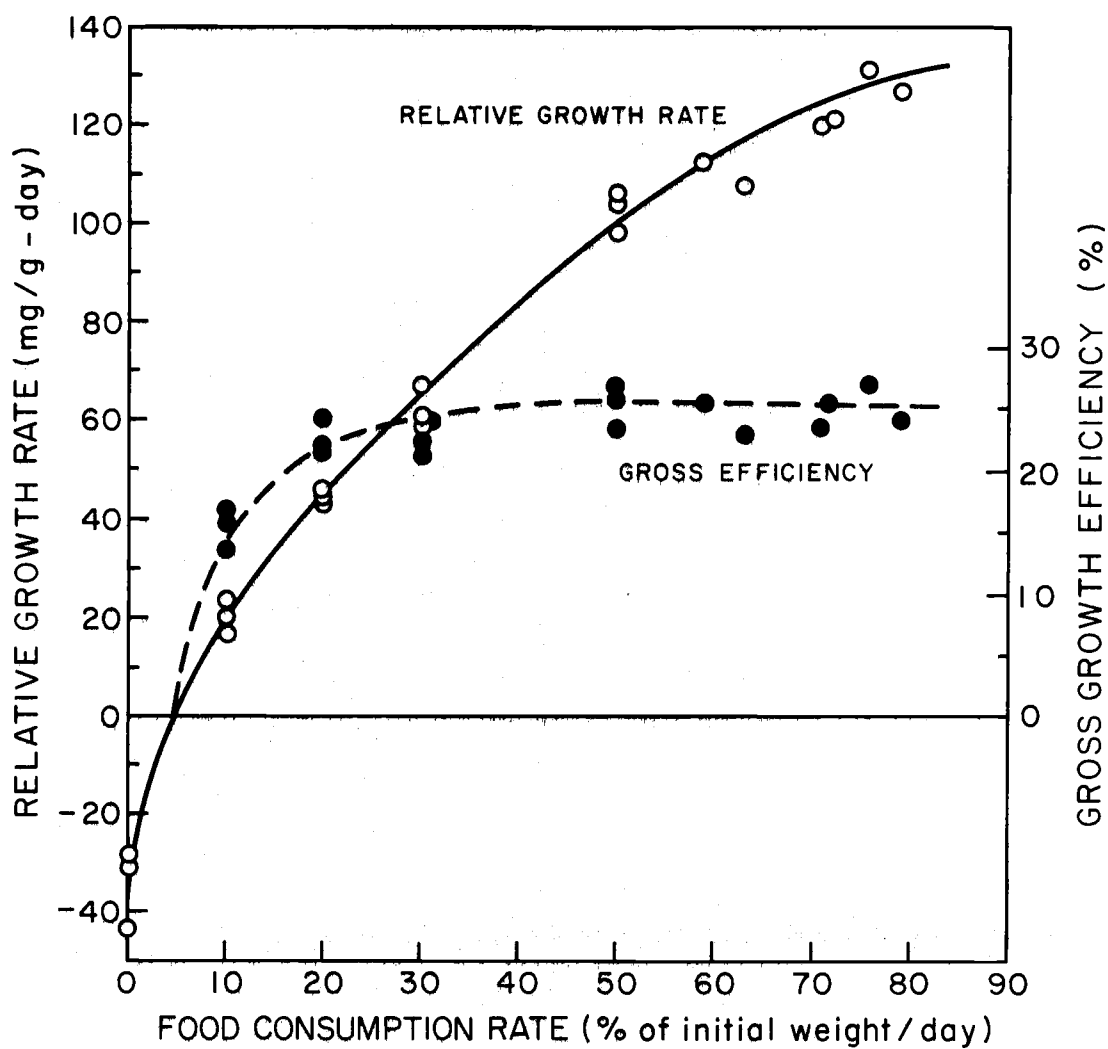


Figure 4. Relationships between food consumption, growth, and gross growth efficiency for the control fish.

of juvenile guppies fed an unrestricted ration were inversely related to their body weight.

In Figure 4, the gross growth efficiency increased asymptotically from 0% at the maintenance ration (approximately 5% of the initial body weight per day) to a maximum efficiency of approximately 25% at the 20% feeding level. Further increases in food consumption did not appreciably affect the efficiency of food utilization. Moreover, gross growth efficiency was not measurably influenced by body weight.

On the basis of Figure 4, a ration equivalent to 20% of the initial body weight of each group of fish was selected as the restricted ration for subsequent studies. This feeding level was chosen because it yielded a relatively high rate of growth and represented a ration that was close to the maximum gross growth efficiency of the fish. Furthermore, the ration was small enough to insure that over the range of toxicant concentrations studied the restricted ration would be entirely consumed.

As previously discussed, the effects of the toxicants and their mixtures on the relative growth rate, relative food consumption and gross growth efficiency were normalized to the responses of control or unexposed fish. When body weight was not a factor, the relative growth rates and gross growth efficiencies of fish exposed to the various toxicants studied were normalized to the mean responses

determined for the unexposed fish. The means and standard errors of the responses of the internal controls determined during each of the experimental bioassays for the individual toxicants and their mixtures are presented in Table 1. As previously mentioned the body weight of guppies fed an unrestricted ration did affect the amount of food consumed and hence their relative growth rates. Simple linear regression equations were calculated for the controls expressing both food consumption and growth as a function of body weight. The average weight of each group of experimental fish was used in the appropriate regression equations for the controls to calculate the group's potential relative growth and food consumption rate in the absence of toxicants. This value was used to normalize the actual growth and food consumption rate of the experimental fish.

Copper-Nickel Mixtures

The effects of the chlorides of copper and nickel and their mixture on the gross growth efficiency, relative growth and food consumption rate of juvenile guppies were studied. On the basis of preliminary experiments, four concentrations of copper and nickel were selected ranging from 2 to 12 $\mu\text{g/l}$ and from 4 to 18 mg/l , respectively.

Table 1. Average values for the gross growth efficiency and relative growth rate of control fish for both the restricted and unrestricted ration. Responses of the experimental fish for each of the toxicants listed below were normalized to the average responses of control fish reported in the following table.

Toxicants	Sample Size (Restricted)	Gross Growth Efficiency (% \pm S. E.)	Relative Growth Rate (mg/g/day \pm S. E.)	Sample Size (Unrestricted)	Gross Growth Efficiency (% \pm S. E.)
Nickel	23	25.1 \pm 0.4	43.0 \pm 0.6	30	24.8 \pm 0.3
Copper	21	26.3 \pm 0.3	44.8 \pm 0.4	19	25.5 \pm 0.3
Cu-Ni Mixture	21	24.5 \pm 0.5	42.1 \pm 0.7	16	24.3 \pm 0.3
Dieldrin	23	24.3 \pm 0.3	41.9 \pm 0.4	11	23.9 \pm 0.3
Dieldrin-Ni Mixture (1:2325)	11	27.4 \pm 0.6	46.3 \pm 0.8	10	26.4 \pm 0.3
Dieldrin-Ni Mixture (1:3850)	14	26.1 \pm 0.5	44.4 \pm 0.8	12	27.4 \pm 0.3

Restricted Ration

Figure 5 shows the effects of copper, nickel and their mixture on the gross growth efficiency of fish relegated to the restricted ration. Regression analysis was used to calculate the regression coefficients for the dose response curves. The slopes of the dose responses curves for copper and nickel (-18.4 and -19.6, respectively) when compared by a t-test were not significantly different. Based upon the assumption of concentration addition, the predicted dose response curve was determined for the mixture at the same proportions used in the interaction bioassay. The copper and nickel data were fit with a common regression coefficient (-18.84) determined by analysis of covariance. This value along with the relative potency ($p = 6.27 \times 10^{-4}$) and proportionality factor ($\pi_{Cu}:\pi_{Ni} = .0011:.9989$) were substituted into equation (1) to calculate the predicted dose response curve for the mixture. The regression equations for copper and nickel fit to the common regression coefficient along with the predicted and observed equations for the mixture are reported in Table 2. Statistical comparison of the predicted and observed dose responses for the mixture depicted in Figure 5 suggested that slopes and the intercepts of the equations were not significantly different. The effects of copper and nickel on the relative growth rate of the fish on the restricted ration are shown in Figure 6.

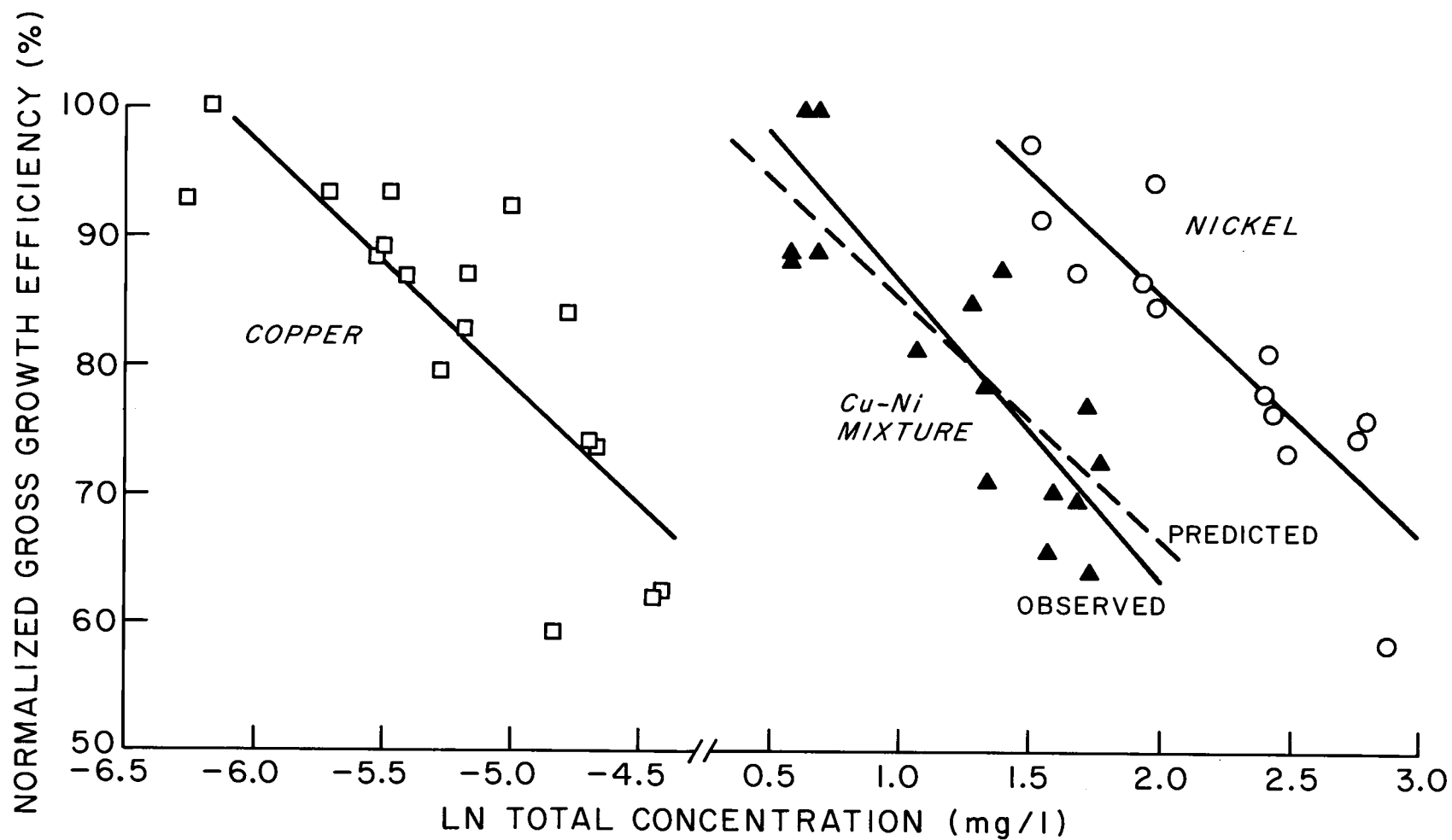


Figure 5. Dose response curves showing effects of copper, nickel, and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (restricted ration study) in Table 1.

Table 2. Regression equations for the copper and nickel dose response curves expressing normalized gross growth efficiency (Y) and relative growth rate (Y) for the restricted ration as a function of toxicant concentration (X).

Toxicants	Sample Size	Regression Equations	
		Gross Growth Efficiency	Relative Growth Rate
Copper	17	$Y = -15.67 - 18.84 \ln X$	$Y = -5.36 - 17.17 \ln X$
Nickel	13	$Y = 123.27 - 18.84 \ln X$	$Y = 121.55 - 17.17 \ln X$
Cu-Ni Mixture (observed)	16	$Y = 109.83 - 23.18 \ln X$	$Y = 109.01 - 20.95 \ln X$
Cu-Ni Mixture (predicted)	-	$Y = 104.19 - 18.84 \ln X$	$Y = 103.97 - 17.17 \ln X$

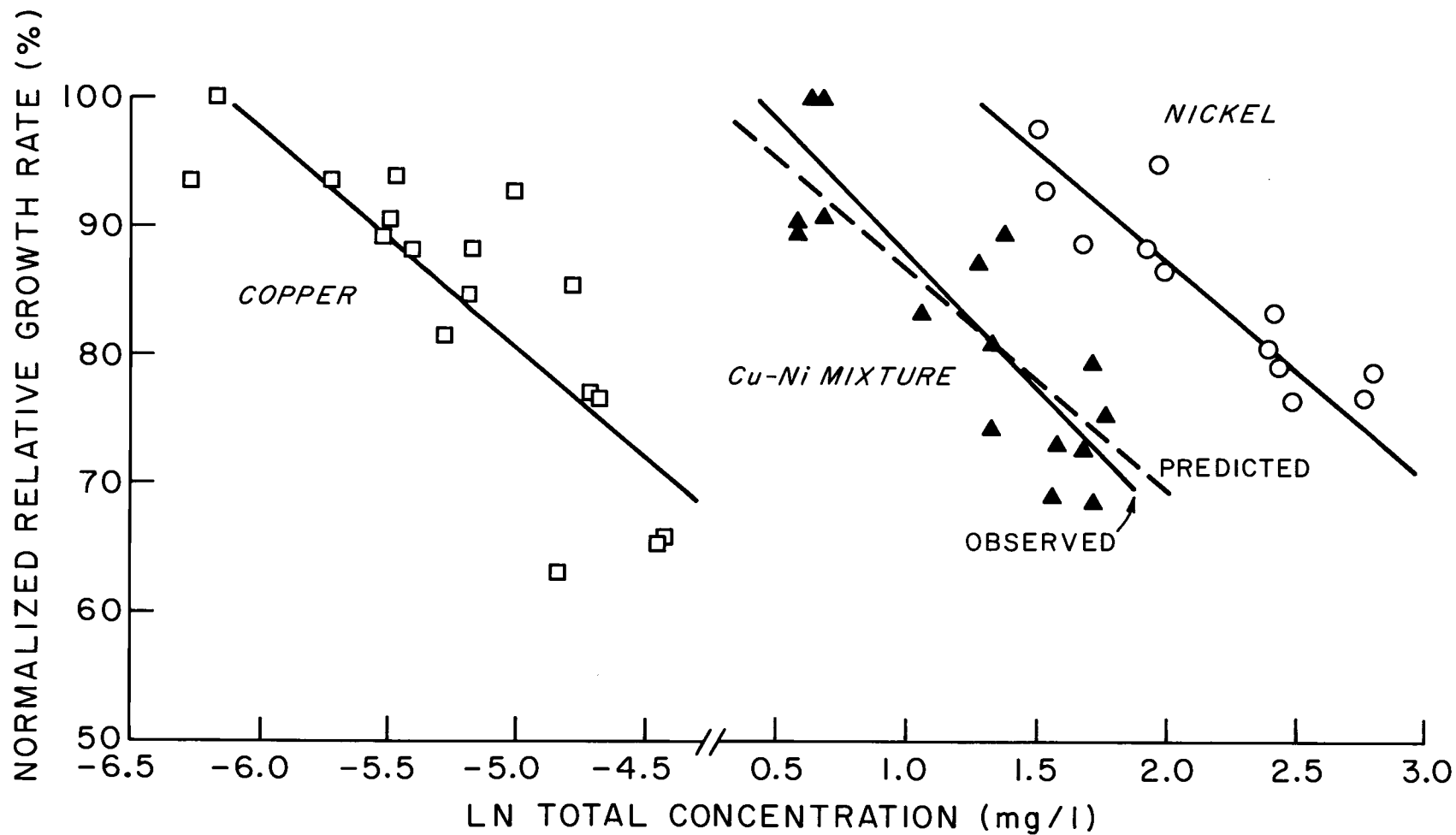


Figure 6. Dose response curves showing effects of copper, nickel, and their mixtures (observed and predicted) on relative growth rate normalized to responses of controls (restricted ration study).

The observed and predicted dose response curves for the mixture are essentially identical to the curves for gross growth efficiency in Figure 5.

Unrestricted Ration

The results of experiments evaluating the effects of copper and nickel on the gross growth efficiency of fish fed an unrestricted ration are shown in Figure 7. Based on a t-test the slopes of the regression lines calculated for copper (-33.2) and nickel (-26.8) were not significantly different. Consequently, concentration addition was predicted for the mixture. Following the procedures outlined for the other interaction studies, the common regression coefficient (-29.34), relative potency ($p = 5.0 \times 10^{-4}$) and proportionality factor ($\pi_{Cu}:\pi_{Ni} = .001:.999$) were substituted into equation (1) to calculate the predicted dose response curve. Statistical comparison of the predicted and observed regression equations in Table 3 supported the assumption that the mixture was concentration additive.

In Figure 7 the data for the copper and nickel mixture showed considerable variability. Inspection of the data indicated that this variability might be due to differences in the weights of the groups of fish. A multiple regression analysis including body weight as a variable confirmed this observation. However, the elimination of groups of fish of low body weight did not significantly change the

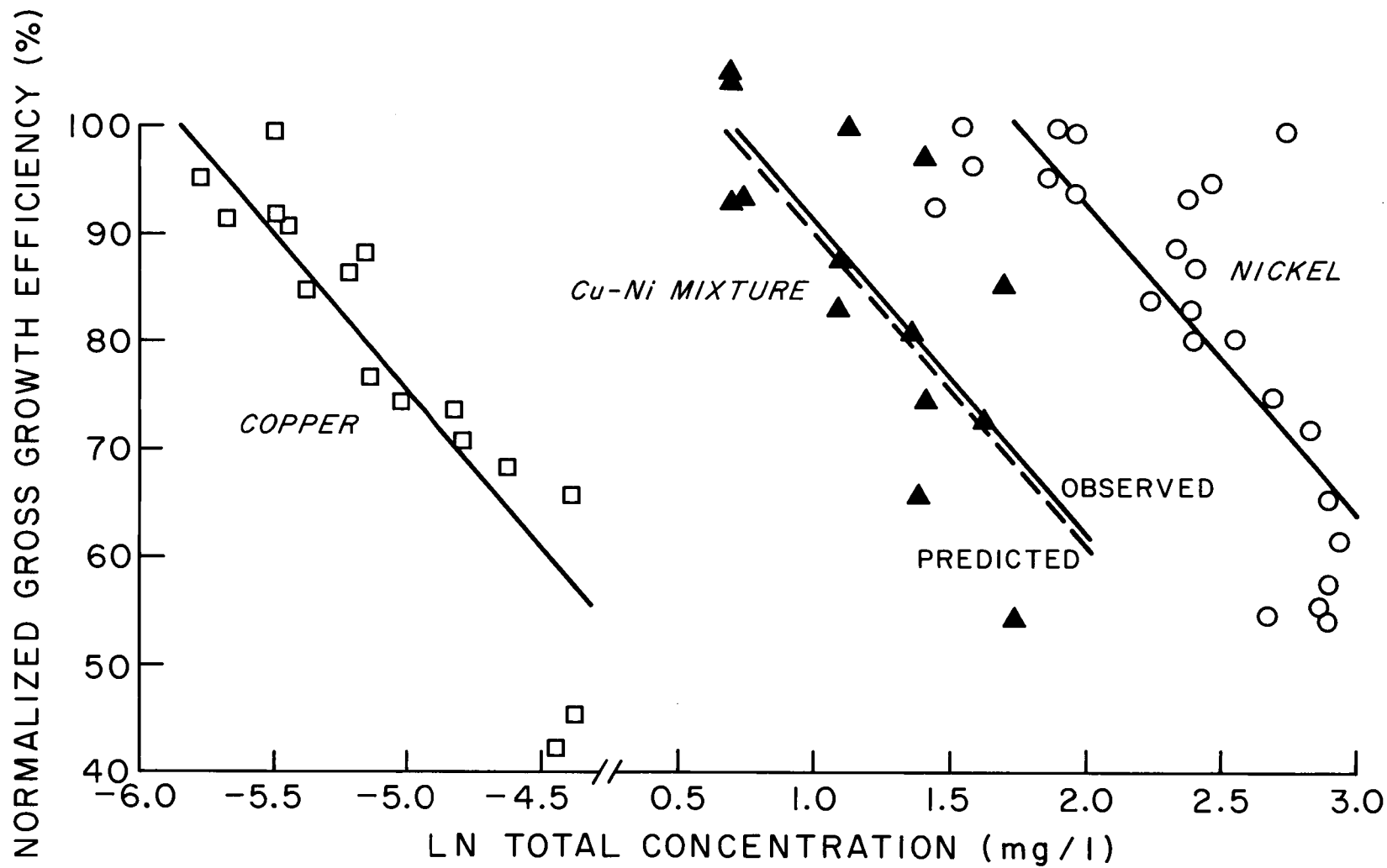


Figure 7. Dose response curves showing effects of copper, nickel, and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (unrestricted ration study).

Table 3. Regression equations of the copper and nickel dose response curves for the unrestricted food ration studies. Normalized gross growth efficiency, food consumption rate and relative growth rate (Y) are expressed as a function of toxicant concentration (X). Predicted equations were calculated based on the assumption of concentration addition for the mixture.

Toxicants	Sample Size	Regression Equations		
		Gross Growth Efficiency	Food Consumption Rate	Relative Growth Rate
Copper	16	$Y = -71.42 - 29.34 \ln(X)$	$Y = -59.09 - 24.14 \ln(X)$	$Y = -137.85 - 37.24 \ln(X)$
Nickel	24	$Y = 151.54 - 29.34 \ln(X)$	$Y = 120.20 - 24.14 \ln(X)$	$Y = 141.68 - 37.24 \ln(X)$
Cu-Ni Mixture (observed)	14	$Y = 120.35 - 28.99 \ln(X)$	$Y = 88.48 - 28.40 \ln(X)$	$Y = 90.32 - 40.08 \ln(X)$
Cu-Ni Mixture (predicted)	-	$Y = 119.37 - 29.34 \ln(X)$	$Y = 96.40 - 24.14 \ln(X)$	$Y = 103.09 - 37.24 \ln(X)$

relationship between the observed and predicted equations for the mixture (Figure 8).

The results of the unrestricted ration bioassays studying the effects of copper and nickel on the food consumption rate of the guppies are presented in Figure 9. As in the previous experiments, the slopes of the regression lines fit to the copper and nickel data (-26.82 and -22.42, respectively) were not significantly different; therefore, concentration addition was predicted for the interaction. Analysis of covariance yielded a common regression coefficient of -24.14. Based on the assumption of concentration addition, the predicted equation was calculated for the mixture in the manner previously discussed. Inspection of the observed and predicted dose response curves in Figure 9 shows that the effect of the copper-nickel mixtures on the food consumption of the fish is slightly supra-additive relative to the curve predicted on the basis of concentration addition. A statistical test comparing the intercepts of the predicted and observed regression equations confirmed this observation; however, the slopes of the two regression equations were essentially the same. The regression equations for the dose response curves depicted in Figure 9 are reported in Table 3.

The results of experiments studying the effect of the mixture on the relative growth rate of the fish fed an unrestricted ration are presented in Figure 10. Based on statistical tests demonstrating that

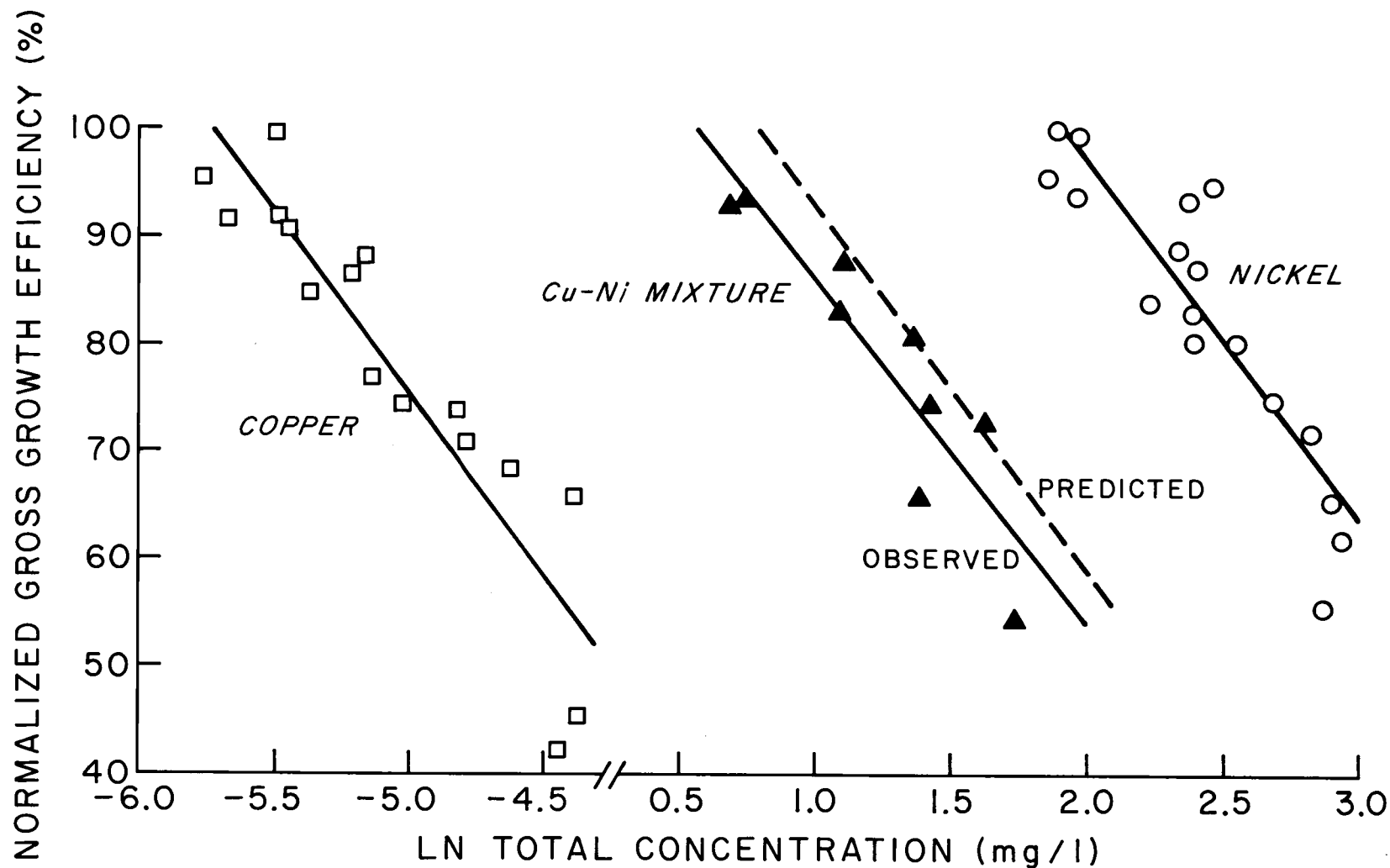


Figure 8. Dose response curves showing effects of copper, nickel and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (unrestricted ration study) after elimination of groups of fish of low body weight.

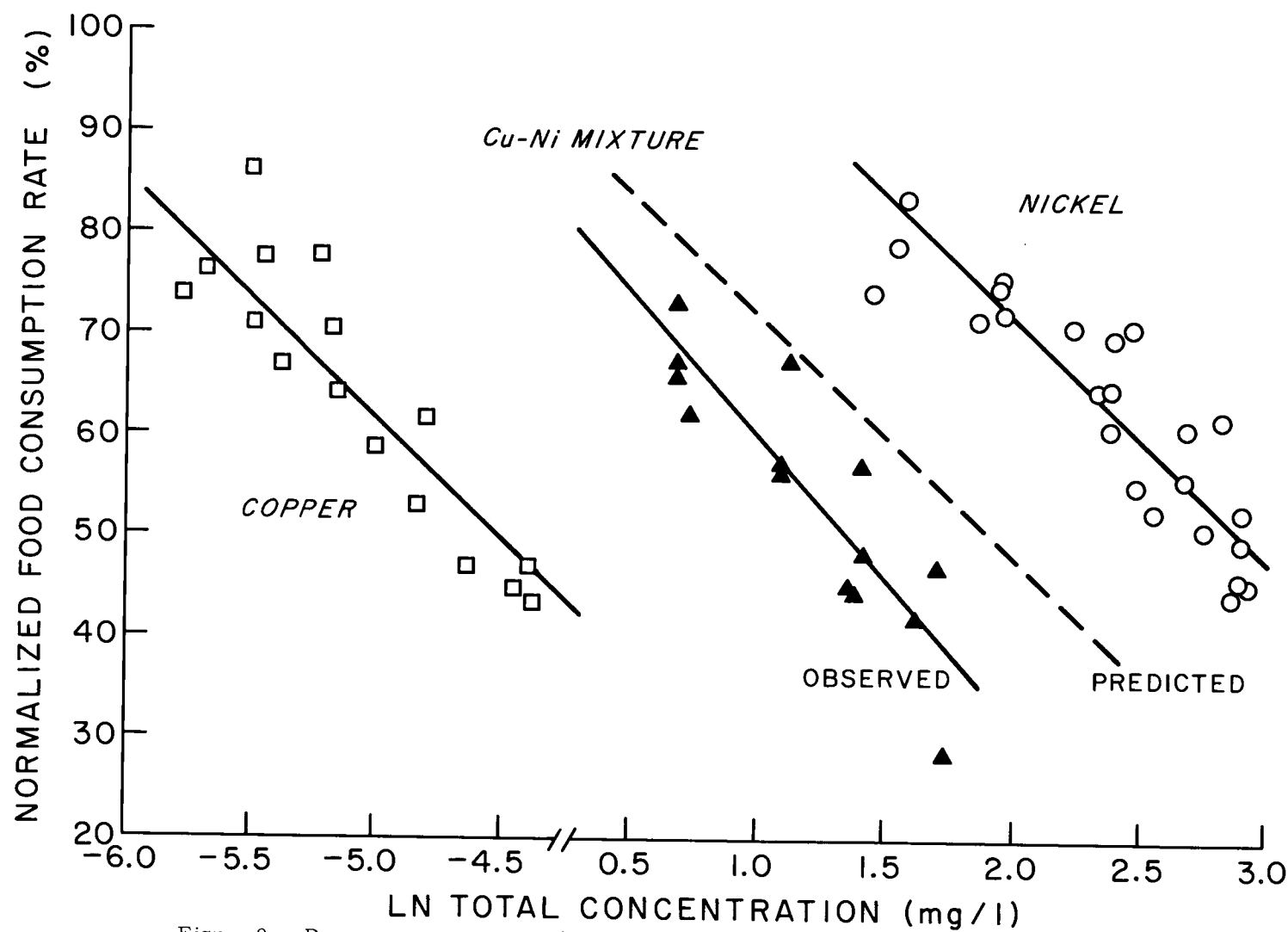


Figure 9. Dose response curves showing effects of copper, nickel and their mixtures (observed and predicted) on food consumption rate normalized to responses of controls (unrestricted ration study).

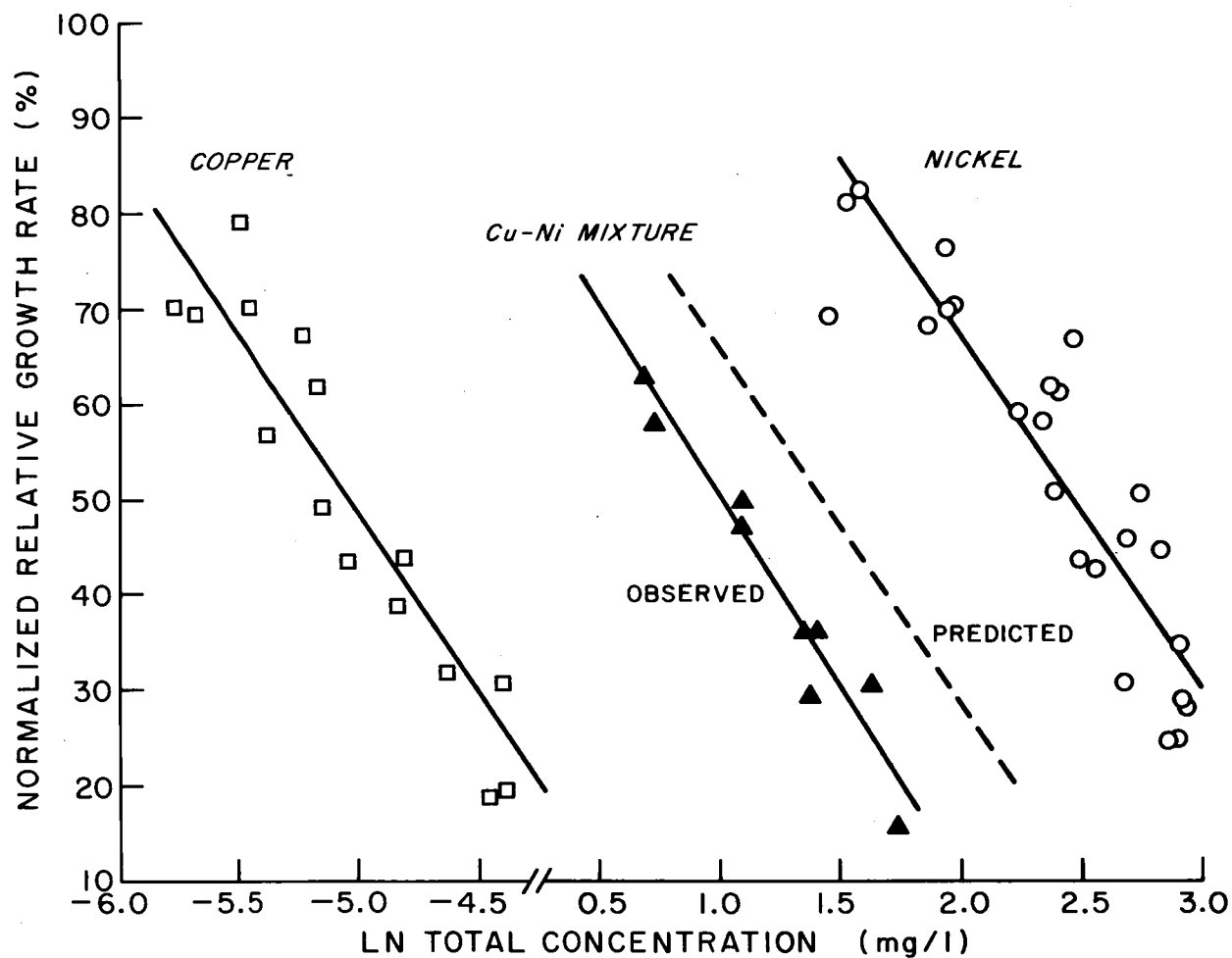


Figure 10. Dose response curves showing effects of copper, nickel and their mixtures (observed and predicted) on relative growth rate normalized to responses of controls (unrestricted ration study).

the slopes of the copper and nickel dose response curves (-40.17 and -35.54, respectively) were not significantly different, concentration addition was predicted for the mixture. Regression equations fit to the common regression coefficient (-37.24) are reported in Table 3. As in the food consumption studies, the effects of a mixture of copper and nickel on the relative growth rate of the guppies was supra-additive relative to the predicted equation.

Dieldrin-Nickel Mixtures

Another set of experiments was performed evaluating the effects of dieldrin and nickel and their mixture on the gross growth efficiency, relative growth and food consumption rates of juvenile guppies. For the derivation of the individual dose response curves, dieldrin concentrations ranged from .4 to 4.0 $\mu\text{g/l}$. The nickel dose response curves were the same as the ones used in the preceding section. Concentrations greater than 4.0 $\mu\text{g/l}$ of dieldrin resulted in fish mortality. After normalization to the responses of controls, the resulting percent response was graphically plotted as a function of the natural logarithm of toxicant concentration.

Two fixed proportions of dieldrin to nickel (1:2325 and 1:3850) were used to study the nature of the interaction between the two compounds. Concentrations for the two mixtures ranged from .9-4.0 $\mu\text{g/l}$ dieldrin and 2.1-9.0 mg/l nickel for the 1:2325 ratio and from

.9-4.0 $\mu\text{g/l}$ dieldrin and 3.3-15.0 mg/l nickel for the 1:3850 mixture.

Inspection of the dose response relationship for dieldrin and the dieldrin-nickel mixtures in the following figures showed that there was considerably more variation in the guppies response to dieldrin than to nickel alone. Whether this variability was due to the toxic properties of dieldrin itself or to experimental error resulting from the low and narrow range of concentrations assayed could not be determined. Regardless of the source of this variability, the quantitative analysis with the procedures utilized for the copper-nickel interaction is not appropriate for this data. Consequently, the presentation and discussion of the results of this interaction study are by necessity more qualitative and descriptive in nature.

Restricted Ration

The effects of dieldrin and nickel on the gross growth efficiency and relative growth rate of guppies fed the restricted ration are presented in Figures 11 and 12. Concentrations of dieldrin between .4 and .6 $\mu\text{g/l}$ resulted in growth enhancement (greater than 100%) relative to the growth of control or unexposed fish. Regression equations fit to the dieldrin and nickel dose response curves are presented in Table 4. Although the slopes of the dose response curves for dieldrin and nickel appear to be different for both gross growth efficiency (-12.43 and -19.59, respectively) and relative growth rate (-11.18

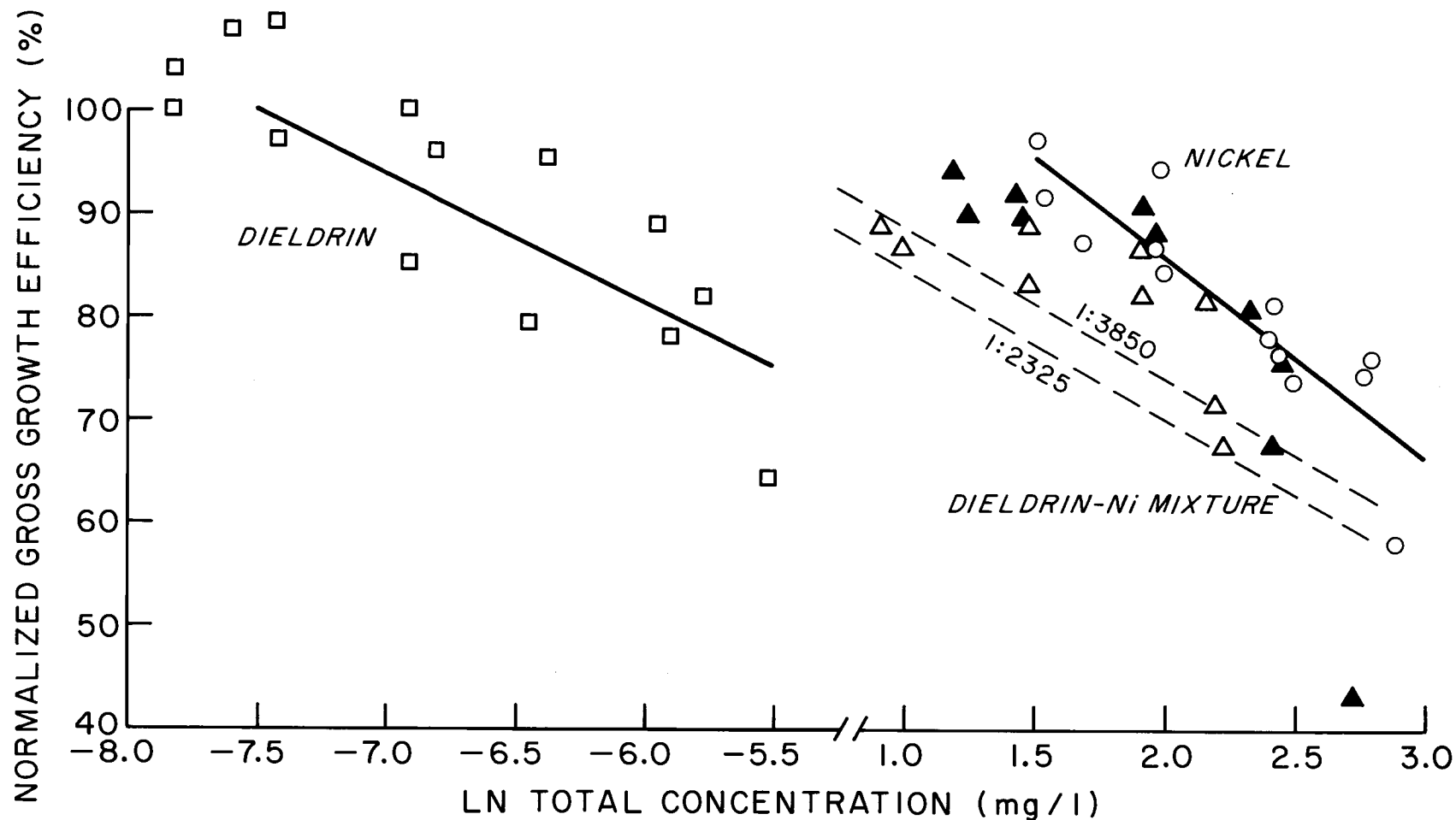


Figure 11. Dose response curves showing effects of dieldrin, nickel and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (restricted ration study). Mixtures of the toxicants in the fixed proportions of 1:2325 (Δ) and 1:3850 (\blacktriangle).

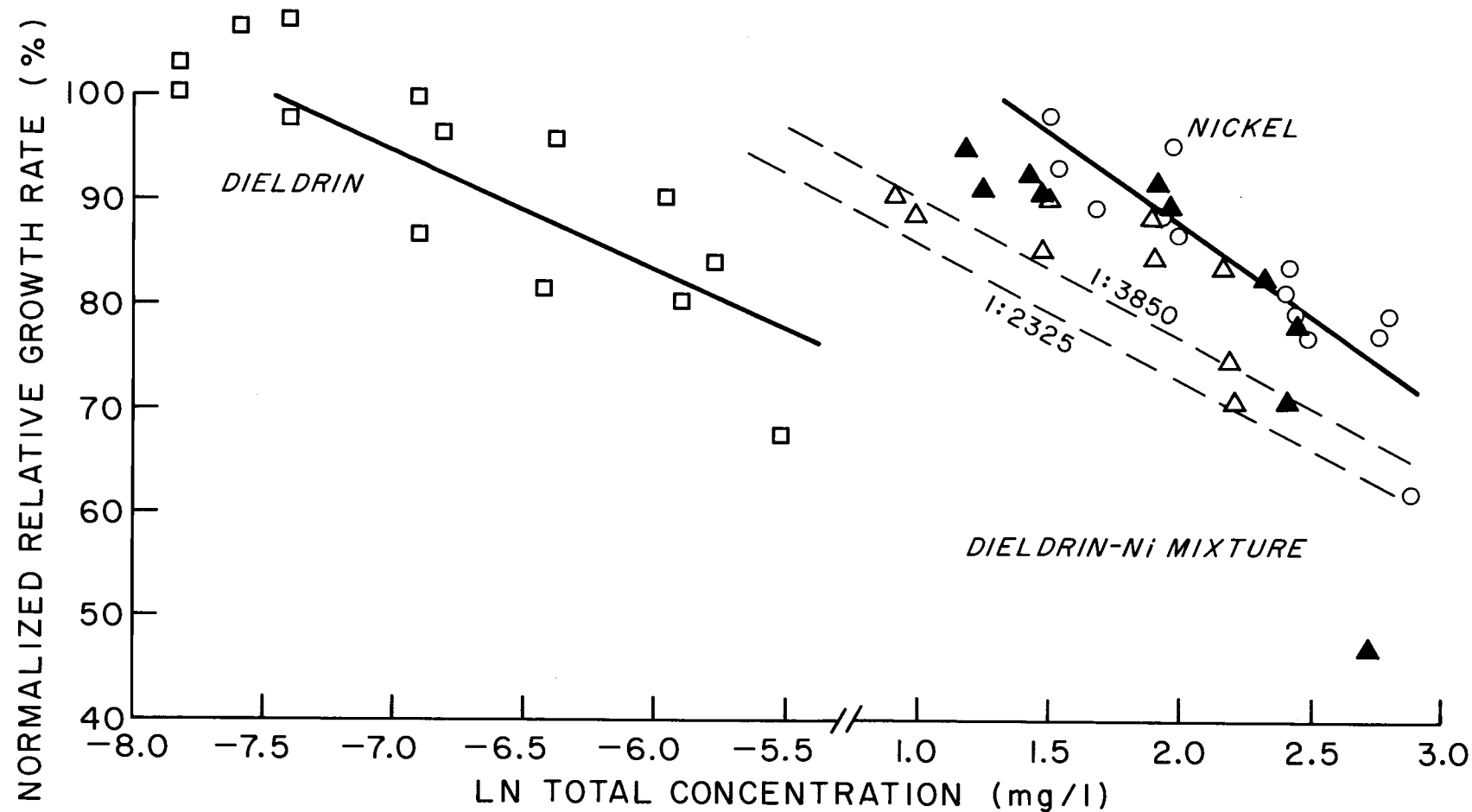


Figure 12. Dose response curves showing effects of dieldrin, nickel and their mixtures (observed and predicted) on relative growth rate normalized to responses of controls (restricted ration study). Mixtures of the toxicant in the fixed proportions of 1:2325 (\triangle) and 1:3850 (\blacktriangle).

Table 4. Regression equations for the dieldrin and nickel dose response curves expressing normalized gross growth efficiency (Y) and relative growth rate (Y) for the restricted ration as a function of toxicant concentration (X). The predicted equations were calculated based on the assumption of concentration addition.

Toxicants	Sample Size	Regression Equations	
		Gross Growth Efficiency	Relative Growth Rate
Dieldrin	14	$Y = 6.83 - 12.43 \ln X$	$Y = 16.24 - 11.18 \ln X$
Nickel	13	$Y = 124.93 - 19.59 \ln X$	$Y = 123.02 - 17.83 \ln X$
1:2325 Mixture (predicted)	-	$Y = 99.17 - 14.82 \ln X$	$Y = 99.62 - 13.46 \ln X$
1:3850 Mixture (predicted)	-	$Y = 103.53 - 14.82 \ln X$	$Y = 103.54 - 13.46 \ln X$

and -17.83, respectively), statistical comparison of the regression coefficients suggested that the slopes were not significantly different within the limits of sampling error. This result might be related to the variability observed in the data for the individual toxicants.

The results of the interaction study for mixtures of dieldrin and nickel at two fixed proportions (1:2325 and 1:3850) are shown in both Figures 11 and 12 for the two responses studied. Over the range of concentrations assayed, the dose response curve for the 1:2325 ratio is shifted to the left relative to the curve for the 1:3850 ratio. This result was expected due to the greater proportion of the more toxic constituent, dieldrin, in the 1:2325 mixtures. Since the slopes of the regression equations for the dieldrin and nickel dose response curves were not significantly different, equations for their mixture were calculated based on the assumption of concentration addition. Although this procedure is of questionable value given the variability observed in the dieldrin and nickel data, the determination of a predicted dose curve provides a frame of reference for making qualitative statements concerning the relative toxicity of the mixture. Based on an analysis of covariance, common regression coefficients were used to calculate the predicted equations reported in Table 4. The resulting equations were plotted in Figures 11 and 12.

The toxicity of the dieldrin-nickel mixtures relative to the predicted dose response curves varied over the range of

concentrations assayed. At low and high concentrations, the interaction of dieldrin and nickel appeared to be additive resulting in a greater reduction in the gross growth efficiency and relative growth rate than would have been expected for either toxicant alone. However, at intermediate concentrations for the mixture (approximately 6 to 10 mg/l), the guppies responded as if dieldrin were not present. Over this range of concentrations, the responses of the fish to the mixture at both of the proportions studied and to nickel alone were similar. Graphical comparison of the dose response curves for the mixtures and the predicted equations indicated that for most of the concentrations assayed the interaction of dieldrin and nickel was infra-additive relative to the dose response curves predicted on the assumption of concentration addition.

Unrestricted Ration

The results of experiments evaluating the effects of dieldrin and nickel on the normalized gross growth efficiency, food consumption rate, and relative growth rate of guppies are shown in Figures 13-15. Regression equations fit to the data for the individual toxicants are presented in Table 5. Statistical comparison of the regression coefficients for each of the responses studied indicated that the slopes of the dose response curves were not significantly different. Predicted equations based on a concentration additive interaction were

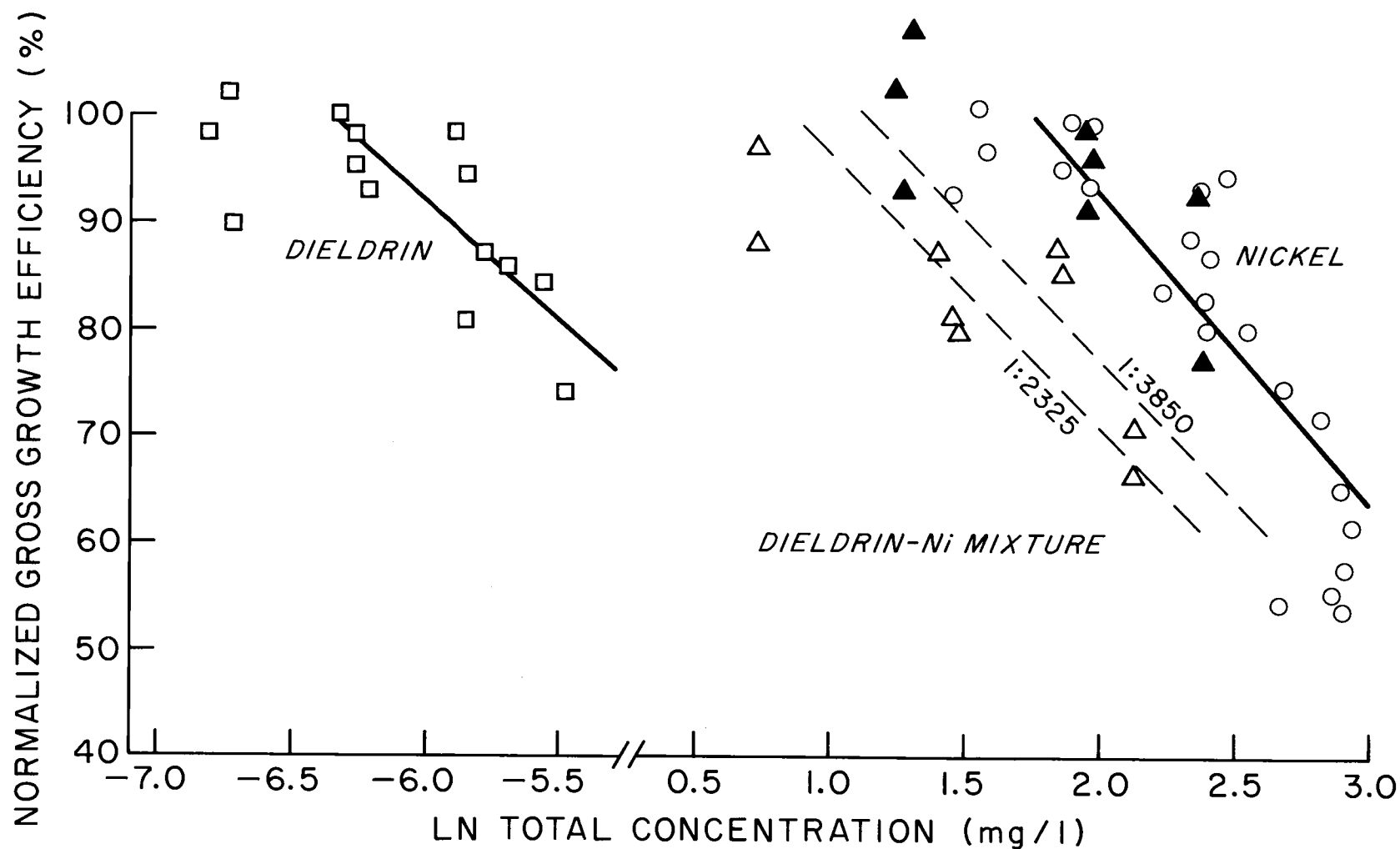


Figure 13. Dose response curves showing effects of dieldrin, nickel and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (unrestricted ration study). Mixtures of the toxicants in the fixed proportions of 1:2325 (Δ) and 1:3580 (\blacktriangle).

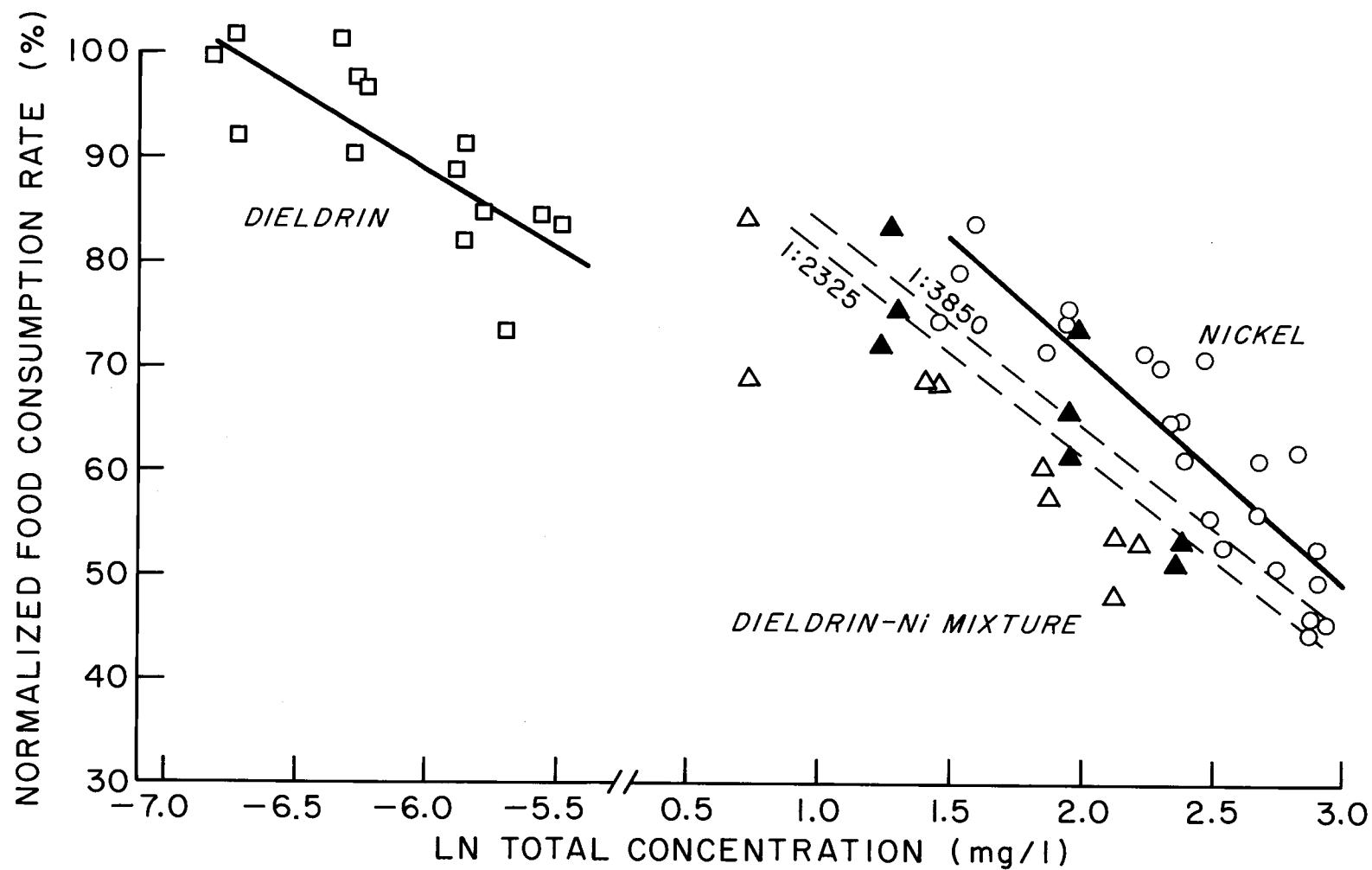


Figure 14. Dose response curves showing effects of dieldrin, nickel and their mixtures (observed and predicted) on food consumption rate normalized to responses of controls (unrestricted ration study). Mixtures of the toxicants in the fixed proportions of 1:2325 (\triangle) and 1:3850 (\blacktriangle).

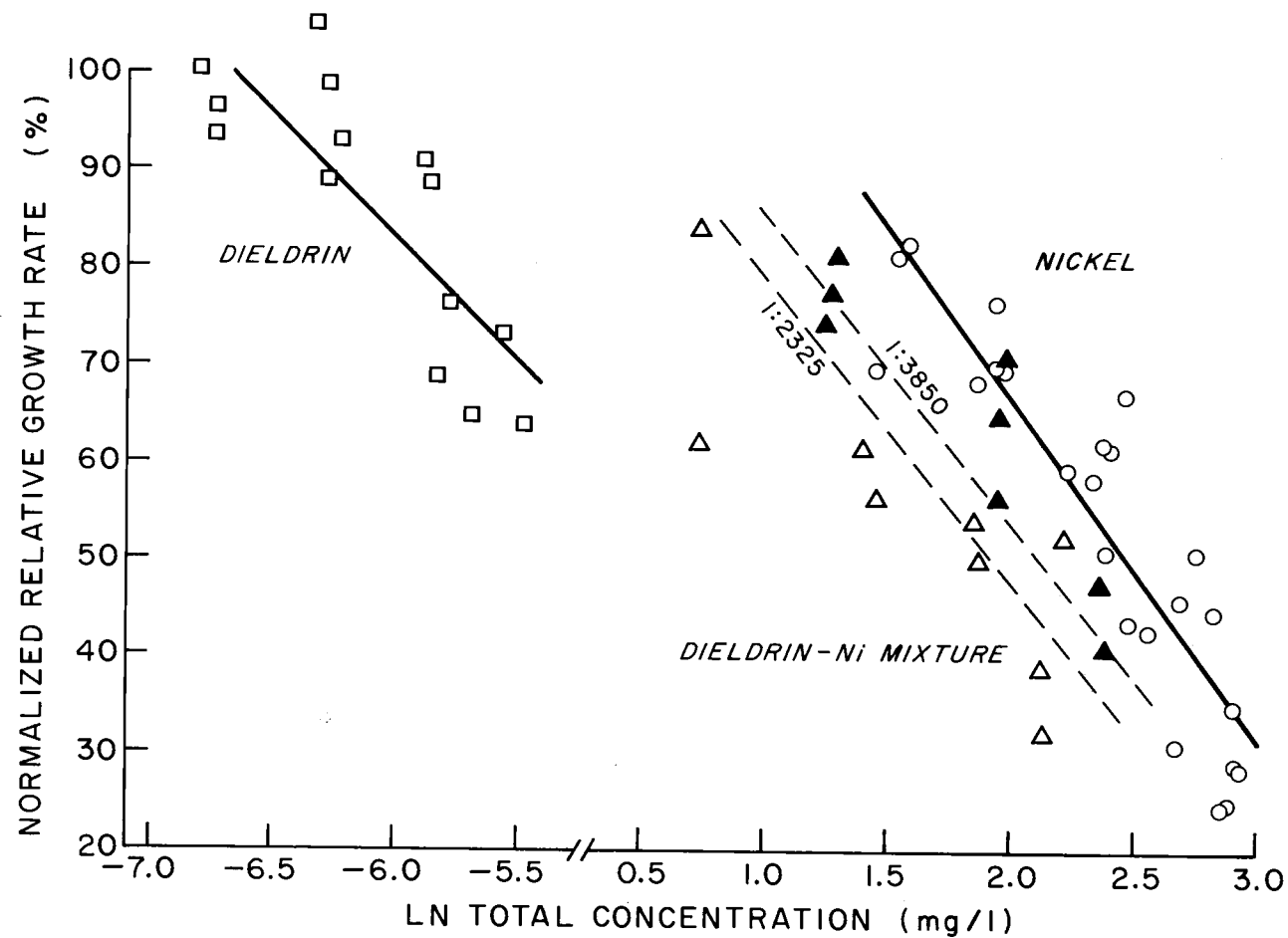


Figure 15. Dose response curves showing effects of dieldrin, nickel and their mixtures (observed and predicted) on relative growth rate normalized to responses of controls (unrestricted ration study). Mixtures of the toxicants in the fixed proportions of 1:2325 (Δ) and 1:3850 (\blacktriangle).

Table 5. Regression equations of the dieldrin and nickel dose response curves for the unrestricted ration. Normalized gross growth efficiency, food consumption rate and relative growth rate (Y) are expressed as a function of toxicant concentration (X). Predicted equations were calculated based on the assumption of concentration addition for the mixture.

Toxicants	Sample Size	Regression Equations		
		Gross Growth Efficiency	Food Consumption Rate	Relative Growth Rate
Dieldrin	14	$Y = -42.11 - 22.36 \ln(X)$	$Y = 0.19 - 14.80 \ln(X)$	$Y = -69.37 - 25.46 \ln(X)$
Nickel	24	$Y = 145.55 - 26.82 \ln(X)$	$Y = 116.10 - 22.41 \ln(X)$	$Y = 137.17 - 35.34 \ln(X)$
1:2325 Mixture (predicted)	-	$Y = 122.71 - 26.12 \ln(X)$	$Y = 101.70 - 19.79 \ln(X)$	$Y = 111.40 - 31.94 \ln(X)$
1:3850 Mixture (predicted)	-	$Y = 129.28 - 26.12 \ln(X)$	$Y = 104.54 - 19.79 \ln(X)$	$Y = 117.28 - 31.94 \ln(X)$

estimated for mixtures of the toxicants by the procedures previously discussed. These equations for both of the proportions studied are also reported in Table 5.

The combined effects of dieldrin and nickel on the gross growth efficiency of guppies fed the unrestricted ration are shown in Figure 13. Although there is considerable scatter in the data, the responses of the fish to the toxicants seem to be quite different for the two proportions studied. Mixtures at the 1:3850 ratio did not appear to be additive with the responses of the fish being similar to the ones observed for nickel alone. However, the combined effects of the toxicants at the 1:2325 ratio were additive as observed from the close association of this data to the dose response curves predicted on the basis of concentration addition.

The results of the study evaluating the effects of the dieldrin-nickel mixture on the rate of food consumption indicate that the combination of the toxicants in the ratios studied is additive (Figure 14). Graphical comparison of the data for the mixtures to the dose response curves predicted for a concentration additive interaction shows that most of the observed responses are slightly supra-additive relative to this form of addition. The combined effects of dieldrin and nickel on the relative growth rate of the fish is depicted in Figure 15. At the 1:2325 ratio the response to the mixture appears to be slightly supra-additive relative to the dose response curve

predicted for this mixture. However, the reductions in the relative growth rate of the fish exposed to concentrations of the toxicants in the 1:3850 are similar to the responses predicted for a concentration additive interaction.

DISCUSSION

Copper-Nickel Mixtures

The dose response curves for copper and nickel were parallel for all the responses studied; consequently, concentration addition was predicted for binary mixtures of the toxicants. Comparison of the predicted and observed dose response curves indicated that the assumption of concentration addition adequately predicts the effects of a copper-nickel mixture on the gross growth efficiency of juvenile guppies fed both a restricted and an unrestricted ration. This type of interaction was also observed by Anderson and Weber (1977) in 96 hour lethality studies evaluating the effects of a copper-nickel mixture on the survival of male guppies.

The dose response curve for mixtures showing the effects of the toxicants on the food consumption rate of the fish was supra-additive relative to the curve predicted on the basis of concentration addition. An explanation for differences in the effects of the toxicants on the gross growth efficiency and food consumption rate of the guppies is beyond the scope of the present study. However, it is not inconceivable that the combined effects of the toxicants on the metabolic state of the fish as reflected in alterations in the gross growth efficiency might be somewhat different than their effects on the physiological and behavioral processes regulating the consumption

of food.

The effects of the mixture on the relative growth rate are similar to the ones observed for gross growth efficiency at the restricted ration (concentration addition) and for food consumption at the unrestricted ration (supra-addition). These results are to be expected because of the dependence of the growth rate on the amount of food consumed. For the restricted ration studies, food consumption was held constant; therefore, the effects of the toxicants on the gross growth efficiency are directly related to toxicant induced changes in the relative growth rate. In the unrestricted ration bioassays, the effects of the toxicants on the relative growth rate reflect the supra-additive interaction observed in the food consumption studies.

For any given response (i. e. gross growth efficiency, relative growth rate, or relative food consumption), it is interesting to note that the ranges of response over the concentrations assayed are similar in magnitude for copper, nickel and the mixture. For example, in the restricted ration studies the maximum percent reduction in growth efficiency is 40% at concentrations of copper and nickel approaching the incipient lethal level. This observation might be expected for mixtures of toxicants interacting in a concentration additive manner. As stated previously, the basic assumption for concentration addition is that the toxicants in the mixture act upon similar biological systems. From this assumption it might be logically supposed that compounds affecting similar systems and

interacting in a concentration additive manner will cause similar magnitudes of effects. Although this is a tempting supposition and appears to be supported for the most part by the nickel and copper data, further corroborative evidence on the modes of action of these compounds and studies for other toxicants acting in a similar manner are necessary before such empirical generalizations can be made. However, it should be pointed out that the effects of the toxicants on the food consumption of the guppies was supra-additive relative to concentration addition even though the magnitude of effects was similar for both toxicants.

Dieldrin-Nickel Mixtures

Dieldrin and nickel were selected for the multiple toxicity studies because it was thought a priori that these compounds might interact in a response additive manner. They are representative of different types of toxicants with greatly different physical and chemical characteristics. Consequently, dieldrin and nickel might be expected to primarily effect different physiological systems. However, the results of the dieldrin-nickel studies are inconclusive regarding the determination of the nature of the interaction for mixtures of these toxicants.

There are several possible reasons for the difficulties encountered in interpreting the results of the dieldrin-nickel interaction.

As previously discussed, the variability in the guppies responses to dieldrin leaves open to question the value of the statistical procedures used to analyze the copper-nickel mixture. Although the slopes of the dose response curves for dieldrin and nickel appear to be different for some of the responses studied, statistical comparison of the slopes indicates that they are similar. Whether this is due to actual similarities in the dose response relationships or due to variability in the data can not be determined. However, as previously implied the slopes of the dose response curves may not be a very reliable criterion to use for predicting the type of interaction for mixtures of toxicants.

A further difficulty in determining the nature of the dieldrin-nickel interaction is implicit to the discussion of the hypothetical dose response curves discussed previously. At certain fixed proportions of the toxicants (i. e. in Figure 1 at ratios of 1:10 and 1:1000), the relative potencies of the mixtures of toxicants acting in either a concentration or a response additive manner are very similar. It is possible that an analogous situation exists for the fixed proportions used in the dieldrin-nickel interaction studies (1:2325 and 1:3850). To test this possibility dose response curves calculated on the assumption of response addition were compared to the predicted dose response curves shown for a concentration additive interaction. The relationship between the predicted dose response curves for mixtures

in the 1:2325 ratio are shown in Figure 16. Similar results were observed at the 1:3850 ratio. Although the form of the predicted dose response curves are different, the relative toxicities for both types of interactions at the fixed proportions assayed are similar.

Another reason that the combined effects of the dieldrin-nickel mixture are difficult to characterize is related to the apparent complexity of the interaction itself. The relative toxicity of the mixture seems to vary not only for the different responses studied (gross growth efficiency, relative growth and food consumption rate) but also for the different fixed proportions and ranges of concentrations assayed. For example, in the restricted ration study the effect of the mixture on the gross growth efficiency is additive at high and low concentrations but at intermediate doses the responses to the mixture are similar to those observed for nickel alone. However, in the unrestricted ration study, mixtures of dieldrin and nickel in a 1:3850 ratio were not additive at any of the concentrations studied but at the 1:2325 ratio the mixture appeared to be concentration additive.

As in the copper-nickel study, the effects of the dieldrin-nickel mixture on the relative growth rate of the guppies reflect the combined effects of the toxicants on the distribution of food energy among the other energy requiring processes (as indicated by changes in their gross growth efficiency) and on the amount of food consumed. For the food consumption studies, a slightly supra-additive interaction was

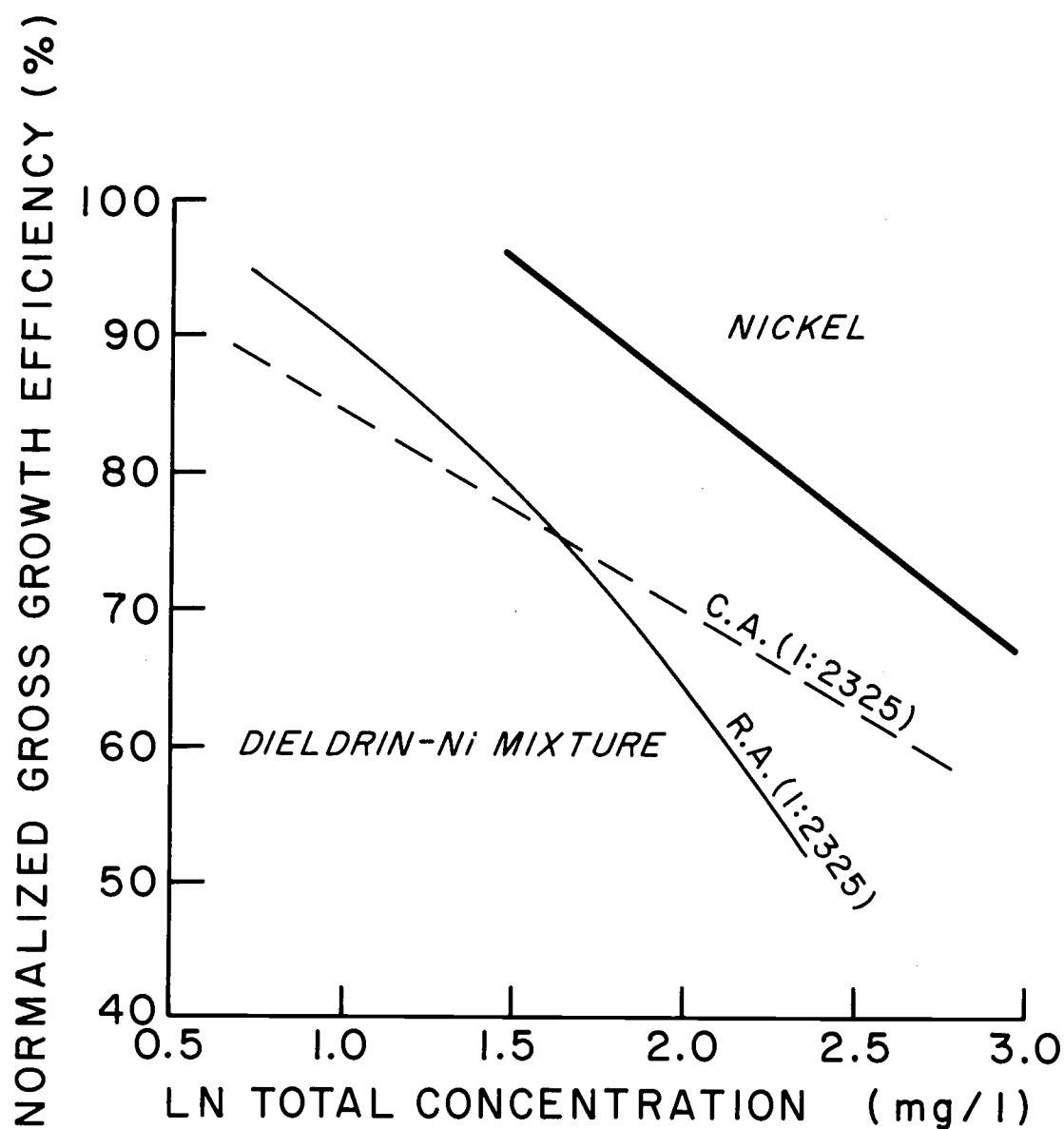


Figure 16. Comparison of dose response curves predicted for a concentration additive (C. A.) and response additive (R. A.) interaction. Dose response curves determined from data in Figure 11 for dieldrin and nickel mixtures in the ratio of 1:2325.

observed for both proportions of the mixture. While the combined effects of the toxicants in the 1:2325 ratio on the relative growth rate is also supra-additive in the unrestricted ration studies, the effects of the 1:3850 ratio appear to be additive or slightly infra-additive. This discrepancy is apparently due to the differential effects of the two ratios of mixtures on the amount of food energy available for growth.

Evaluation of the Approach

The proposed approach provides a methodology for studying the effects of mixtures of environmental toxicants on the performances of whole organisms. The results indicate that the assumption and concentration addition adequately predicts the effects of a copper-nickel mixture on the gross growth efficiency of guppies. A previous study (Anderson and Weber 1977) demonstrated a similar interaction evaluating the effects of the toxicants on the survival of male guppies. However, it should not be inferred from these results that the type of joint toxicity observed when organisms are exposed to high, rapidly lethal concentrations of mixtures will necessarily occur in cases where organisms are subjected to sublethal levels of the same toxicants. In other words, the nature of the toxicant interaction can only be meaningfully described in relation to the particular response under consideration. In both the copper-nickel and dieldrin-nickel studies,

different types of interactions were observed for the different responses (i. e. relative growth and food consumption rate) studied. In the interpretation of the effects of toxicant mixtures evaluated at one fixed proportion, it should also be considered that at different fixed proportions and different levels of response other types of interactions are possible.

Although this approach appears to offer a method for evaluating the effects of combined toxicants, its limitations should not be overlooked. One major limitation is inherent to all statistical explanations. By means of the statistical tests used in the analysis of the data, it was possible to state whether the observed responses to the mixture agreed with those predicted within the limits of sampling error. However, statistical analysis can only provide contradictory or permissive evidence but not indicative evidence (Hewlett and Plackett 1950). For example, an implication of the mathematical model for concentration addition is that the toxicants in a mixture act primarily upon similar biological systems. Statistical agreement of the observed dose response curves to the curves predicted on the basis of concentration addition does not necessarily mean that the toxicants in the mixture acted upon similar biological systems but only that they appeared to do so.

To insure the success of a species in nature, it is necessary to evaluate the effects of potentially hazardous toxicant mixtures on

whole organism performances such as survival, growth, and reproduction. The proposed approach provides a methodology for assessing the toxicity of mixtures of environmental toxicants at this level of biological organization. However, to offer explanations as to why mixtures of environment toxicants interact in a particular manner requires knowledge of the effects of combined toxicants on underlying biochemical processes and physiological functions. Such studies will be useful for evaluating the assumptions of the proposed approach and in suggesting other possible types of toxicant interaction.

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