DOSE AND TIME RELATED RESPONSE PATTERNS IN TEST POPULATIONS OF *BRACHYDANIO RERIO* EXPOSED TO COPPER, CADMIUM AND MERCURY IN PURE SOLUTIONS AND IN BINARY MIXTURES

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ABSTRACT

DOSE AND TIME RELATED RESPONSE PATTERNS IN TEST POPULATIONS OF BRACHYDANIO RERIO EXPOSED TO COPPER, CADMIUM AND MERCURY IN PURE SOLUTIONS AND IN BINARY MIXTURES.

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Dose and time-response analyses were conducted to quantify the lethal toxicity of copper (Cu), cadmium (Cd) and mercury (Hg), individually, and in certain binary mixtures to zebrafish, Brachydanio rerio. Both forms of analysis indicated the following order of potency for discrete metal solutions; Hg>Cu>Cd (240 hr LC_{50}=0.15, 0.26, 5.82 mg/l, respectively). Furthermore, an incipient lethal level was observed for copper (0.26 mg/l) and cadmium (5.82 mg/l) but not for mercury within the 10 day period studied.

Lethal bioassays conducted with $CuSO_4$ and $CdCl_2$ in pure solutions and mixtures indicated that the combined effect of these metals was more than-additive (2.01 x). An incipient lethal level was observed for the mixtures of Cu and Cd as with their pure solutions.

Preliminary bioaccumulation studies of copper and/or cadmium, into fish, indicated a reciprocal enhancement of uptake rates into gill tissue. This may explain the greater than additive effect observed at the lethal level.

The interactive effect of mixtures containing $HgCl_2$ and $CdCl_2$ varied from less-than-additive (48 hr) through additive (96 hr) to more-than-additive (2.03x, 240 hr). There was no evidence of an incipient lethal level for these mixtures.

The results of a sequential exposure study suggested that the more-than-additive effect of Cd-Hg mixtures may result from a Cd induced sensitivity to Hg toxicity.

The results are discussed with reference to the usefulness of various approaches for assessing criteria to safeguard aquatic organisms against the hazard of multiple toxicity.

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INTRODUCTION

The exposure of cell surface membranes to heavy metals[1] is known to result in protein denaturation and a subsequent loss of structural and or functional capacity (Passow et al., 1961). Because of this, heavy metals pose a particular hazard to fish through their ability, as waterborne contaminants, to react directly with the respiratory epithelium of gill surface membranes. In fact, many authors have suggested that the toxicity of various heavy metals is mediated through a common capacity to interfere with gill function (Lloyd, 1960, 1962; Matthiessen and Brafield, 1973). Therefore it is not surprising that some investigators (Sprague, 1970; Seba, 1975) have concluded that the principle of strict addition, which implies similar action (Bliss, 1939), adequately describes the lethal toxicity of heavy metal mixtures i.e., each constituent of a mixture contributes to the toxicity in proportion to its respective potency. However, while strict addition has often proved suitable for predicting median lethal tolerance limits exceptions have been reported (Sprague, 1970; Calamari and Marchetti, 1973; Eaton, 1973; Anderson and Weber, 1975).

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[1] Heavy metals are defined as those with a specific gravity greater than 5 (Passow et al. 1961).

Herbert and Shurben (1964) reported that survival time was unpredictable in mixtures which were rapidly lethal despite the fact that the lethal thresholds were strictly additive. Sprague and Ramsay (1965) noted a similar non additive effect i.e. shorter than predicted survival times for salmonid fish exposed to rapidly lethal mixtures of heavy metals. This finding confirmed the earlier work of Doudoroff and Katz (1953) who exposed minnows to mixtures of certain heavy metals. These reports would suggest that the form of interaction between fish and heavy metal mixtures may be different at various points in time.

The purpose of this thesis is to determine whether the combined lethal effect on fish of binary mixtures containing Copper (Cu), Cadmium (Cd) or Mercury (Hg) varies through exposure time. Furthermore, if the form of interaction does change is it a function of differences in the time-response patterns of the constituents (accumulative-non accumulative) or a consequence of the methodology employed to analyze the data.

Background

In recent years it has become increasingly apparent that mans' various industrial, municipal and agricultural activities discharge a wide range of waste products into aquatic environments. This "at the pipe" or point source dumping has lead to the situation where aquatic organisms inhabiting polluted waters encounter an array of chemical contaminants (Anderson and D'Appolonia, 1978). Pollutants may also enter aquatic habitates through indirect means i.e. rain, snow, fallout, ground water

leachings and surface water runoff. Barton (1978) has estimated that these latter non point sources contribute more than fifty per cent of the toxicants which contaminate natural waterways. Furthermore if has been shown that pollutants which enter the aquatic environment from various point and non point sources are mobile, resulting in the formation of "coincidental" mixtures. In fact, the in situ chemical monitoring of aquatic ecosystems has confirmed the virtual ubiquity of pollutant mixtures within both the ambient environment and the tissues of organisms (FAO, 1972; Kerr and Vass, 1973; Larkin, 1974). With respect to the toxicity of mixtures the evidence gathered to date indicates that unique forms of toxicity can be ascribed to the concurrent or sequential exposure of organisms to two or more pollutants (Sprague, 1970). The significance of these facts becomes apparent when it is realized that operating discretely and at prevailing levels, each constituent within a mixture may be harmless yet collectively these pollutants may cause toxic effects.

In view of this information it would appear that a need clearly exists for the development of adequate procedures for the toxicity testing of mixtures.

Rationale

Various models have been proposed for predicting the toxicity of mixtures from the individual toxicities of the separate contaminants. However, two methodologies predominate in the literature and will be used in this study to examine additive, greater than and or lesser than additive interactions between

constituents of mixtures. Utilizing these methodologies will allow for the identification of any changes in the form of the interaction throughout the exposure period.

The simplest approach has been promoted by researchers at the British Water Pollution Research Laboratory in Stevenage (Lloyd, 1961; Herbert 1962; Herbert and Shurban, 1964; Herbert and Van Ryke, 1964; Brown, 1968; Brown and Dalton, 1970). In practical terms this approach computes the toxicity of a mixture by adding the toxicity of each component. Each contaminant's contribution to the toxicity of the mixture is expressed as a fraction of its lethal threshold concentration (incipient LC_{50}[1]) or more commonly an approximation such as the 96 hour LC_{50}[2]. Sprague and Ramsay (1965) proposed that this fraction be called a toxic unit:

$$\text{Toxic unit} = \frac{\text{actual concentration in solution}}{\text{96-Hr } LC_{50}} \quad (1)$$

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1 The incipient LC_{50} is that concentration of toxicant below which 50% of a test population will not respond.

2 The 96 hour LC_{50} is that concentration which will result in 50% response in a test population at 96 hours of exposure.

Thus, if the sum of the toxic unit values calculated for each component of a mixture equals 1.0 then the predicted joint toxicity will result in 50% mortality at 96 hours. Should the "toxic unit" value of a mixture be greater than 1.0 then more than 50% mortality is predicted. If less than 1.0, then less than 50% mortality is predicted.

Anderson and Weber (1975) utilizing the concepts of Bliss (1939) have promoted a slightly more involved system of predicting the toxicity of mixtures. They suggest that two additive manifestations of pollutant interaction exist.

(A) Concentration Addition: This form of interaction is predicted for constituents of a pollutant mixture which presumably have qualitatively similar toxic effects. The similarity of toxic effects is determined by:

(i) the structural similarity of toxicants and presumed modes of action and

(ii) parallelism between the dose-response curves developed for each toxicant and their mixture.

Thus, each similarly acting constituent of a mixture will contribute to the toxicity of the mixture in an amount equal to its concentration multiplied by its relative potency. To calculate the toxicity of a mixture the respective contributions in dosage of each contaminant are added. It should be noted that this method of calculating joint toxicity is basically the same as the toxic unit method. The essential difference being that the toxic unit method only identifies increases or decreases in the toxicity of a mixture relative to a single predicted level of

response i.e. 50%. The method of Anderson and Weber will identify increases or decreases in the toxicity of a mixture relative to the entire range of responses i.e. 0 to 100%.

(B) Response Addition: This form of interaction is predicted for constituents of a pollutant mixture which have qualitatively dissimilar toxic effects. The dissimilarity of toxic effects is determined by:

(i) structural dissimilarity of toxicants and presumed different modes of action and

(ii) non parallelism between the dose-response curves defined for each toxicant.

Such toxicants are presumed to act through different physiological systems yet contribute to a common response. In its simplest terms the toxicity of a mixture is computed to be the sum of the relative contributions of each toxicant above its respective threshold value. (A more detailed description in which the correlation of susceptability of the organisms to each toxicant is taken into account appears in the materials and methods).

The use of the preceeding methodologies will allow for the identification of any changes in the interactive effect of the mixtures through time. In addition a comparison may be made of each models' ability to define the hazard presented by the mixtures.

Selection of Toxicants

Cadmium, copper and mercury have been selected for the present study because the salts of these heavy metals are prevalent pollutants of aquatic environments. (U.S. Council of Environment Quality, 1971). Furthermore, it has been determined that each of these toxicants are often mobilized within natural waterways in amounts which approach toxic levels (Chapman, 1978).

Cadmium salts are considered to be significant water pollutants not only because of their toxicity in the 0.01 mg/liter range but also because of their ability to be incorporated into the food chain by aquatic organisms and plants (Cearly et al, 1973). Studies have shown that freshwater fish can concentrate cadmium 10 to 1000 times higher than the concentration within the ambient environment (Fleischer et al, 1974). Although the toxic effects of cadmium in man have been described (Friberg et al., 1971) little is known about the biochemical and physiological effects of this metal in aquatic vertebrates.

Large amounts of cadmium are used by industrialized countries for electroplating, production of batteries and many other technologic purposes. The production of the metal is principally derived from the byproducts of refining zinc, lead, and copper ores. Both Canada and the United States are significant producers of cadmium (Simons, 1967).

Copper salts generally enter aquatic environments through ground water erosion of mineral deposites. However, there are significant localized anthropogenic inputs as a byproduct of such industrialized processes as tanning, dyeing, and pigment manufacture. (Mckee and Wolf, 1963; Spear and Pierce, 1979).

Another source of copper pollution arises from its use as an algicide in reservoirs and streams. It has been determined that in Canadian surface waters copper levels, while wide spread, rarely exceed 5 µg/liter (Spear and Pierce, 1979).

Mercury is the most toxic heavy metal for many aquatic organisms (Jaakkola et al.,1972). In addition to the extreme toxicity of mercury which results from assimilation directly from the ambient environment it has also been shown that mercury can be accumulated through the food chain (Hannerz, 1968; Jernelov and Lann, 1971; Fagerstrom et al, 1974).

Overall 5000 tons of mercury are discharged each year into the atmosphere by fossil fuel combustion (Krenkel, 1970). A further 800 tons per year come from the natural weathering of rock (Gravis et al, 1972). Recent surveys have identified concentrations of mercury in certain aquatic ecosystems as high as 20 ppm (Krenkel, 1971). Significant concentrations of mercury have been reported in Canadian fish from the Saskatchewan river system (Wobeser et al, 1970) as well as other areas (Bligh, 1970, 1971; Uthe et al., 1971; Fimreti, 1970).

As previously stated each of the individual heavy metals, copper, cadmium and mercury, is mobilized in the ambient environment in amounts which approach their respective toxic level (Chapman, 1978). Thus it is apparent that physiological interactions which would enhance their toxicities when encountered as constituents of a mixture may well create a hazard to aquatic organisms. Therefore, it would seem that the form of interaction exhibited by mixtures containing these specific heavy metals should be carefully examined.

A further advantage to the choice of these metals relates to their modes of accumulation. Evidence gathered in the literature would suggest that accumulation of metal resulting in an acutely lethal response is limited to the initial 96 hours of exposure for copper and cadmium while accumulation continues through to and beyond 240 hours for mercury. (Lloyd, 1960; Sprague and Ramsay, 1965; Macleod and Pessah, 1973). The length of the exposure time for this study was 240 hours hence copper and cadmium are termed non-accumulative while mercury is termed accumulative. Therefore, the selection of these heavy metals allows for the testing of two types of binary combinations i.e. non-accumulative plus non-accumulative and non accumulative plus accumulative. This will offer an opportunity to examine whether these properties are determinants of the toxicity exhibited by the mixtures during the exposure time.

Selection of Test Organism

The zebrafish, Brachydanio rerio, (Hamilton-Buchanan, 1822-1823) is a small tropical fish of the family Cyprinidae (Laale, 1977), and was chosen for the present study. Fogels and Sprague (1976) proposed that the zebrafish be selected as a standard test species of the International Standards Organization. At present rainbow trout are the most popular bioassay species in Canada. While zebrafish are not native to cold water areas such as Canada it is of note that neither are rainbow trout indigenous to all areas. In comparative testing zebrafish were found to be on average 2.6 times more tolerant than rainbow trout to certain toxicants (Fogels and Sprague, 1976). These facts plus the relative ease of laboratory maintenance favoured the use of the zebrafish in this study.

Summary

The objectives of this study are:

(1) To determine if the form of the combined lethal effect on fish of binary mixtures containing certain heavy metals changes through the exposure time.

(2) To determine whether changes in the interactions, if present, are a function of

(a) differences in the accumulation patterns of the constituents i.e. accumulative or non-accumulative or

(b) a consequence of the methodology employed to analyze the data.

The results will be discussed in relation to possible mechanisms of interaction. Conclusions will be drawn with respect to the applicability and usefulness of the various approaches to access the hazard of mixtures.

MATERIALS AND METHODS

Test Organisms

Adult zebrafish, Brachydanio rerio, (Hamilton-Buchanan, 1822-1823) (Figure 1), were purchased, when required, from Riverview Water Gardens in Sebastian, Florida. The fish were quarantined for two weeks and any found with visible signs of disease were rejected. All accepted fish were allowed to acclimate to laboratory conditions for a minimum of three weeks. During the acclimation period the fish were held in large 350 liter aquaria of either polyethylene or fiberglass. Acclimation tanks were supplied with a continuous flow of dechlorinated (<0.02 ppm Cl^-) and aerated tap water.

Periodic monitoring of certain chemical characteristics of the water was conducted (Table 1). The water was 85-95% saturated with oxygen and the temperature was maintained at $27^oC \pm 1^oC$.

The fish were fed twice daily with Tetramin Staple tropical fish food. Wastes and unconsumed food were removed regularly. A photoperiod of 12 hours (8:30 - 20:30) was provided by fluorescent light fixtures.

Dosing Apparatus

Stock solutions of copper were prepared by dissolving reagent grade $CuSO_4 \cdot 5H_2O$ (Fisher) in an acetate buffer (APHA et al., 1965). Stock solutions of cadmium and mercury were prepared

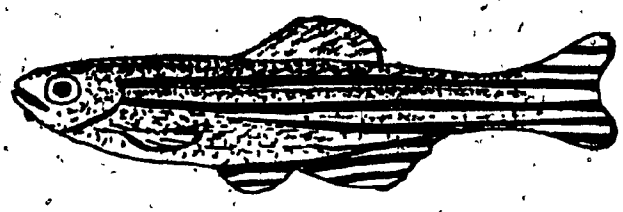

Figure 1. Diagram of zebrafish Brachydanio rerio
Ratio 2:1

Table 1. Water Quality Data

ANALYSIS OF LABORATORY WATER USED IN EXPERIMENTS		
Dissolved Oxygen	% SAT	88±5
pH		7.65±0.11
Temperature	^{o}C	27±1
Total Hardness	mg/l as $CaCO_3$	125±3

ANALYSIS OF SOURCE WATER 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^{+++}	0.3
Detergents	LAS	0.017

by mixing reagent grade $CdCl_2 \cdot 2\frac{1}{2} H_2O$ (Fisher) and $HgCl_2$ (Fisher) with de-ionized, glass distilled H_2O. Stock solutions made for each experiment were placed into individual Mariotte bottles (Grenier, 1960).

The stock solutions were released at a rate of 1 ml per minute into a serial dilution apparatus. Figure 2 is a schematic diagram indicating the physical arrangement of the dilutor.

A constant drip of stock solution was achieved by maintaining a fixed vertical displacement between the point of outflow, a rotatory faucet, and the surface of the solution within the Mariotte bottle. The constant hydraulic head within the Mariotte bottle was created by inserting a stand pipe which would allow air to enter the bottle. When the system was functioning the solution/air interface at the lower end of the stand pipe was the "functional surface" within the Mariotte bottle (Figure 3). As toxicant solution dripped from the bottle a partial vacuum was created causing air to enter through the stand pipe to replace the fluid lost. The "functional surface" was unaffected by this and therefore, a constant hydraulic head was maintained.

The flow rate out of the mariotte could be adjusted by rotating the glass faucet (Figure 4), thereby changing the vertical displacement between the surface of the stock solution and the point of outflow.

Stock solution so released entered a multiple stage serial dilutor designed to supply test solutions to twelve exposure tanks (Figure 5). The initial dilution was collected in a large mixing chamber beneath the mariotte. The once diluted toxicant was

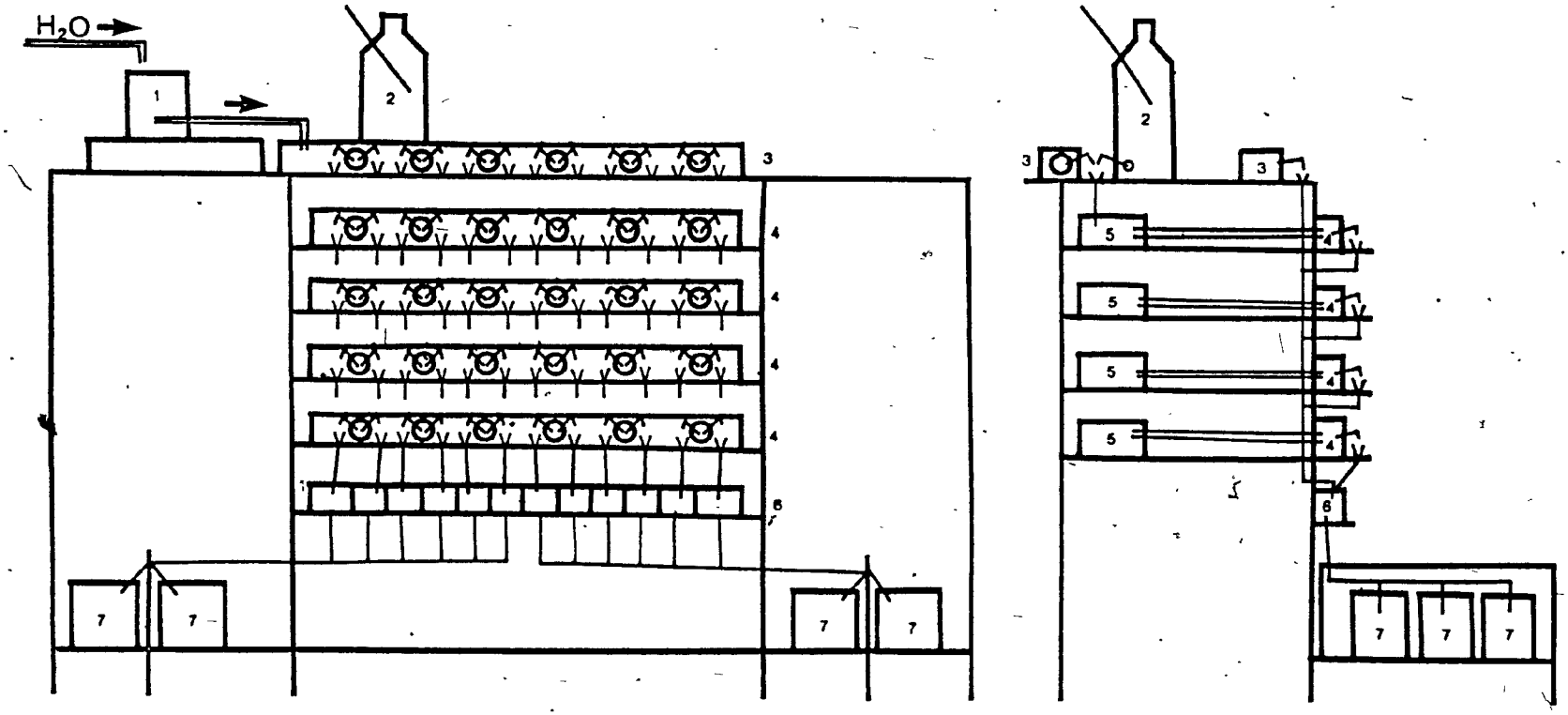

Figure 2. Schematic diagram of the bioassay apparatus - Front view and side view. (1) Headbox, (2) Mariotte, (3) Distribution chamber for toxicant free water, (4) Distribution chamber for each toxicant, (5) Mixing chamber, (6) Collecting chambers for each exposure tank, (7) Exposure chambers.

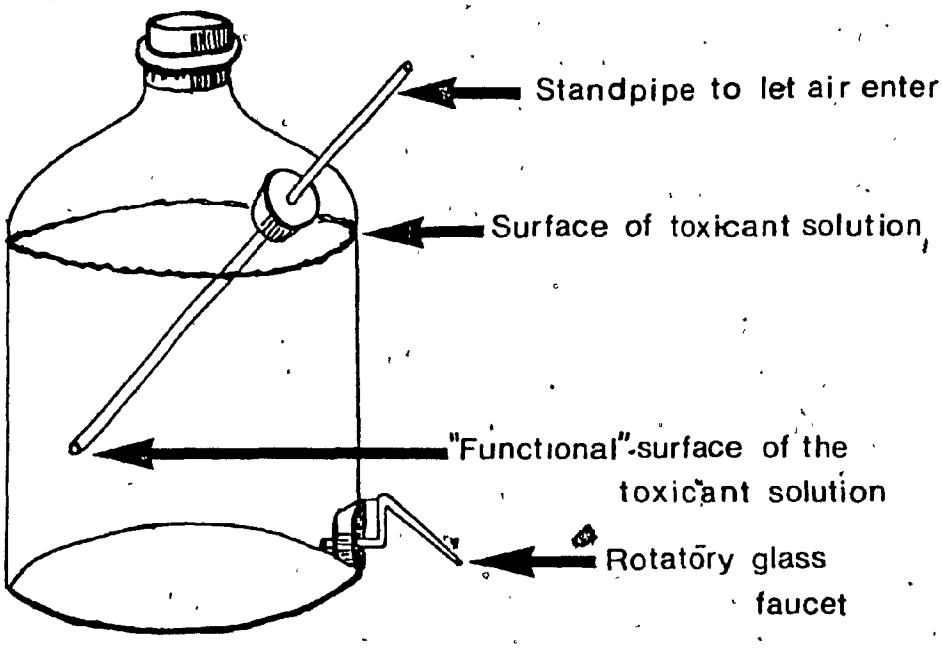

Figure 3. Diagram showing the design of a Mariotte bottle.

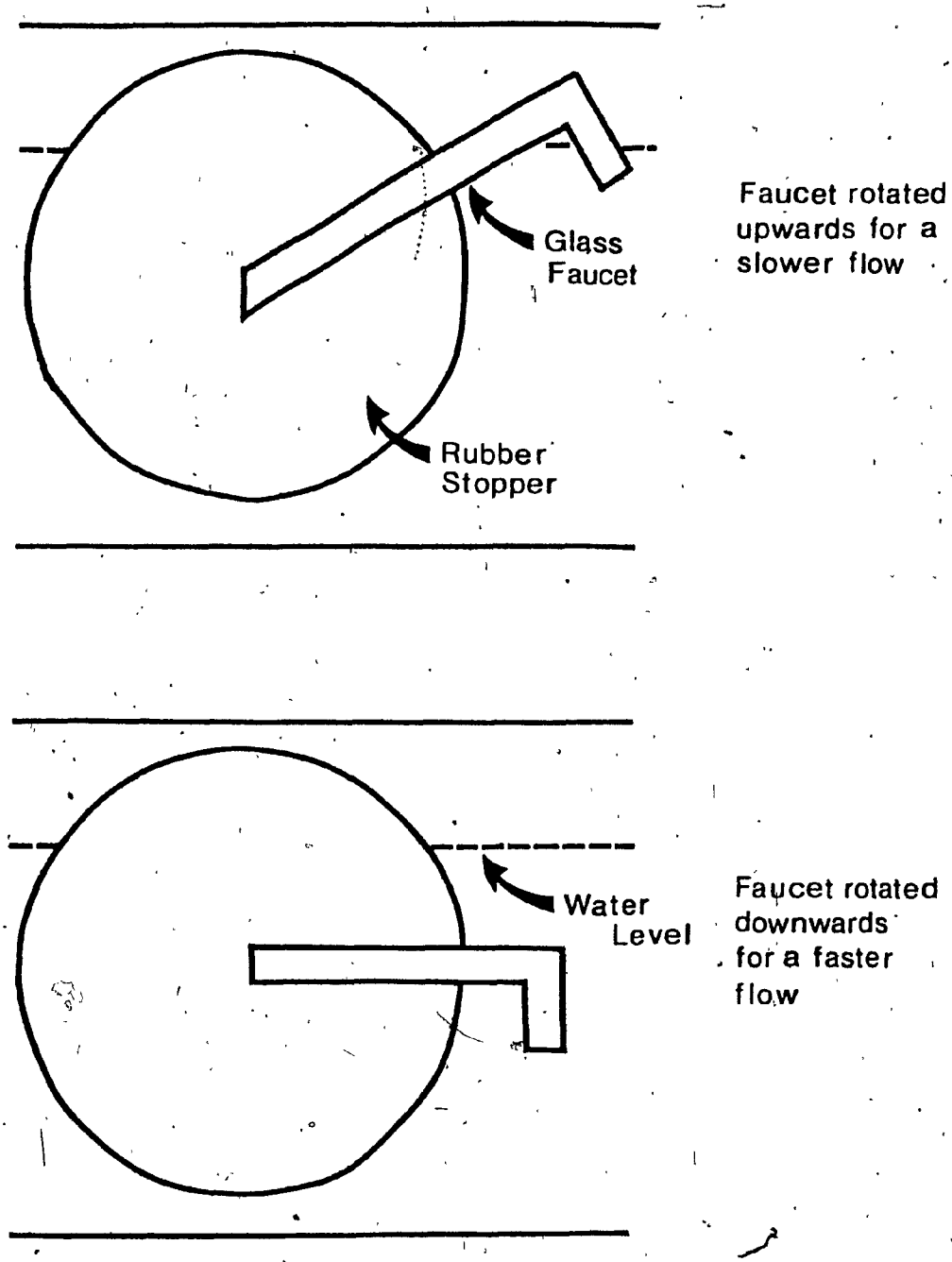

Figure 4. Diagram showing method used for obtaining various flow rates from the rotatory glass faucets.

transferred via a p.v.c. pipe to a distribution chamber (Figure 2, 5 and 6).

In each chamber an overflow standpipe was used to drain excess toxicant solution thereby maintaining a constant hydraulic head. Rotatory glass faucets, located in series at the front of each chamber, controlled the flow of the toxicant into the collecting chamber (Figure 2). The relative proportions of toxicant solution and water entering simultaneously from a separate chamber determined the final concentrations obtained in the collecting chambers. Each of the 12 collecting chambers were connected via tygon and glass tubing to their respective exposure tanks.

When more than one toxicant was mixed, the procedure was identical to that previously decribed, however, each toxicant was diluted by the total volume of all other toxicants entering the collecting chamber and not just water.

The flow rate for each exposure tank during the lethal dose response experiments was 300 ml per minute resulting in a 90% replacement within 2 hours (Sprague, 1973). The flow rate for each exposure tank during the tissue uptake experiment was 650 ml per minute resulting in a 90% replacement within one hour (Sprague, 1973).

Laboratory Conduct of Lethal Response Experiment

Fish from the holding tanks were sorted into lots of 14 individuals, three days prior to an experiment. Each lot of 14 fish represented a discrete weight class i.e. 0.10 $\pm$ 0.05 grams.

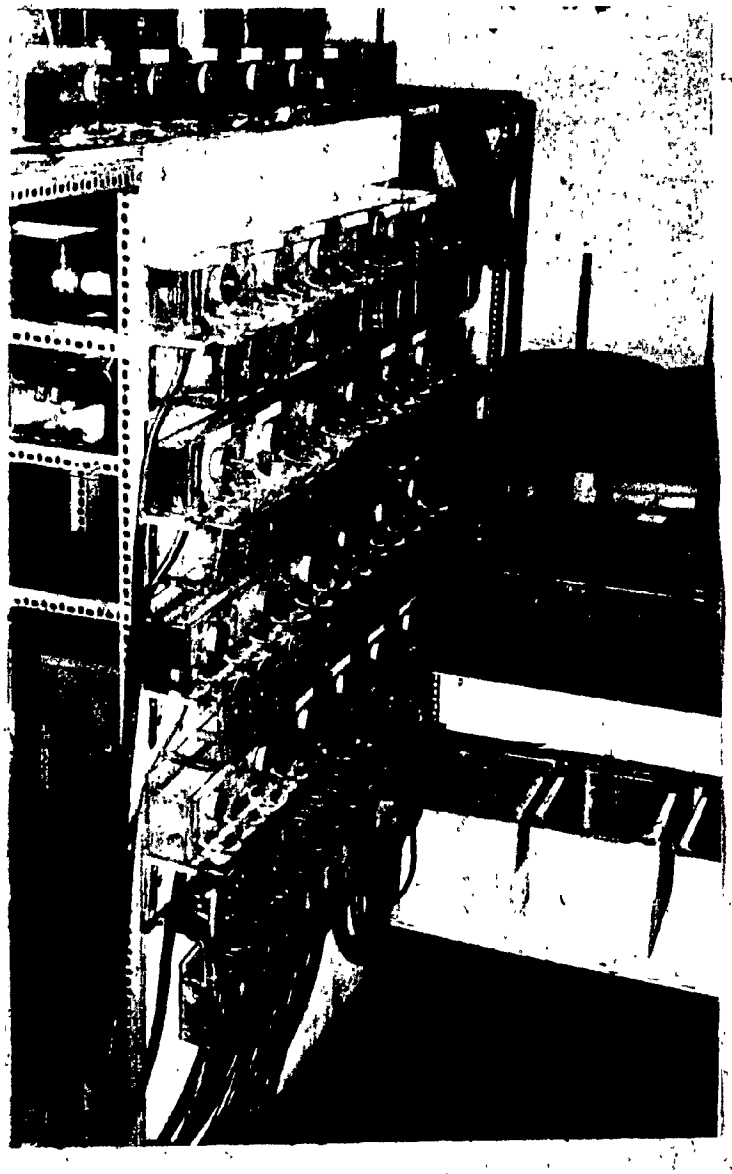

Figure 5. Photograph of Bioassay Apparatus, Front view.

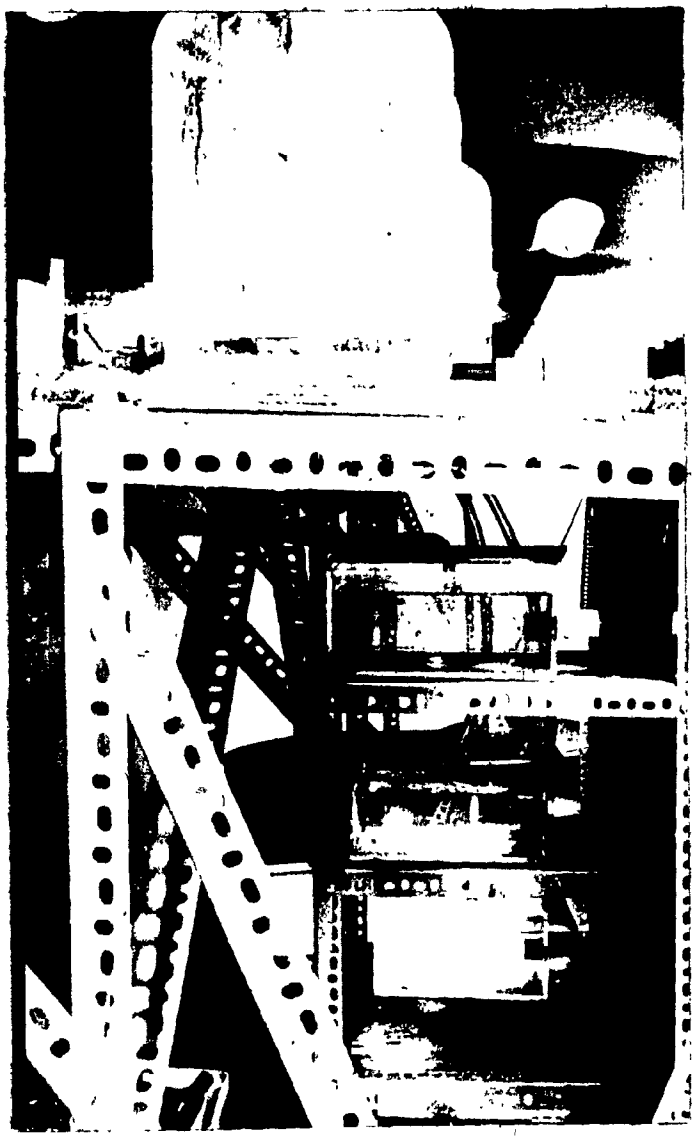

Figure 6. Photograph of Bioassay Apparatus side view.

Feeding was suspended 24 hours prior to exposure and resumed on day 5 of the exposure period following which time they were fed at two day intervals e.g. day 7 and 9. This feeding schedule was adopted as a balance between continuous feeding, which might have lead to significant toxicant assimilation via the ingestion of food, and no feeding, which might have lead to adverse effects resulting from starvation.

The flow of toxic solutions through the exposure tanks was established 24 hours prior to the introduction of the test organisms. This was done in order that an equilibrium between heavy metal ions in solution and those adsorbed to the walls of the exposure tanks could be established. Each lot of 14 test fish was transferred to a separate exposure tank between 2100 hours and 2200 hours. A random number table was used to ensure that weight class groups were distributed to exposure tanks in an unbiased manner. Each tank was considered as a separate experiment which commenced the moment it received its full complement of 14 fish.

Each test lasted exactly 240 hours during which time observations were made every four hours throughout the initial 96 hours and every six hours throughout the subsequent 144 hours. Fish were examined for visible evidence of toxicity. The toxic end point measurement for these experiments was death as determined by a 10 minute observation period during which no visible signs of gill or heart movement were noted. Fish were removed from the exposure tank upon death and discarded.

Temperature, pH and dissolved oxygen measurements were made on days 1, 3, 5, 7 and 9. Water samples were removed on days 1, 2, 3, 4, 6, 8 and 10 for metal content analysis.

Analysis of Water Samples from Lethal Response Experiments

Water samples for determinations of total metal content were collected in 60 ml polypropylene tubes, acidified with 0.5 ml concentrated HCl and refrigerated until analysis could be conducted.

The total metal content of each sample was determined using a Perkin-Elmer 503 atomic absorption spectrophotometer. Cadmium was assayed using a flame atomizer, mercury was measured by a flameless technique and copper content was determined using a graphite furnace method. The operating procedures used for measuring metal content are detailed in the operation manual for the Perkin-Elmer 503.

Dissolved oxygen content was measured using an azide modification of the Winkler method (APHA et. al.,1965). Hydrogen ion concentration was assayed using a Corning Scientific pH meter model # 10 and following standard operating procedures.

Laboratory Conduct of the Tissue Uptake Experiment

The tissue uptake experiment involved the exposure of fish to various pure solutions of copper and cadmium and their mixtures. All methods and procedures were identical to those outlined for the lethal dose-response study. However, only one weight class of test fish (0.35 ± 0.05 grams) was used.

Fish were introduced to each of the exposure regimens in groups of thirty. Randomly selected groups of five fish were removed from each tank at 4, 5, 6, 8, 12 and 20 hours post exposure and allowed to swim in a toxicant free tank for 10 minutes. The swim in contaminant free water was necessary to

to remove non-absorbed metal from the external body surfaces and the opercular cavity. Subsequently the test organisms were sacrificed and the complete set of gill arches and respiratory filaments (Hughes and Shelton, 1962) were removed.

Analysis of Water Samples from the Tissue Uptake Experiment

A water sample was obtained from each exposure tank at the end of the experiment. The same procedure as previously described was employed in the handling of samples and determination of metal content, pH and temperature.

Tissue Metal Content Analysis

All glassware employed in the determination was washed for 2 weeks in reagent grade nitric acid mixed 1:5 with glass distilled water. This was followed by five rinses with glass distilled water.

Gill and body tissue was prepared separately for analysis of metal content following the same procedure. Initially tissues were placed in a drying oven at 37° for seven days. Subsequently, tissues were mascerated with a glass pestle and placed in a tarred 50 ml beaker and weighed. The beakers were placed in a muffle furnace preheated to 50°C in increments of 50°C every 30 minutes. The tissues were left at 500°C for 12 hours after which the beaker were placed in a desiccator until cool. To each beaker was added 10 ml of a solution containing 2/3 nitric acid and 1/3 perchloric acid. The beakers were then heated and the liquid allowed to evaporate. The fine powder remaining was dissolved in 10 ml of distilled H_2O and stored in a polypropylene tube pending analysis.

The cadmium and copper content of each sample, including control blanks (i.e. no tissue), was determined using a Perkin-Elmer 503 absorption spectrophotometer and employing graphite furnace technique.

Methods of Analysing Lethal Response Study Data

(I) Derivation of Linear regressions representing the lethal response to individual metals.

The data collected from the lethal response experiments for cadmium, copper and mercury were fitted to linear functions following the probit analysis method of Finney (1971). This method fits a regression to coordinates which represent response in terms of probits and stimulus in terms of log concentration.

Data are systematically weighted to give the highest degree of confidence to those points arising in the 50% response region. The general mathematical definition of the linear function is given by the equation:

$$Y = a + b(x) \quad . \qquad (2)$$

where Y = % mortality as probits,

a = the Y intercept

b = the slope of the regression

x = concentration in log units.

The assumption that the dose-response data were adequately described by a linear function was tested using a chi-square test for heterogeneity (Finney, 1971)

(II) Analysis of data representing the lethal response to combinations of metals.

Bliss (1939) proposed that certain forms of multiple toxicity resulted from the interaction of toxicants at or through target sites in an organism. He defined two additive forms of multiple toxicity which were termed "independent" and "similar joint action". Independent joint action is exhibited by those mixtures whose constituents act at different target sites yet contribute to a common response. Similar joint action is exhibited by those mixtures whose constituents act at the same or similar target sites and thereby contribute to a common response.

It has been assumed by many authors including Bliss that if two toxicants have parallel dose-response curves they are similarly acting. This premise is based on the assumption that the variation in susceptibility of individual organisms is completely and positively correlated for similarly acting toxicants. While this has been shown to be true for some toxicant mixtures, Plackett and Hewlett (1952) presented dose-response data for structurally related (and presumably similarly acting) pesticides which were non-parallel. They have therefore pointed out that complete correlation of individual susceptabilities and hence parallelism is not a necessary prerequisite for similar joint action.

Bliss (1939) did however, offer an empiracle criterion by which the two models could be differentiated. Bliss suggested that only similar joint action could explain additive responses elicited when both constituents of a mixture were below threshold.

Despite the tenuous the association between similar joint action and parallelism of dose-response lines, it does provide a useful starting point for data analysis. Where the dose-response lines are

parallel a dose response curve for the mixture can be calculated employing the equation (Finney, 1971):

$$Ym = a_1 + b \ln (\pi_1 + p\pi_2) + b \ln x \quad (3)$$

where Ym = % response in probit units

a_1 = Y intercept of the reference toxicant

b = common slope

π_1 = proportion of the first toxicant

π_2 = proportion of the second toxicant

p = potency of the second toxicant relative to the first

x = concentration of the mixture.

To avoid the assumptions which imply a knowledge of sites and mechanisms of toxicant action Anderson and Weber (1975) introduced the terms concentration and response addition which are mathematically analogous to similar and independent joint action. In this respect their terminology is superior to that of Bliss and will be adopted henceforth.

A chi-square test for heterogeneity was used to determine whether the observed dose-response for the mixtures significantly differed from the predicted dose-response ($P \leq 0.05$). If the predicted regression adequately described the response to the mixture then the concentration addition model was accepted.

In those cases where the dose-response lines are not parallel or when parallel but theoretical reasons suggest constituents of a mixture will have different modes of action, response addition may be predicted. In order to determine the proportion of organisms which

will respond it is not only necessary to know how many would respond to each toxicant alone, but also to what degree the susceptability of the organism to one toxicant is correlated to the other toxicant.

Plackett and Hewlett (1948) developed mathematical models which take into account the correlation of individual tolerances. If the correlation is completely negative, r = -1, then the proportion of individuals responding can be determined using the following equation:

$$P_m = P_a + P_b \text{ (if } P_a + P_b \leq 1) \quad (4)$$

where P_m = The proportion of individuals responding

P_a = The proportion of individuals responding to toxicant A

P_b = The proportion of individuals responding to toxicant B.

Where no correlation in tolerances exists, R = 0, the proportion responding may be determined by equation:

$$P_m = P_a + P_b (1 - P_a) \quad (5)$$

Where a completely positive correlation in tolerances exists, R = +1, the proportion responding may be determined by equation:

$$P_m = P_a \text{ (if } P_a \geq P_b) \quad (6)$$

$$P_m = P_b \text{ (if } P_b \geq P_a)$$

A chi-square test for heterogeneity was used to determine if the predicted and observed responses were significantly different ($P \leq 0.05$). When there were no significant departures from the expected responses the response addition model was accepted.

When dose response patterns observed are not adequately described by either response or concentration addition models we assume non-additive interactions have occurred at the biokinetic or biodynamic level.

Biokinetic processes are those which involve the uptake, distribution and elimination of the toxicant i.e. those factors which determine the biological availability of the toxicant at the critical target site. Biodynamic processes are those which modulate the induction of the effect in the target tissue. (Figure 7).

In the case of non-additive responses it is impossible to predict, from a knowledge of the toxicity of the individual constituents, what the potency of the mixture will be. Following the terminology of Anderson and Weber (1975), a mixture which is less toxic than additivity would predict is referred to as Infra-additive and by analogy a mixture which is more toxic than additivety would predict is referred to as Supra-additive.

Method of Analysing Heavy Metal Accumulation Kinetics into Fish Tissue.

Accumulation kinetics of copper, cadmium and their mixture into the gills and body during a 20 hour exposure period were determined. Logarithmic linear regressions were fitted to data representing the change in metal content during the 4 to 20 hour period by the method of least squares.

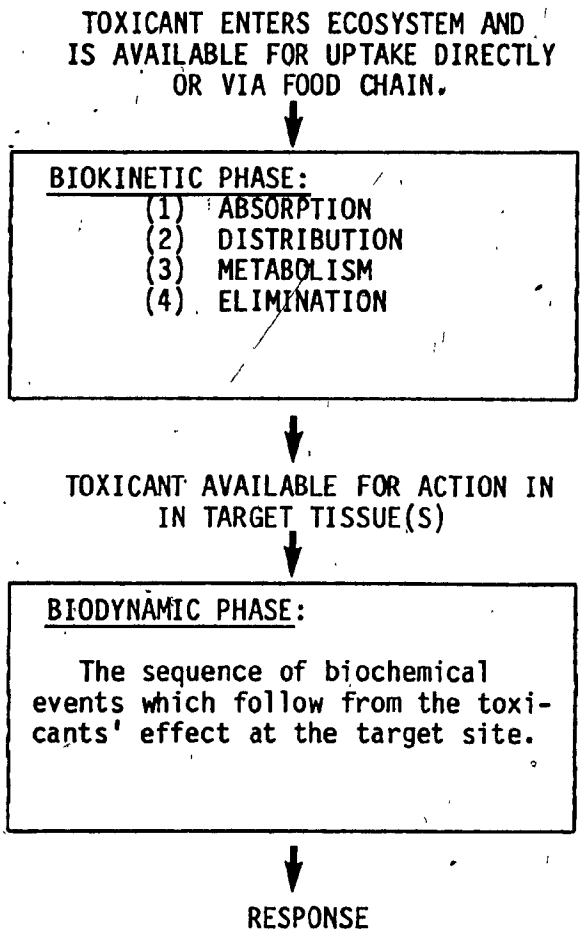

FIGURE 7. PHASES OF INTERACTIONS BETWEEN CHEMICAL CONSTITUENTS OF POLLUTANT MIXTURES (MODIFIED FROM ANDERSON AND D'APPOLONIA, 1978).

RESULTS

Lethal Response Studies-Discrete Heavy Metal Solutions

Dose-response data from the lethal bioassays in which zebrafish were exposed to pure solutions of either copper, cadmium or mercury are presented in tables 2,3,4 and 5. These tables detail mean concentrations of each metal to which fish were exposed and the per cent of the test population which responded at each of these concentrations. The tables also list the number of fish exposed and the mean weight class value for each test lot. The duration of each experiment was 240 hours and analysis of dose response was conducted for 96 and 240 hours. The dose response data for cadmium and copper are presented only for 96 hours exposure as no mortality was recorded after that time. Linear functions were fitted to each set of data following the probit analysis method (Finney, 1971). The probit regression equations describing each of the dose-response relationships are presented in table 6. A graphic representation of the dose response data and associated linear regression lines are presented in figures 8,9,10 and 11.

The results of a student t-test indicated that none of the dose-response regression coefficients were significantly different ($p>0.05$).

Time to Response-Discrete Heavy Metal Solutions

Cummulative mortality (in probit units) was plotted relative to log time for fish exposed to copper, cadmium or mercury. Median mortality times (LT_{50}) were computed for each exposure concentration and these are presented in tables 7,8 and 9. The distribution of LT50 versus concentration of each toxicant is

Table 2. Lethal response data for zebrafish exposed to copper for 96 hours.

Mean Assayed Concentration of Copper (mg/L ± S.D.)	# of Fish Tested	Mean Wet Weight of Fish (g ± S.D.)	Observed % Mortality in 96 Hours.
0.05 ± 0.02	10	0.29 ± 0.03	0.0
0.09 ± 0.02	10	0.42 ± 0.04	0.0
0.13 ± 0.03	10	0.29 ± 0.03	10.0
0.15 ± 0.04	10	0.29 ± 0.03	20.0
0.17 ± 0.05	10	0.29 ± 0.03	30.0
0.22 ± 0.08	10	0.42 ± 0.04	30.0
0.24 ± 0.07	10	0.42 ± 0.04	40.0
0.27 ± 0.04	10	0.42 ± 0.04	50.0
0.31 ± 0.06	10	0.42 ± 0.04	60.0
0.35 ± 0.05	10	0.42 ± 0.04	80.0

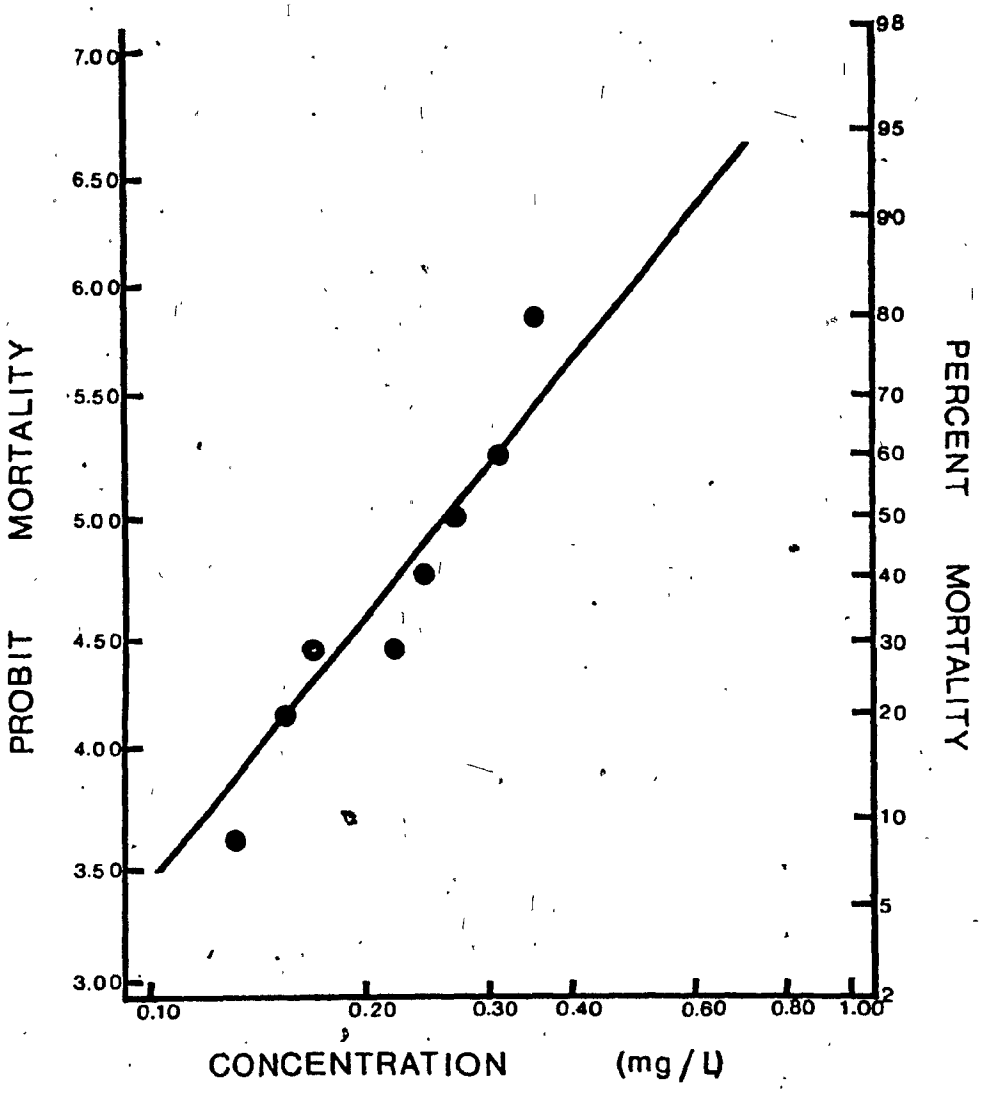

Figure 8. The lethal response curve for zebrafish exposed to copper for 96 hours.

Table 3. Lethal response data for zebrafish exposed to cadmium for 96 hours.

Mean Assayed Concentration of Cadmium (mg/L ± S.D.)	# of Fish Tested	Mean Wet Weight of Fish (g ± S.D.)	Observed % Mortality in 96 Hours.
3.59 ± 0.19	14	0.29 ± 0.03	7.1
4.03 ± 0.05	14	0.29 ± 0.03	7.1
4.56 ± 0.28	14	0.39 ± 0.04	7.1
5.05 ± 0.23	14	0.29 ± 0.03	21.4
5.41 ± 0.21	14	0.29 ± 0.03	42.9
5.83 ± 0.23	14	0.29 ± 0.03	50.0
6.32 ± 0.17	12	0.39 ± 0.04	100.0
7.60 ± 0.11	12	0.39 ± 0.03	91.7
9.79 ± 0.23	12	0.30 ± 0.03	100.0
13.19 ± 0.31	12	0.39 ± 0.04	100.0
17.22 ± 0.27	12	0.30 ± 0.03	100.0
19.19 ± 0.16	12	0.30 ± 0.03	100.0

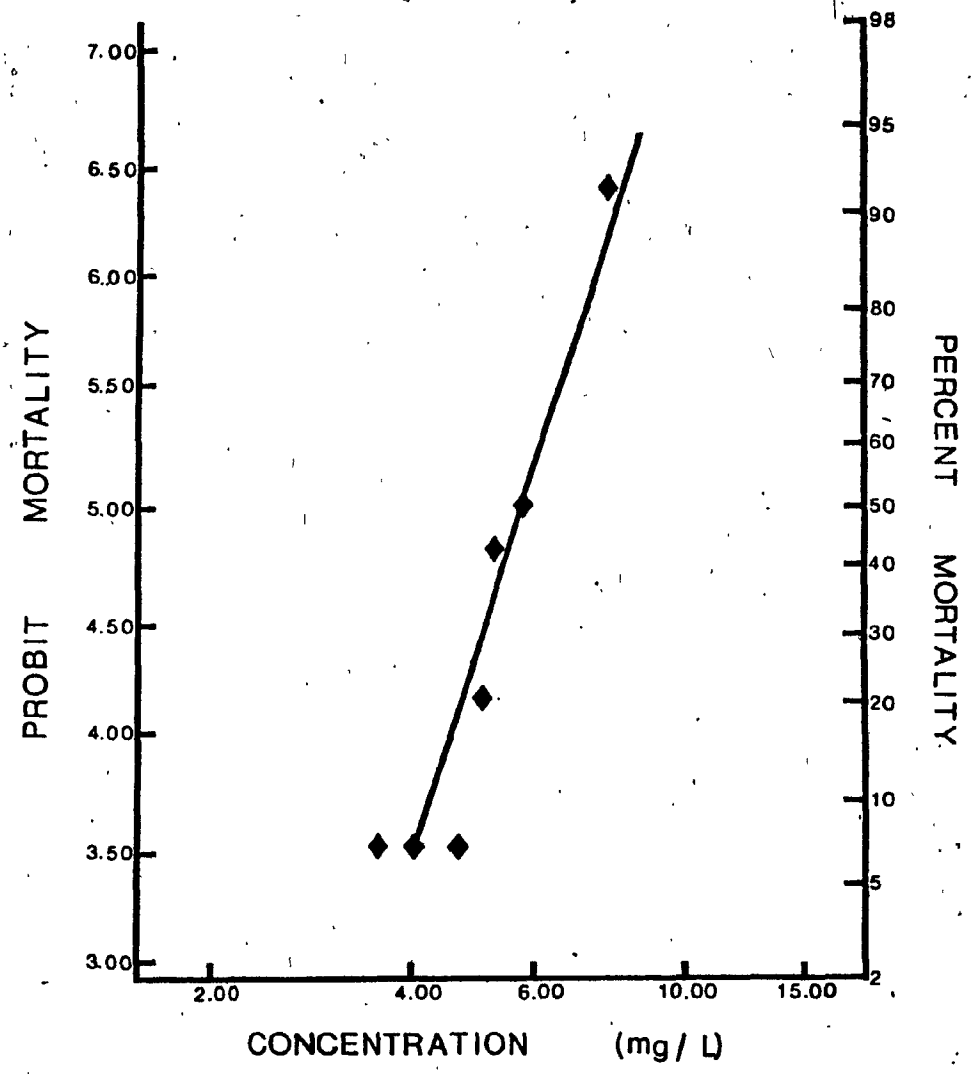

Figure 9. The lethal response curve for zebrafish exposed to cadmium for 96 hours.

Table 4. Lethal response data for zebrafish exposed to mercury for 96 hours.

Mean Assayed Concentration of Mercury (mg/L ± S.D.)	# of Fish Tested	Mean Wet Weight of Fish (g ± S.D.)	Observed % Mortality in 96 Hours.
0.017 ± 0.002	14	0.35 ± 0.03	0.0
0.085 ± 0.029	14	0.35 ± 0.03	0.0
0.138 ± 0.021	14	0.35 ± 0.03	0.0
0.193 ± 0.018	14	0.35 ± 0.03	7.1
0.303 ± 0.036	14	0.35 ± 0.03	7.1
0.317 ± 0.025	14	0.45 ± 0.03	42.9
0.383 ± 0.031	14	0.35 ± 0.03	50.0
0.447 ± 0.020	14	0.45 ± 0.03	100.0
0.512 ± 0.027	14	0.45 ± 0.03	100.0
0.543 ± 0.035	14	0.54 ± 0.03	100.0
0.585 ± 0.042	14	0.54 ± 0.03	100.0

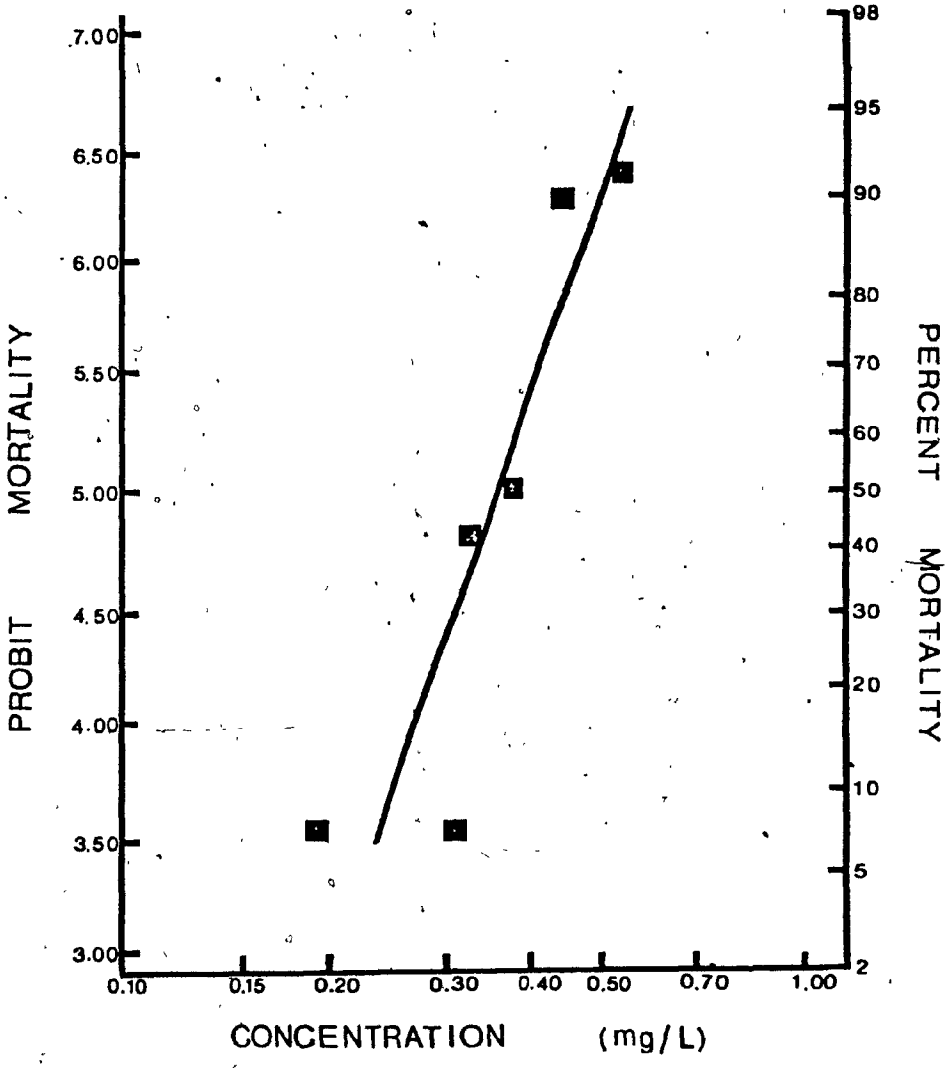

Figure 10. The lethal response curve for zebrafish exposed to mercury for 96 hours.

Table 5. Lethal response data for zebrafish exposed to mercury for 240 hours.

Mean Assayed Concentration of Mercury (mg/L ± S.D.)	# of Fish Tested	Mean Wet Weight of Fish (g ± S.D.)	Observed % Mortality in 240 Hrs.
0.018 ± 0.002	14	0.35 ± 0.03	0.0
0.088 ± 0.046	14	0.35 ± 0.03	0.0
0.152 ± 0.019	14	0.35 ± 0.03	57.1
0.200 ± 0.010	14	0.35 ± 0.03	71.4
0.303 ± 0.004	14	0.35 ± 0.03	92.9
0.317 ± 0.035	14	0.45 ± 0.03	100.0
0.392 ± 0.026	14	0.35 ± 0.03	100.0
0.447 ± 0.004	14	0.45 ± 0.03	100.0
0.503 ± 0.013	14	0.45 ± 0.03	100.0

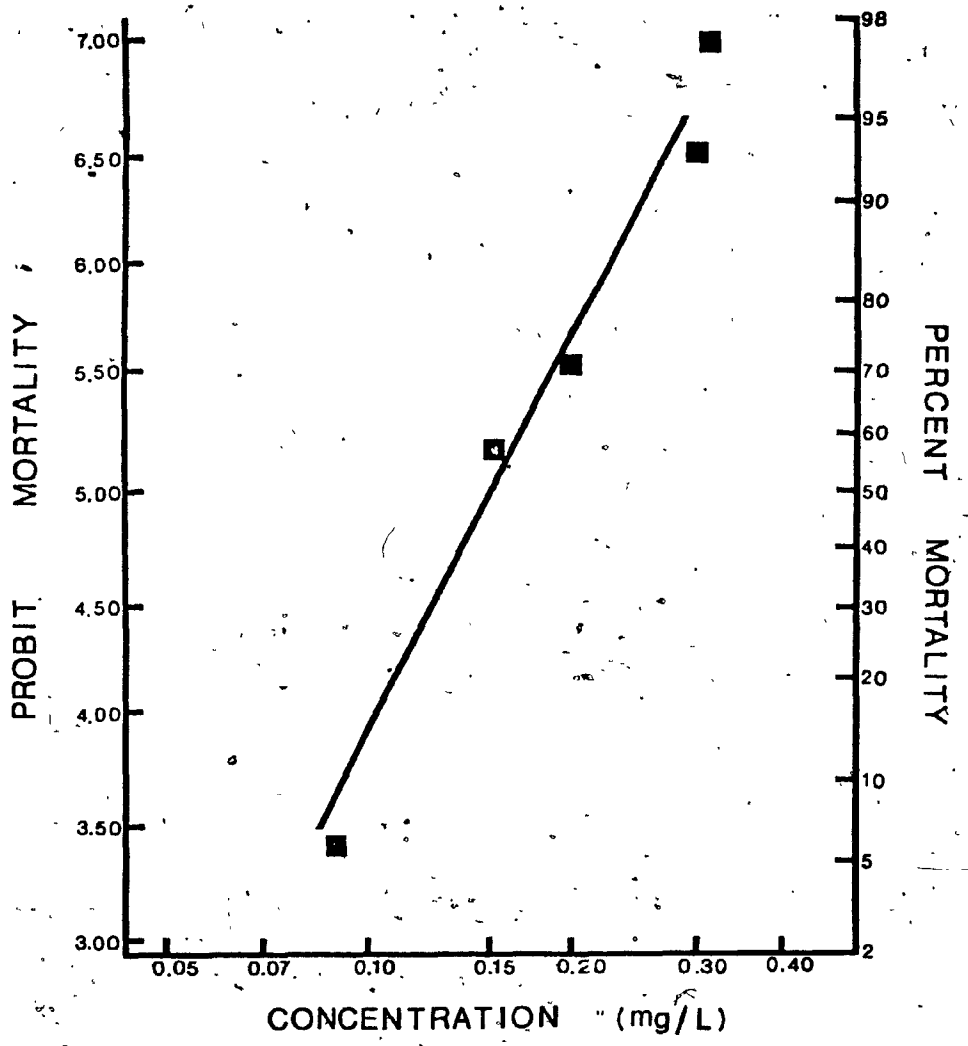

Figure 11. The lethal response of zebrafish exposed to mercury for 240 hours.

Table 6. Linear regressions fitted to concentration - response data for zebrafish exposed to copper, cadmium or mercury at 96 hours or 240 hours exposure.

Metal	Exposure Time (Hours)	Regression Equation	LC_{50} (mg/L)	95% Fiducial Limits (mg/L)
Copper	96 Hr	Y= 7.325 + 4.022(x)	0.26	0.22 - 0.35
Cadmium	96 Hr	Y= -1.989 + 9.137(x)	5.82	5.39 - 6.58
Mercury	96 Hr	Y= 8.835 + 8.123(x)	0.36	0.32 - 0.39
	240 Hr	Y= 9.700 + 5.773(x)	0.15	0.13 - 0.18

Where Y = % response in probit units

x = concentration of metal in log units

shown in table 10. The toxicity curves for Log LT_{50} versus concentration are shown in figures 12, 13 and 14. Linear regressions and their respective correlation coefficients were derived to represent Log LT_{50} versus Log concentration and are listed in table 11, figures 15, 16 and 17.

Lethal Response Studies - Mixtures of Heavy Metal Solutions

Cadmium and Copper: Test for Concentration Addition

The linear functions describing the response of zebrafish exposed to copper or cadmium were tested for parallelism. The regression coefficients for the two sets of data were not found to be significantly different. The similarity of the regression coefficients suggests that the metals, as constituents of mixtures, may be similarly acting and therefore the effect of their mixtures may be predicted by the empirical model of concentration addition (Anderson and Weber, 1975).

The concentration addition hypothesis was tested and the lethal response data for the mixture are presented in table 12 and figure 18. The results of chi-square tests indicates that the discrepancy between expected and observed responses are not attributable to random fluctuations about the predicted relation ($P > 0.05$). Therefore, the hypothesis that these metals are concentration additive is rejected.

Cadmium and Copper: Test for Response Addition

The concentration additive model does not adequately describe the interaction of cadmium and copper. Therefore, the dose-response data were tested to determine the suitability of modeling according to response addition theory. Table 13 lists the effective concentration of each mixture along with the observed responses and those predicted on the assumption that the metals are response

Table 7. Cumulative mortality - time data for zebrafish exposed to copper.

% Mortality	Copper (mg/L)	0.27	0.27	0.31	0.35	0.38	0.65	0.68
					Response Time (Minutes)			
7.1		-	1562	-	-	1075	-	-
10.0		1370	-	2005	1643	-	-	-
14.3		-	-	-	-	1560	1272	1244
20.0		1745	-	2200	1688	-	-	-
21.4		-	1772	-	-	-	1557	1615
28.6		-	2252	-	-	1770	-	-
30.0		1895	-	-	-	-	-	-
35.7		-	2852	-	-	2250	-	1765
40.0		-	-	2875	1868	-	-	-
42.9		-	3107	-	-	2370	1767	1885
50.0		3185	4562	-	2393	2745	-	-
57.1		-	-	3235	3248	-	2247	2245
60.0		-	-	-	-	-	-	-
64.2		-	-	-	-	-	2367	-
70.0		-	-	-	3548	-	-	-
71.4		-	-	-	-	-	2577	2575
78.6		-	-	-	-	-	2687	-
80.0		-	-	-	4058	-	-	-
85.7		-	-	-	-	-	3102	2805
90.0		-	-	-	-	-	-	-
92.9		-	-	-	-	-	-	-
100.0		-	-	-	-	-	3717	3100
Intercept		- 6.44	-4.34	-18.60	- 9.21	- 7.82	-15.23	-17.84
Slope		3.28	2.59	6.78	4.16	3.73	6.13	6.92
LT50		3977	4055	3029	2600	2724	2007	1990

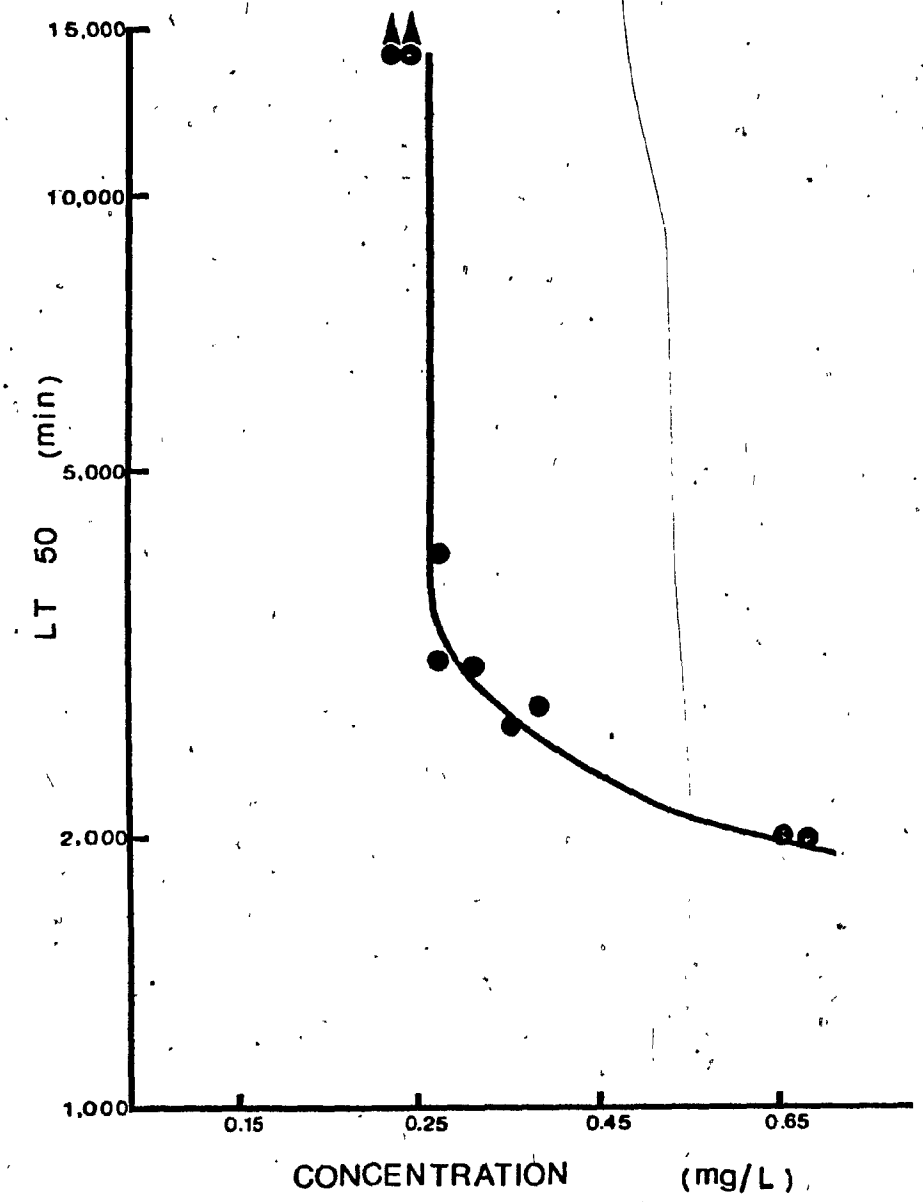

Figure 12. The median mortality-time curve (LT_{50}) for zebrafish exposed to copper (Log-Normal scale).

Table 8. Cumulative mortality - time data for zebrafish exposed to cadmium.

% Mortality	Cadmium (mg/L)	5.83	7.60	9.79	13.19	17.22	19.19
				Response Time (Minutes)			
7.1		2390	-	-	-	-	-
8.3		-	2561	2529	1792	1340	-
14.3		2530	-	-	-	-	1548
16.7		-	2651	2634	1972	-	-
21.4		3395	-	-	-	-	-
25.0		-	2786	2649	2032	1550	-
28.6		-	-	-	-	-	-
33.3		-	3626	2694	2257	1790	1602
35.7		3875	-	-	-	-	-
41.7		-	-	2784	2417	-	-
42.9		4660	-	-	-	-	
50.0		5240	-	-	2437	1970	-
58.3		-	5006	-	2632	2100	1962
66.7		-	5276	2874	2647	2235	2052
75.0		-	5456	2949	2662	2270	2092
83.3		-	-	3114	2782	2330	2262
91.7		-	5756	3264	3022	2405	2322
100.0		-	-	3714	3337	2495	2502
Intercept		-9.46	-15.96	-84.35	-36.58	-28.76	-34.23
Slope		3.90	5.81	25.90	12.30	10.29	12.03
LT_{50}		5146	4063	2822	2401	1905	1830

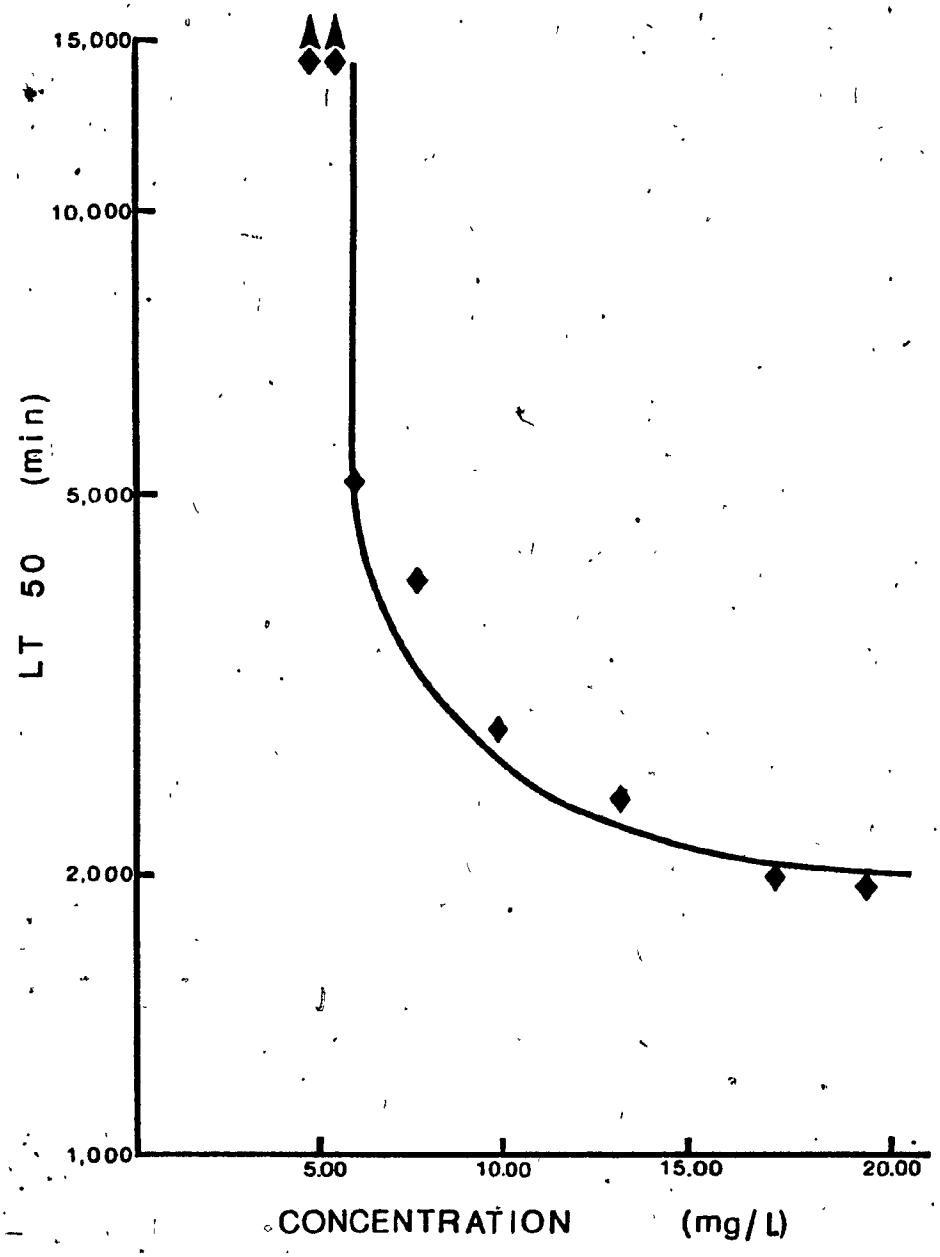

Figure 13. The median mortality-time curve (LT_{50}) for zebrafish exposed to cadmium (Log-Normal scale)

Table 9. Cumulative mortality - time data for zebrafish exposed to mercury.

%Mortality \ Mercury (mg/L)	0.15	0.20	0.30	0.32	0.39	0.45	0.50	0.53	0.58
					Response Time (Minutes)				
7.1	9249	3802	5735	4908	1576	934	807	-	668
14.3	-	6812	-	5478	-	1229	882	670	-
21.4	10194	7552	6605	5568	-	967	805	-	
28.6	-	-	-	5628	-	1574	-	-	-
35.7	-	-	7010	-	2536	-	1227	880	878
42.9	10764	7822	7205	4906	-	-	1045	963	
50.0	11964	-	7420	5778	5206	1784	-	-	1043
57.1	12234	8242	7460	5898	-	2024	1392	1225	1133
64.3	12729	8452	8150	6153	-	2144	-	1780	-
71.4	-	10762	8240	6603	6151	3914	1782	2220	1223
78.6	-	-	8445	7818	6806	4319	-	2530	1388
85.7	-	-	11960	-	7321	4424	2022	2845	1568
92.9	-	-	12170	8328	8236	-	-	-	-
100.0	-	-	-	8918	8326	5354	3022	3700	1778
Intercept	-43.65	-16.49	-26.47	-41.23	-6.90	-6.66	-14.19	-5.12	-16.52
Slope	11.95	5.47	8.11	12.21	3.32	3.51	6.13	3.26	7.14
LT_{50}	11773	8527	7573	6104	3863	3258	1353	1274	1036

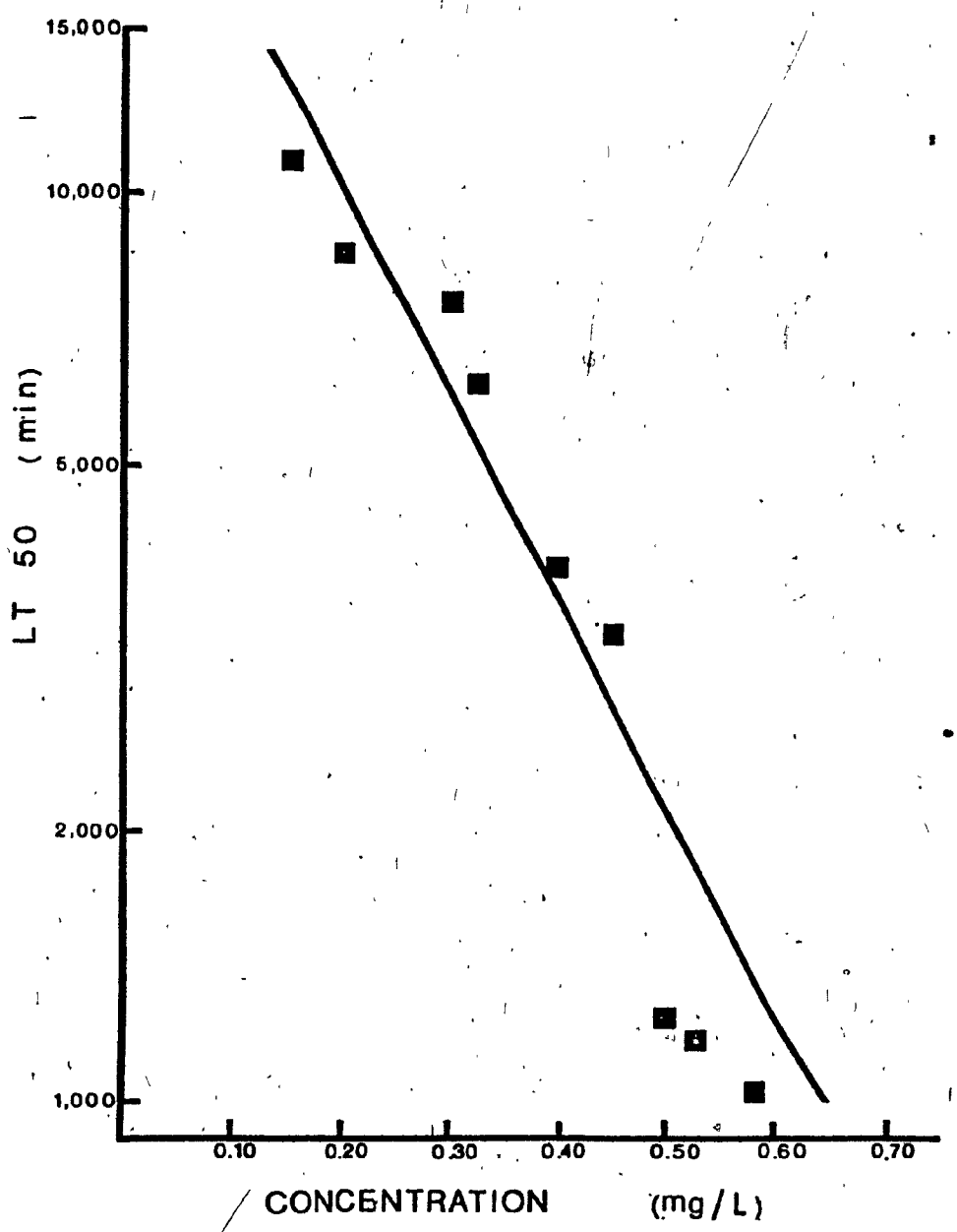

Figure 14. The median mortality-time curve (LT_{50}) for zebrafish exposed to mercury (Log-Normal scale).

Table 10. Median mortality times (LT_{50}) of zebrafish exposed to copper, cadmium or mercury.

Metal	Concentration (mg/L)	LT_{50} (Minutes)
Copper	0.27	3077
	0.27	4055
	0.31	3029
	0.35	2600
	0.38	2724
	0.65	2007
	0.68	1990
Cadmium	5.83	5146
	7.60	4063
	9.79	2822
	13.19	2401
	17.22	1905
	19.19	1830
Mercury	0.15	11773
	0.20	8527
	0.30	7573
	0.32	6104
	0.39	3863
	0.45	3258
	0.50	1353
	0.53	1274
	0.58	1036

Table 11. Linear regression representing the relationship between log LT_{50} and log concentration for zebrafish exposed to copper, cadmium, or mercury.

Metal		Equation of Regression	Correlation Coefficient
Copper	A	Y = 3.19 - 0.60 (x)	- 0.925*
Cadmium	B	Y = 4.37 - 0.88 (x)	- 0.990*
Mercury	C	Y = 2.73 - 1.82 (x)	- 0.921*

Where Y = Log LT_{50}

x = Log concentration of metal.

* Corr. Coef. significant at $P<0.01$

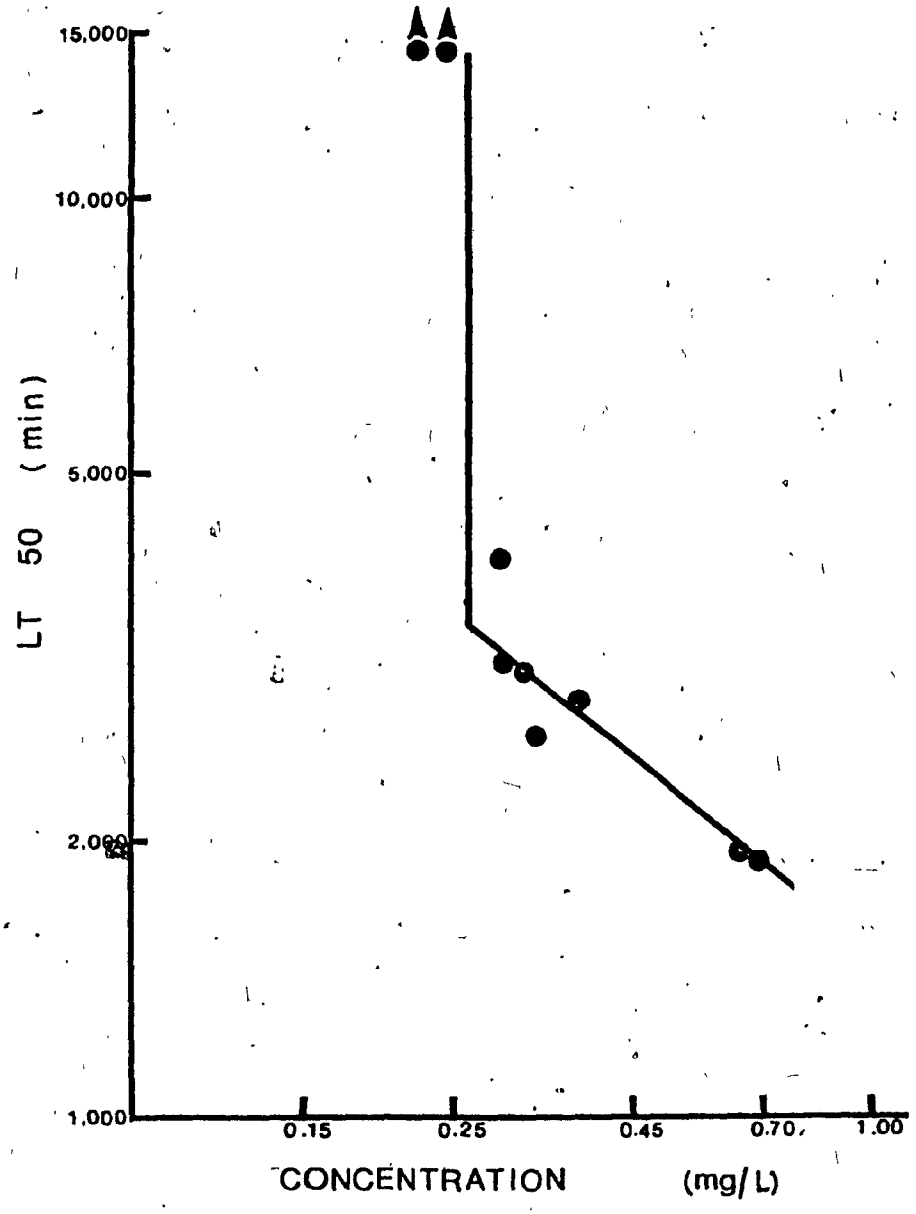

Figure 15. Median mortality-time curve (LT_{50}) for zebrafish exposed to copper (Log-Log scale).

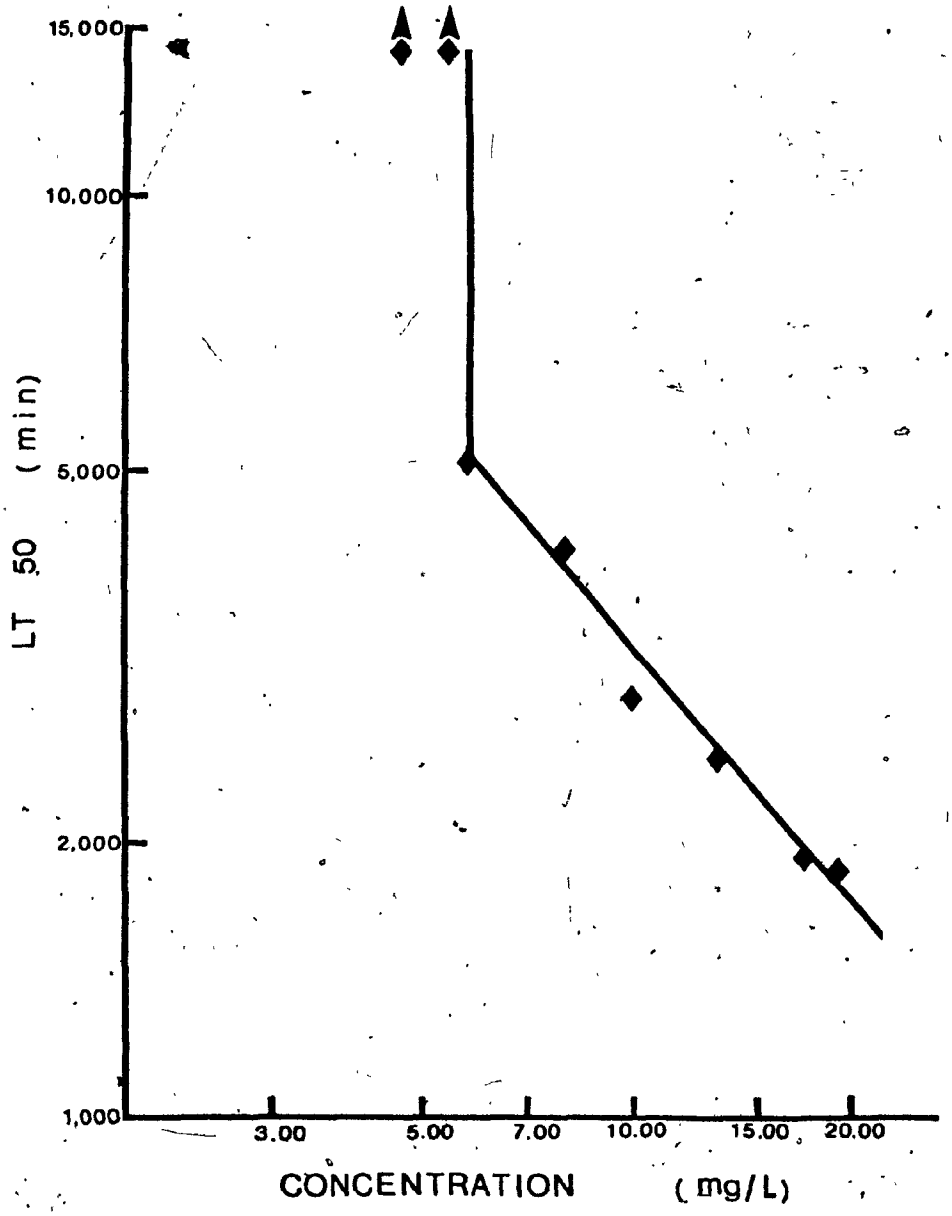

Figure 16. Median mortality-time curve (LT_{50}) for zebrafish exposed to cadmium (Log-Log scale).

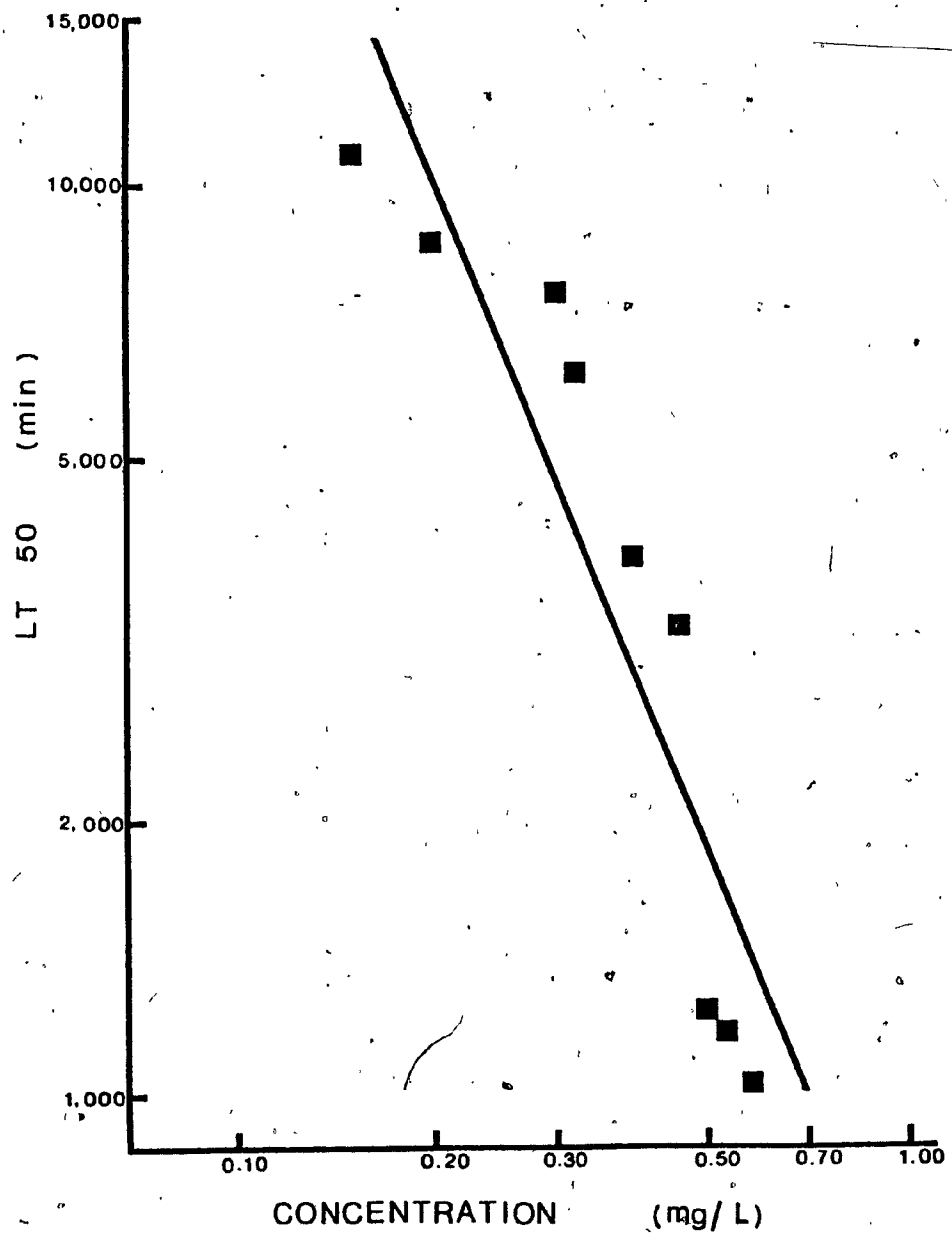

Figure 17. Median mortality-time curve (LT_{50}) for zebrafish exposed to mercury (Log-Log scale).

Table 12. Lethal response data for zebrafish exposed to mixtures of cadmium and copper for 96 hours : Concentration Addition Model.

Mean Assayed Concentration of Copper. (mg/L ± S.D.)	Mean Assayed Concentration of Cadmium (mg/L ± S.D.)	Copper Concentration expressed as equiv.units of Cadmium (mg/L)	Concentration of mixture as Cadmium (mg/L)	Observed Mortality 96 Hr. %	Observed Mortality 96 Hr. Probit	Predicted Mortality %	Predicted Mortality Probit
0.007 ± 0.001	0.803 ± 0.153	1.178	1.981	0.00	3.54*	0.1	0.72
0.015 ± 0.005	1.150 ± 0.208	1.647	2.797	42.86	4.82	0.2	2.09
0.016 ± 0.006	1.310 ± 0.253	1.694	3.004	92.86	6.47	0.5	2.38
0.029 ± 0.009	1.395 ± 0.007	2.201	3.596	92.86	6.47	2.8	3.09
0.033 ± 0.008	1.437 ± 0.290	2.333	3.770	92.86	6.47	4.3	3.28
0.050 ± 0.019	1.685 ± 0.064	2.798	4.483	78.57	5.79	15.0	3.96
0.065 ± 0.033	1.623 ± 0.326	3.140	4.763	71.43	5.57	21.4	4.21
0.058 ± 0.016	1.955 ± 0.064	2.987	4.942	92.86	6.47	25.8	4.35
0.097 ± 0.100	1.395 ± 0.018	3.747	5.142	100.00	7.05*	31.2	4.51
0.061 ± 0.013	2.135 ± 0.035	3.058	5.193	100.00	7.05*	32.6	4.55

Equation of Probit Regression: $Y = 2.512 + 5.791 (\log x)$ LC_{50} = 2.689 mg/L
95 % Fiducial limits 2.24 to 3.01 mg/L

* Mortality data fitted according to Litchfield & Wilcoxin (1949)

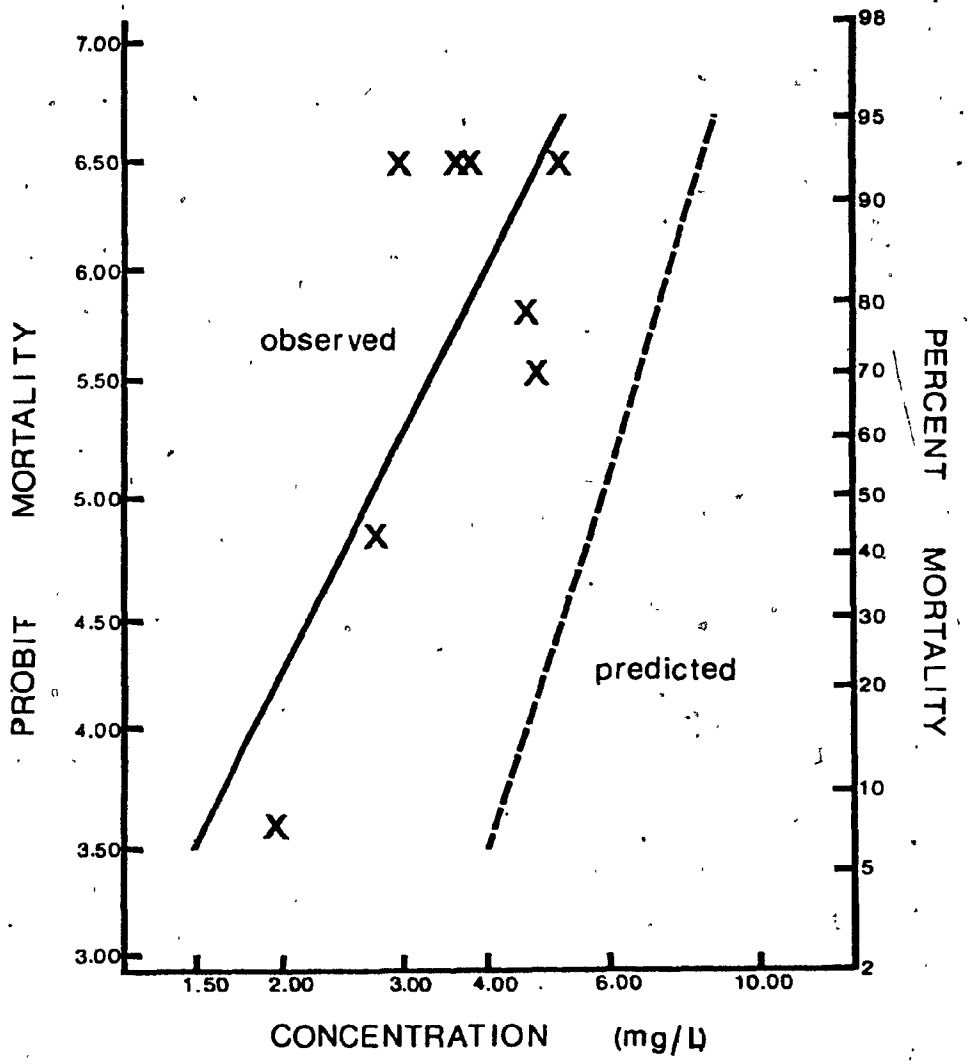

Figure 18. The lethal response curve for zebrafish exposed to mixtures of cadmium and copper (expressed as equivalent units of cadmium) for 96 hours (X , ———). The lethal response curve predicted on the basis of concentration addition (- - - - -) is depicted for reference.

Table 13. Lethal response data for zebrafish exposed to mixtures of cadmium and copper for 96 hours: Response Addition Model.

Mean assayed Concentration of Copper (mg/L ± S.D.)	Mean assayed Concentration of Cadmium (mg/L ± S.D.)	Observed % Mortality 96 Hr.	Predicted % Mortality if r = -1	r = 0	r = +1
0.007 ± 0.001	0.803 ± 0.153	0.00	<1	<1	<1
0.015 ± 0.005	1.150 ± 0.208	42.86	<1	<1	<1
0.016 ± 0.006	1.310 ± 0.253	92.86	<1	<1	<1
0.029 ± 0.009	1.395 ± 0.007	92.86	<1	<1	<1
0.033 ± 0.008	1.437 ± 0.290	92.86	<1	<1	<1
0.050 ± 0.019	1.685 ± 0.064	78.57	<1	<1	<1
0.065 ± 0.033	1.623 ± 0.326	71.43	<1	<1	<1
0.058 ± 0.016	1.955 ± 0.064	92.86	<1	<1	<1
0.097 ± 0.100	1.395 ± 0.018	100.00	<4	<4	<4
0.061 ± 0.013	2.135 ± 0.034	100.00	<1	<1	<1

additive. The predicted responses are clearly not consistent with the observed responses ($P > 0.05$). Therefore, the hypothesis that these metals are response additive is rejected.

Cadmium and Copper: Supra-Additive Synergism

The lethal response data for zebrafish exposed to mixtures of cadmium and copper indicates the toxicity is greater than predicted assuming additivity. Responses which are significantly greater than predicted are referred to as supra-additive. The magnitude of the increased response, relative to that predicted for concentration addition, can be appreciated through the calculation of a relative potency factor (Anderson and Weber, 1975). In this case it was determined that copper and cadmium, when in combination, are 2.011 ± 0.488 times more potent than predicted. The computation of the relative potency factor is found in table 14.

Cadmium and Mercury: Test for Concentration Addition

The tolerance distribution describing the response of zebrafish exposed to cadmium or mercury were tested for parallelism. The regression coefficients for the two sets of data were not found to be significantly different for either 96 or 240 hours exposure. The similarity of the regression coefficients suggests that these metals, when in combination, will be concentration additive throughout the 240 hour exposure period.

The concentration addition hypothesis was tested for 96 hours exposure and the lethal response data are presented in table 15 and figure 19. The results of chi square tests indicated that there were no significant differences between observed and predicted responses. Therefore, the concentration addition model adequately

Table 14. Computation of the relative potency factor, for copper and cadmium mixture versus discrete toxicants.

Observed % Mortality (probits)	A	B	A/B
4.82	5.562	2.797	1.989
6.47	8.429	3.004	2.806
6.47	8.429	3.596	2.344
6.47	8.429	3.770	2.236
5.79	7.101	4.483	1.584
5.57	6.719	4.763	1.411
6.47	8.429	4.942	1.706

$x = 2.011 \pm 0.488$

A = the antilog of the abscissal value determined from the lethal dose response regression line for Cadmium corresponding to the observed mortality.

B = the total concentration of the mixture in terms of cadmium.

Table 15. Lethal response data for zebrafish exposed to mixtures of Cadmium and mercury for 96 hours: Concentration Addition Model.

Mean Assayed Concentration of Mercury. (mg/L ± S.D.)	Mean Assayed Concentration of Cadmium (mg/L ± S.D.)	Mercury Concentration expressed as equiv.units of Cadmium (mg/L)	Concentration of mixture as Cadmium (mg/L)	Observed Mortality 96 Hr. %	Observed Mortality 96 Hr. Probit	Predicted Mortality %	Predicted Mortality Probit
0.128±0.01	1.711±0.02	2.322	4.033	7.14	3.53	7.4	3.55
0.193±0.01	2.085±0.01	3.362	5.447	28.57	4.43	39.8	4.74
0.214±0.02	2.085±0.03	3.687	5.772	42.86	4.82	48.7	4.97
0.226±0.01	2.485±0.05	3.869	6.354	64.29	5.37	63.7	5.35
0.296±0.01	2.714±0.03	4.931	7.645	92.86	6.47	86.0	6.08

Equation of Probit Regression: $Y = -3.543 + 11.127 (\log x)$

$LC_{50} = 5.86$ mg/L

95% Fiducial limits = 5.41 to 6.33 mg/L.

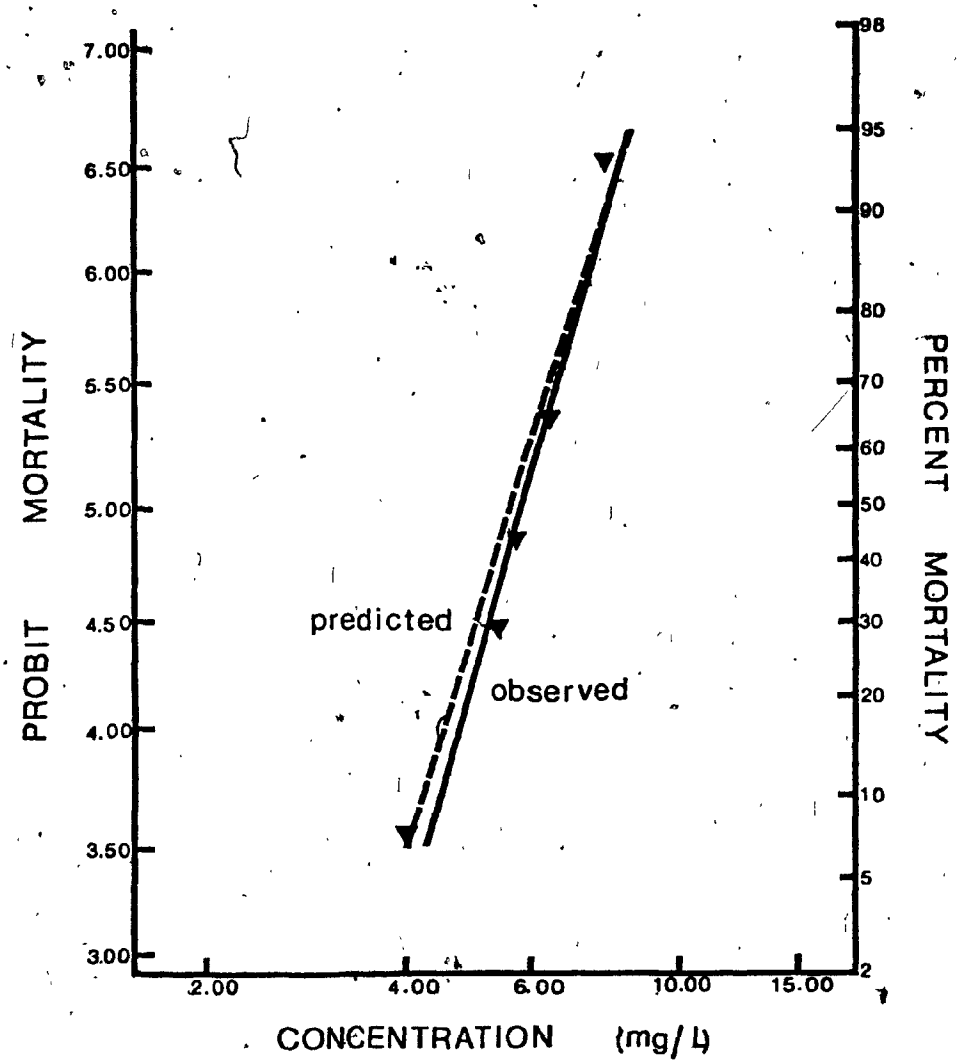

Figure 19. The lethal response curve for zebrafish exposed to mixtures of cadmium and mercury (expressed as equivalent units of cadmium) for 96 hours (▼ , ———). The lethal response curve predicted on the basis of concentration addition (- - - -) is depicted for reference.

describes the dose response of zebrafish exposed to mixtures of cadmium and mercury at 96 hours. The graphical relationship of the observed and predicted regression line is presented in figure 19.

Lethal response data for the mixture at 240 hours are presented in table 16 and figure 20. The hypothesis that these data are representative of a concentration addition model was tested and the hypothesis rejected. ($P>0.05$).

Cadmium and Mercury 240 hours: Test for Response Addition

The concentration addition model does not adequately describe the interaction of cadmium and mercury at 240 hours exposure. Therefore the dose response data was tested to determine the suitability of modeling according to response addition theory. Table 17 lists the effective concentration of each mixture along with the observed and predicted responses presuming the metals to be response additive. The predicted responses are clearly not consistent with the observed responses ($P>0.05$). Therefore, the hypothesis that response addition will predict the interaction between cadmium and mercury, at 240 hours exposure, is rejected.

Cadmium and Mercury, 240 Hours: Supra-Additive Synergism

The response of zebrafish exposed to mixtures of cadmium and mercury is supra-additive at 240 hours. A relative potency factor can be computed comparing the toxicity of the mixture to that predicted for concentration addition (Anderson and Weber, 1975). The potency of the mixture of cadmium and mercury, relative to that predicted, was found to be 2.030 ± 0.227 times greater. The computation of the relative potency factor is found in table 18.

Table 16. Lethal response data for zebrafish exposed to mixture of cadmium and mercury for 240 hours: Concentration Addition Model

Mean Assayed Concentration of Mercury. (mg/L ± S.D.)	Mean Assayed Concentration of Cadmium (mg/L ± S.D.)	Mercury Concentration expressed as equiv.units of Cadmium (mg/L)	Concentration of mixture as Cadmium (mg/L)	Observed Mortality 96 Hr. %	Observed Mortality 96 Hr. Probit	Predicted Mortality %	Predicted Mortality Probit
0.0177 ± 0.0027	1.11 ± 0.03	1.487	2.597	35.71	4.63	0.1	1.80
0.0261 ± 0.0104	1.28 ± 0.03	1.899	3.179	50.00	5.00	0.8	2.60
0.0268 ± 0.0030	1.36 ± 0.03	1.929	3.289	85.71	6.07	1.2	2.74
0.0293 ± 0.0031	1.45 ± 0.05	2.045	3.495	85.71	6.07	2.2	2.98
0.0318 ± 0.0014	1.60 ± 0.01	2.151	3.751	64.29	5.37	4.1	3.26
0.0341 ± 0.0042	1.75 ± 0.04	2.248	3.998	92.86	6.47	6.8	3.51
0.0492 ± 0.0100	1.85 ± 0.02	2.832	4.682	100.00	-	19.4	4.14

Equation of Probit Regression: $Y = 1.089 + 8.497 (\log x)$

$LC_{50} = 2.89$ mg/L

95% Fiducial limits = 2.27 to 3.14 mg/L

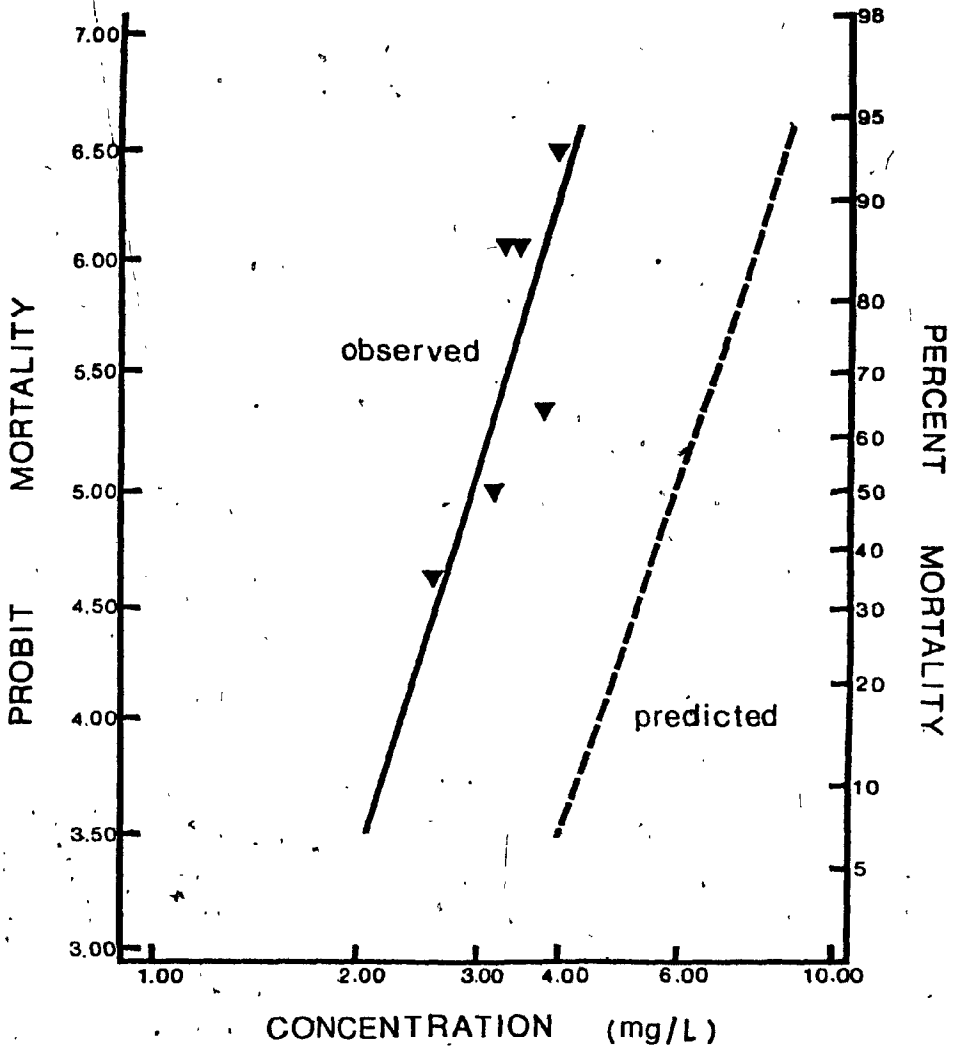

Figure 20. The lethal response curve for zebrafish exposed to mixtures of cadmium and mercury (expressed as equivalent units of cadmium) for 240 hours (▼ , ——). The lethal response curve predicted on the basis of concentration addition (— — —) is depicted for reference.

Table 17. Lethal response data for zebrafish exposed to mixtures of cadmium and mercury for 240 hours: Response Addition Model.

Mean assayed Concentration of Mercury (mg/L ± S.D.)	Mean assayed Concentration of Cadmium (mg/L ± S.D.)	Observed % Mortality	Predicted % Mortality if r = -1	r = 0	r = +1
0.0177 ± 0.0027	1.11 ± 0.03	35.71	<1	<1	<1
0.0261 ± 0.0104	1.28 ± 0.03	50.00	<1	<1	<1
0.0268 ± 0.0030	1.36 ± 0.03	85.71	<1	<1	<1
0.0293 ± 0.0031	1.45 ± 0.05	85.71	<1	<1	<1
0.0318 ± 0.0014	1.60 ± 0.01	64.29	<1	<1	<1
0.0341 ± 0.0042	1.75 ± 0.04	92.86	<1	<1	<1
0.0492 ± 0.0100	1.85 ± 0.02	100.00	<1	<1	<1

Table 18. Computation of the relative potency factor, for cadmium and mercury mixture versus discrete toxicants.

Observed % Mortality (Probits)	A	B	A/B
4.63	5.302	2.597	2.042
5.00	5.820	3.179	1.831
6.07	7.621	3.289	2.317
6.07	7.621	3.495	2.181
5.37	6.389	3.751	1.703
6.47	8.429	3.998	2.108
			$\bar{x} = 2.030 \pm 0.227$

A = the antilog of the abscissal value determined from the lethal dose response regression line for cadmium corresponding to the observed mortality.

B = the total concentration of the mixture in terms of cadmium.

Time to Response - Mixtures of Heavy Metal Solutions

Cadmium and Copper: The relationship between the dose-response curves for cadmium and copper in pure solutions was found to be constant through time. Therefore, the concentration of metal in each mixture was calculated from the 96 hour dose-response equations. Cumulative mortality - time data were recorded for each mixture and are presented in table 19. Table 19 also lists the median mortality times (LT_{50}) which were computed as previously described. The distribution of LT_{50} versus the concentration of each mixture is shown in table 20. The toxicity curve for Log LT_{50} versus the concentration of the mixture is presented in figure 21. The relationship of log LT_{50} versus Log concentration is shown in figure 22.

Cadmium and Mercury: The relationship betwen the dose response curves determined for cadmium and mercury in discrete solutions was not constant with respect to time. Therefore, calculation of the concentration for each mixture was based upon the dose response data, of the discrete metal solutions, corresponding to the median mortality time, i.e. if the LT_{50} for an exposure regimen was determined to be 8000 minutes then the concentration of the mixture was computed using the dose-response data of the discrete metal solutions at 8000 minutes. Tables 21 and 22 list the LT_{50} and computed concentration for each exposure regimen. The toxicity curve for Log LT_{50} versus the concentration of the mixture is presented in figure 23. The rectilinear relationship of log LT_{50} versus log concentration is shown in figure 24.

Table 19. Cumulative mortality - time data for zebrafish exposed to mixtures of cadmium and copper.

% Mortality	Cu+Cd as Cd (mg/L)	3.004	3.596	3.770	4.483	4.763	4.942	5.142	5.193
		Response Time (Minutes)							
7.1		1535	-	1591	935	1229	858	1593	796
14.3		-	1223	1741	-	1349	-	-	891
21.4		1745	1343	2221	1215	-	038	1743	-
28.6		-	-	-	-	-	-	-	1061
35.7		-	1583	2411	1515	1529	1063	1923	-
42.9		2105	1733	-	1575	1589	1218	-	1216
50.0		-	2093	2531	-	-	-	2103	1336
57.1		2225	-	-	1725	1739	-	-	-
64.3		2415	2303	2826	2085	2219	-	2223	-
71.4		-	2523	2891	2175	2529	-	2533	-
78.6		2695	2883	-	2395	-	-	2693	-
85.7		-	-	3116	-	-	1518	2828	-
92.9		3120	3108	-	-	-	-	-	1516
100.0		-	-	3341	-	-	1733	2893	1576
Intercept		-25.24	-12.23	-25.03	-12.51	-14.03	-26.38	-28.31	-26.20
Slope		9.06	5.24	8.84	5.41	5.82	10.22	10.01	10.14
LT_{50}		2176	1930	2501	1711	1856	1177	2122	1193

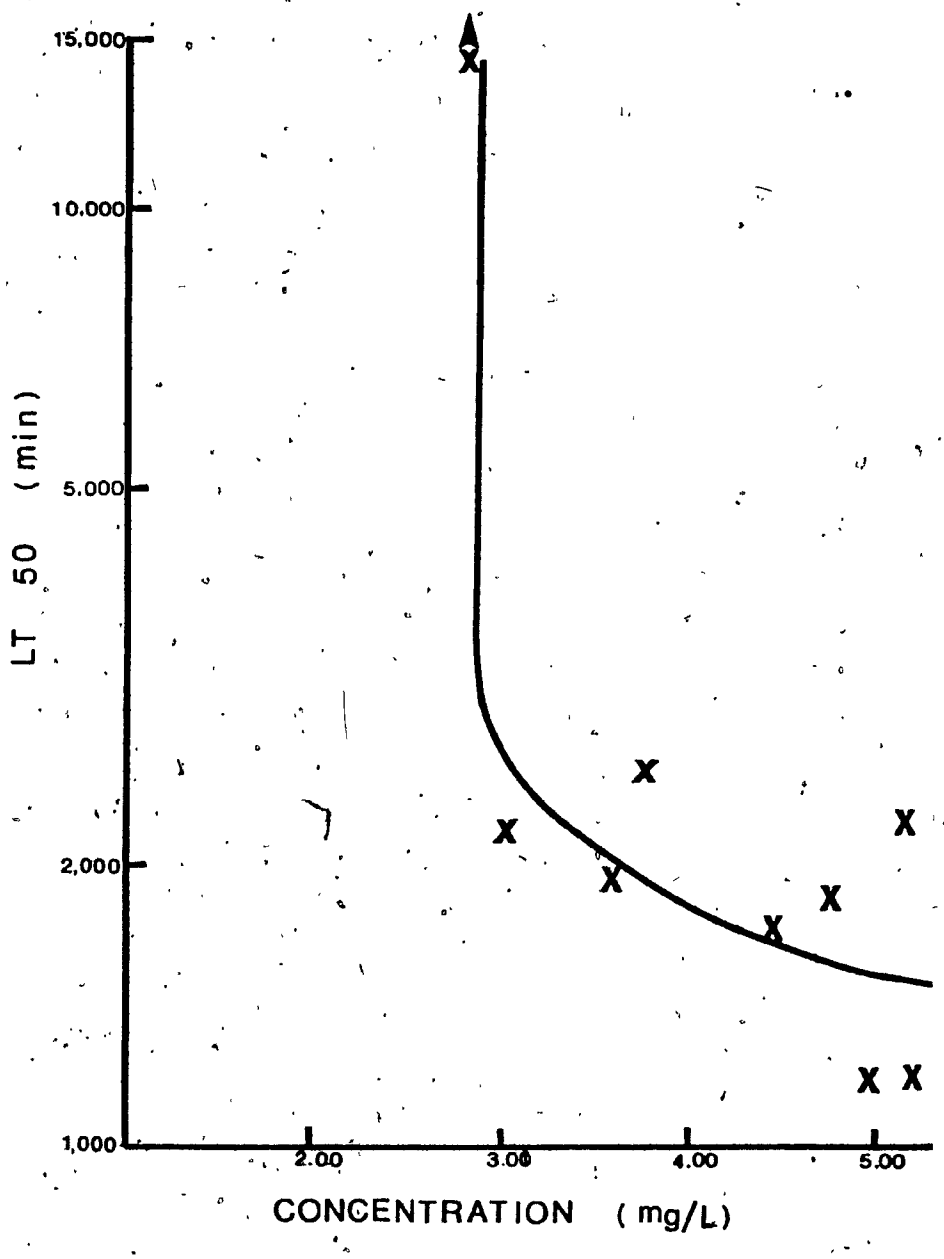

Figure 21. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and copper, expressed as equivalent units of cadmium (Log-Normal scale).

Table 20. Median mortality - times (LT_{50}) of zebrafish exposed to cadmium and copper mixtures.

Metal	Concentration of Mixture expressed as equivalent units of Cadmium. (mg/L)	LT_{50} (Minutes).
Cadmium & Copper	3.004	2176
	3.596	1930
	3.770	2501
	4.483	1711
	4.763	1856
	4.942	1177
	5.142	2122
	5.193	1193

Equation of Regression

$Y = 3.78-0.83(x)$ CORR: -.602

where Y = Log LT_{50}

x = Log Concentration

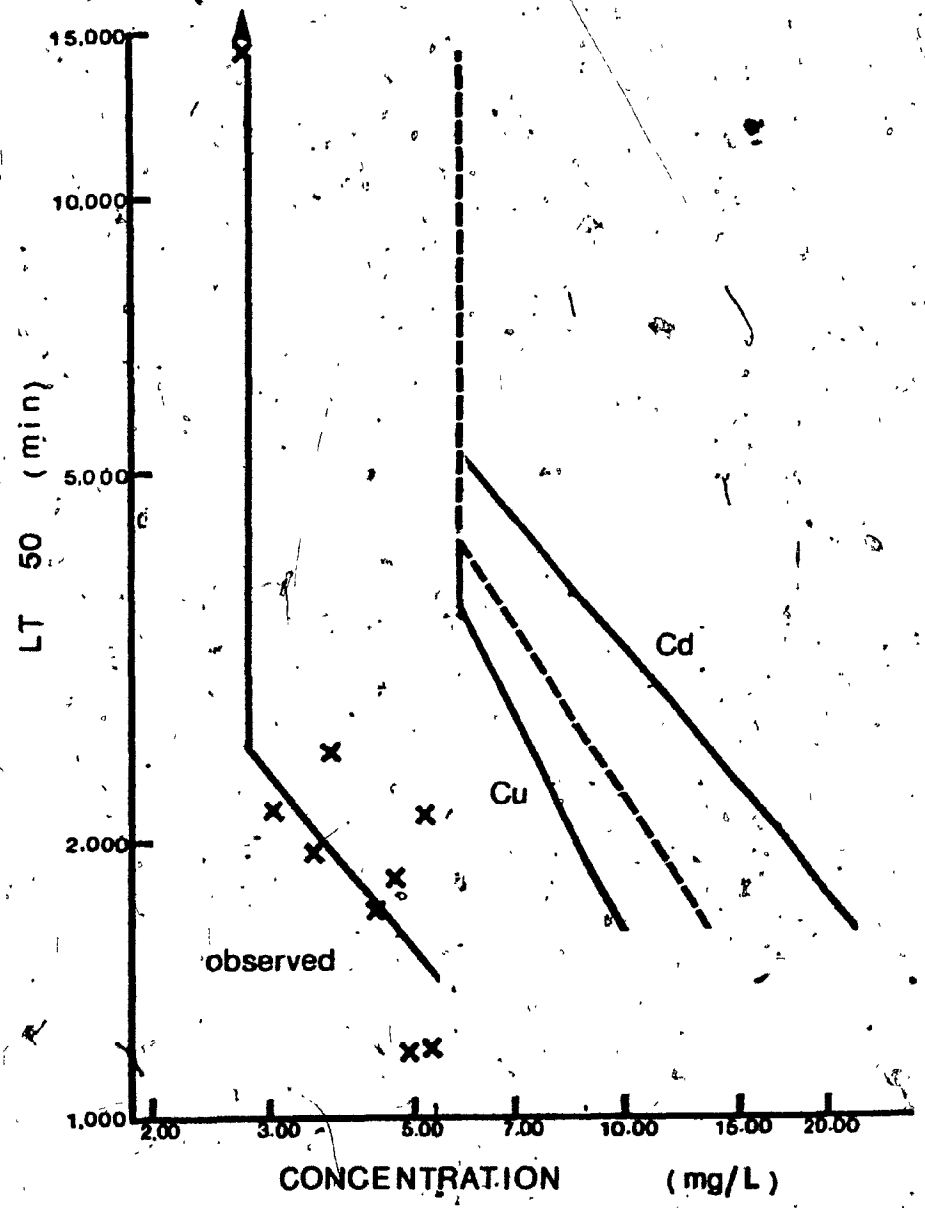

Figure 22. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and copper, expressed as equivalent units of cadmium (Log-Log scale). The median mortality-time curve predicted on the basis of concentration addition (———) is depicted for reference.

Table 21. Cumulative mortality-time data for zebrafish exposed to mixtures of cadmium and mercury.

% Mortality	Hg&Cd as Hg (mg/L) 0.059	0.062	0.069	0.077	0.085	0.125	0.235	0.306	0.431	0.452	0.626
	Response time (Minutes)										
7.1	7484	-	7630	8508	8106	6994	4145	-	3022	2520	2160
14.3	9029	6552	8510	9563	-	7624	5786	3896	-	-	2235
21.4	10034	-	9910	-	8506	-	-	-	3890	-	2295
28.4	11619	9027	10960	10958	9906	-	6483	4016	4063	3235	2525
35.7	13844	9892	11235	11233	10026	8104	6543	-	4152	-	-
42.9	14049	10512	11425	11613	10506	9019	6627	6487	5425	-	3030
50.0	14384	-	-	-	11231	9559	7822	6545	-	5400	3658
57.1	-	10962	-	12538	11611	9904	-	7241	-	-	-
64.3	-	12542	-	13838	11966	10024	-	7825	5876	5700	3836
71.4	-	13842	12540	-	12536	10504	9090	-	6550	5876	3896
78.6	-	14037	12720	-	-	10954	9459	8400	-	-	4723
85.7	-	14382	12950	-	13836	11229	9600	-	7830	6491	-
92.9	-	-	-	-	14041	11609	-	-	8013	6635	5430
100.0	-	-	-	-	-	11964	10020	9465	8502	7246	6503
Intercept	-14.29	-19.77	-38.83	-32.78	-36.35	-44.19	-26.25	-14.61	-19.31	-16.86	-17.14
Slope	4.61	6.15	10.81	9.24	10.24	12.40	8.09	5.18	6.56	5.99	6.29
Lt_{50}	15356	10717	11374	12340	10935	9240	7301	6127	5106	4470	3301

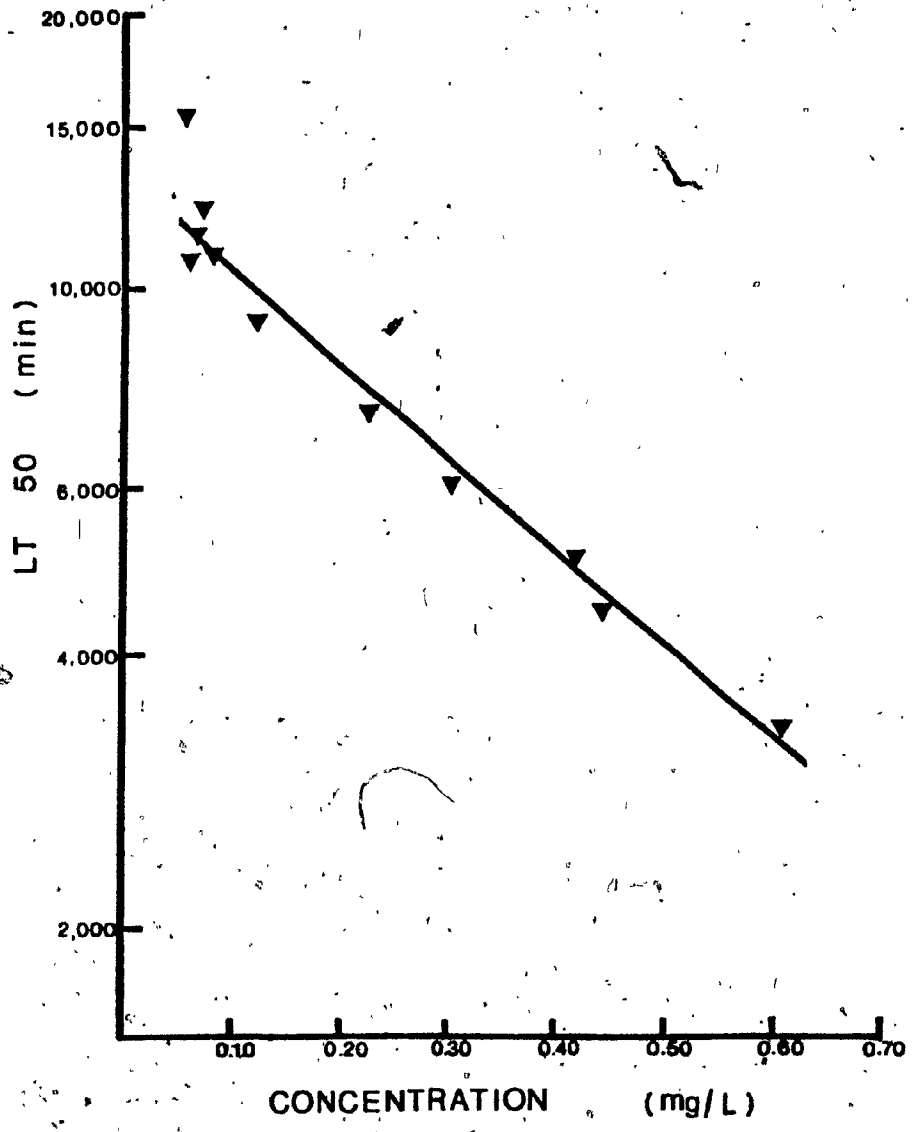

Figure 23. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and mercury, expressed as equivalent units of cadmium (Log-Normal scale).

Table 22. Median mortality - times (LT_{50}) of zebrafish exposed to cadmium and mercury mixtures.

Metal	Concentration of Mixture expressed as equivalent units of Mercury. (mg/L)	LT_{50} (Minutes).
Mercury & Cadmium	0.059	15356
	0.062	10717
	0.069	11374
	0.077	12340
	0.085	10935
	0.125	9240
	0.235	7301
	0.306	6127
	0.431	5106
	0.452	4470
	0.626	3301

Equation of Regression

$Y = 3.48 - 0.52(x)$ CORR: -.973*

where $Y = \text{Log } LT_{50}$

$x = \text{Log concentration}$

* Significant at $P<0.01$

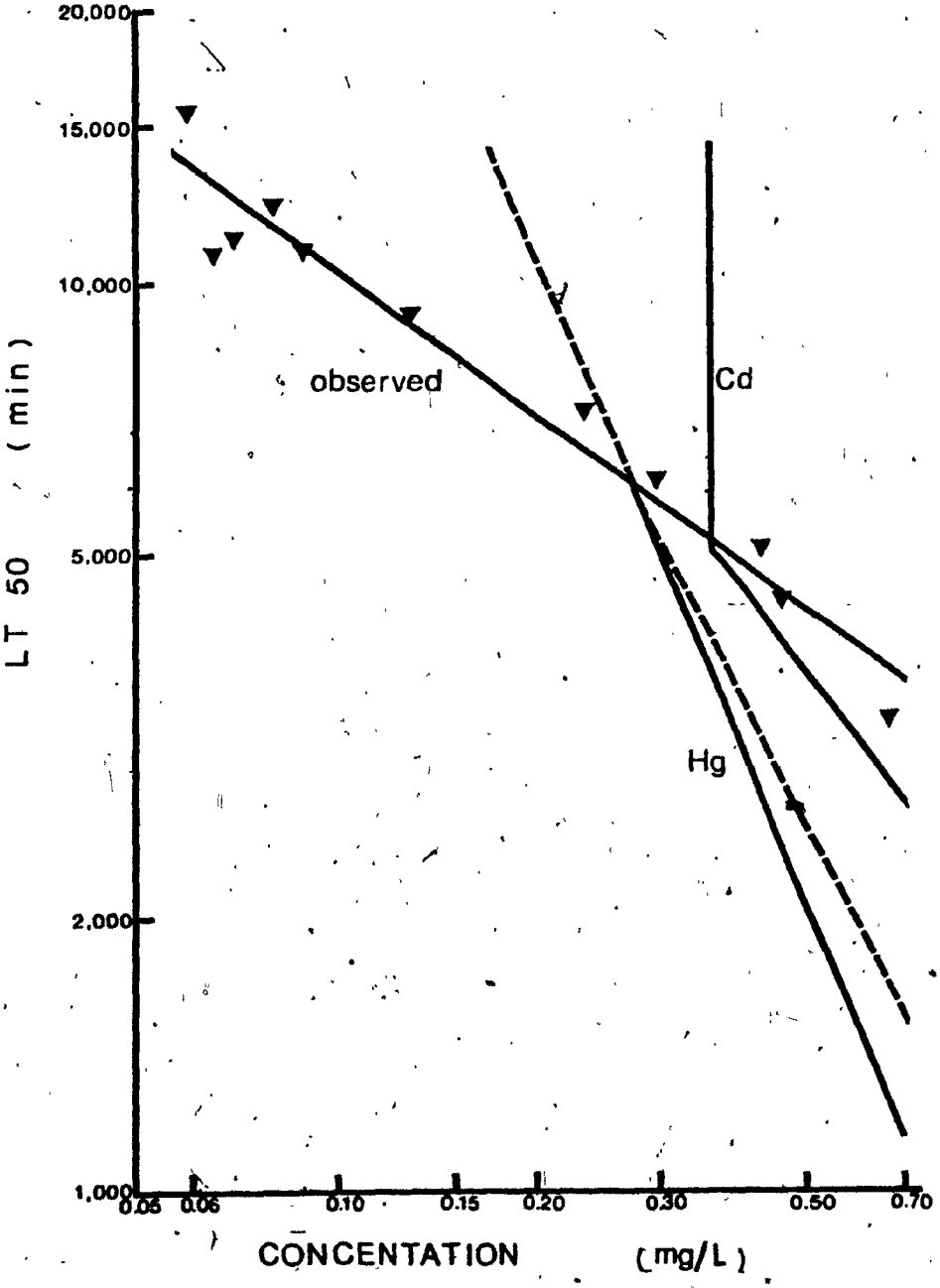

Figure 24. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and mercury, expressed as equivalent units of cadmium (Log-Log scale). The median mortality-time curve predicted on the basis of concentration addition (—·—) is depicted for reference.

Bioaccumulation Study

Copper and cadmium content was determined for gills and degilled bodies of zebrafish exposed for various time intervals to cadmium and or copper solutions. The time course of quantitative change in metal content of gills is given in tables 23 and 24 and that for the degilled bodies in tables 25 and 26. The accumulation kinetics are, in a number of cases, well described by a rectilinear regression as shown by significant correlation coefficients. Regression equations are presented in tables 27 and 28 for log mean metal content versus log exposure time.

In summary it was found that only the gills were sites of significant metal accumulation during the 20 hours of exposure. With respect to copper accumulation in the gills there was a positive dose related increase in uptake when test organisms were exposed to discrete solutions. When exposure to copper was accompanied with cadmium the dose related increase in uptake was still evident however, the absolute quantities were significantly greater. With respect to cadmium accumulation in the gills there was a positive dose related increase in uptake when the test organisms were exposed to discrete solutions. When exposure to cadmium was accompanied with copper the absolute quantity absorbed was greater however, the dose related uptake was not evident. This suggests that cadmium uptake was maximized, at the lowest concentration, under these test conditions.

Table 23. Mean copper content of fish gills from zebrafish exposed to cadmium and or copper for various time intervals.

Concentration of Metal (mg/L)		Mean copper content of fish gills (µg/g dry tissue) Time of exposure (Hr)					
Copper	Cadmium	4	5	6	8	12	20
0.022	0.000	11.576	14.384	5.789	14.568	18.337	14.89
0.043	0.000	14.820	15.327	16.265	10.187	18.814	16.893
0.084	0.000	12.96	15.259	12.357	17.000	20.155	18.616
0.021	1.880	10.410	13.118	11.938	18.340	22.956	24.340
0.043	2.626	13.336	17.039	20.362	35.086	35.765	38.545
0.088	3.565	10.350	12.969	20.513	32.023	38.469	41.956
0.000	1.982	12.402	12.308	10.182	13.250	12.217	14.259
0.000	2.718	13.366	11.237	15.483	14.205	13.235	12.333
0.000	3.555	10.143	14.298	13.442	9.177	11.183	12.263

Table 24. Mean cadmium content of fish gills from zebrafish exposed to cadmium and or copper for various time intervals.

Concentration of Metal (mg/L)		Mean cadmium content of fish gills (µg/g dry tissue) Time of exposure (Hr)					
Copper	Cadmium	4	5	6	8	12	20
0.022	0.000	0.308	0.259	0.243	0.288	0.386	0.278
0.043	0.000	0.391	0.538	0.362	0.391	0.261	0.408
0.084	0.000	0.314	0.278	0.316	0.495	0.263	0.313
0.021	1.880	0.802	1.251	1.711	1.927	2.350	2.084
0.043	2.626	0.875	0.805	1.583	1.708	1.882	1.650
0.088	3.565	1.242	1.466	1.871	2.097	2.079	2.188
0.000	1.982	0.363	0.497	0.508	0.552	0.842	0.713
0.000	2.718	0.288	0.677	0.632	0.588	0.821	0.812
0.000	3.555	0.313	0.843	0.753	0.912	1.278	1.221

Table 25. Mean copper content of degilled fish bodies from zebrafish exposed to cadmium and or copper for various time intervals.

Concentration of Metal (mg/L)		Mean copper content of degilled bodies (µg/g dry tissue) Time of exposure (Hr)					
Copper	Cadmium	4	5	6	8	12	20
0.022	0.000	7.231	5.141	12.512	4.047	11.830	4.894
0.043	0.000	14.254	13.652	7.039	6.123	8.511	10.195
0.083	0.000	10.807	12.311	12.969	12.381	15.761	16.168
0.021	1.880	9.255	15.582	8.195	10.451	14.786	16.456
0.043	2.626	8.097	7.849	8.656	14.482	14.572	18.355
0.088	3.565	9.124	14.170	10.670	15.203	13.853	13.547
0.000	1.982	11.230	13.120	12.948	11.157	12.640	7.963
0.000	2.718	8.760	11.132	15.600	7.269	5.350	12.313
0.000	3.555	13.330	14.430	9.550	5.720	11.075	11.464

Table 26. Mean cadmium content of degilled fish bodies from zebrafish exposed to cadmium and or copper for various time intervals.

Concentration of Metal (mg/L)		Mean cadmium content of degilled bodies (µg/g dry tissue) Time of exposure (Hr)					
Copper	Cadmium	4	5	6	8	12	20
0.022	0.000	0.205	0.300	0.182	0.278	0.308	0.186
0.043	0.000	0.273	0.358	0.270	0.161	0.243	0.210
0.083	0.000	0.305	0.259	0.213	0.213	0.170	0.170
0.021	1.880	0.201	0.192	0.273	0.267	0.318	0.291
0.043	2.626	0.340	0.273	0.386	0.392	0.338	0.368
0.088	3.565	0.216	0.282	0.292	0.373	0.343	0.393
0.000	1.982	0.287	0.136	0.209	0.252	0.282	0.220
0.000	2.718	0.371	0.130	0.237	0.293	0.268	0.232
0.000	3.555	0.204	0.206	0.228	0.179	0.309	0.270

Table 27. Linear regression equations describing the quantitative change in metal content through time in fish gills excised from zebrafish exposed to various concentrations of cadmium and or copper.

Concentration of Metal in exposure tank (mg/L)		Linear regression equations			
Copper	Cadmium	Copper kinetics	r	Cadmium kinetics	r
0.022	0.000	Y= 0.837 +0.289(x)	.427		
0.043	0.000	Y= 1.091 +0.100(x)	.287		
0.083	0.000	Y= 0.958 +0.268(x)	.815*		
0.021	1.880	Y= 0.702 +0.563(x)	.949**	Y= -0.280 +0.541(x)	.821*
0.043	2.626	Y= 0.787 +0.679(x)	.902*	Y= -0.280 +0.457(x)	.743
0.088	3.565	Y= 0.545 +0.910(x)		Y= -0.041 +0.328(x)	.848*
0.000	1.982			Y= -0.631 +0.423(x)	.872*
0.000	2.718			Y= -0.642 +0.473(x)	.735
0.000	3.555			Y= -0.717 +0.700(x)	.808*

where r = correlation coefficient.

Y = Log metal content

X = Log time in hours.

Significance of the r value

* = $P<0.05$

** = $P<0.01$

Table 28. Linear regression equations describing the quantitative change in metal content through time in degilled fish bodies from zebrafish exposed to various concentrations of cadmium and or copper.

Concentration of Metal in exposure tank (mg/L)		Linear regression equations			
Copper	Cadmium	Copper kinetics	r	Cadmium kinetics	r
0.022	0.000	Y= 1.047 - 0.240(x)	-.283		
0.043	0.000	Y= 0.967 + 0.050(x)	.073		
0.083	0.000	Y= 0.900 + 0.252(x)	.933**		
0.021	1.880	Y= 0.805 + 0.308(x)	.615	Y= -0.841 +0.271(x)	.782
0.043	2.626	Y= 0.534 + 0.586(x)	.940**	Y= -0.531 +0.081(x)	.371
0.088	3.565	Y= 0.940 + 0.177(x)	.542	Y= -0.795 +0.317(x)	.857*
0.000	1.982			Y= -0.726 +0.085(x)	.181
0.000	2.718			Y= -0.623 +0.010(x)	.017
0.000	3.555			Y= -0.854 +0.236(x)	.684

where r = correlation coefficient

Y = Log metal content

x = Log time in hours

Significance of the r value

* = $P<0.05$

** = $P<0.01$

Sequential Exposure Study

Test organisms were exposed for 96 hours to a sublethal concentration of cadmium (1.45 ± 0.03 mg/l). The fish were subsequently transferred to exposure tanks containing various concentrations of mercury. The lethal dose-response data are presented in table 29 and figure 25. The results of a student t-test indicated no significant differences between the regression coefficient of the response equation and that for 96 hours exposure to mercury previously determined suggesting no change in the mode of action. There was however, a significant increase in potency (2.62 ± 0.16 x) as observed by the shift of the response line to the left, relative to the line derived for 96 hours exposure to mercury — ($P<0.05$).

Table 29. 96 Hr Lethal response data for zebrafish exposed to mercury after having been exposed to cadmium (1.45 ± 0.03 mg/L) for 96 Hrs.

Mean Assayed Concentration of Mercury (mg/L ± S.D.)	# of Fish Tested	Mean Wet Weight of Fish (g ± S.D.)	Observed % Mortality 96 Hours
0.090 ± 0.002	14	0.343 ± 0.029	4.70*
0.108 ± 0.001	14	0.343 ± 0.029	28.57
0.125 ± 0.003	14	0.438 ± 0.028	35.71
0.162 ± 0.004	14	0.343 ± 0.029	64.29
0.193 ± 0.004	14	0.438 ± 0.028	93.30*
0.224 ± 0.003	14	0.343 ± 0.029	95.90*
0.410 ± 0.004	14	0.438 ± 0.028	100.00

* Mortality data fitted according to Litchfield + Wilcoxcin (1949)
Equation of Probit Regression
$Y = 11.785 + 7.843(x)$
$LC_{50} = 0.136$ mg/L
95% Fiducial Limits = 0.12 to 0.15 mg/L

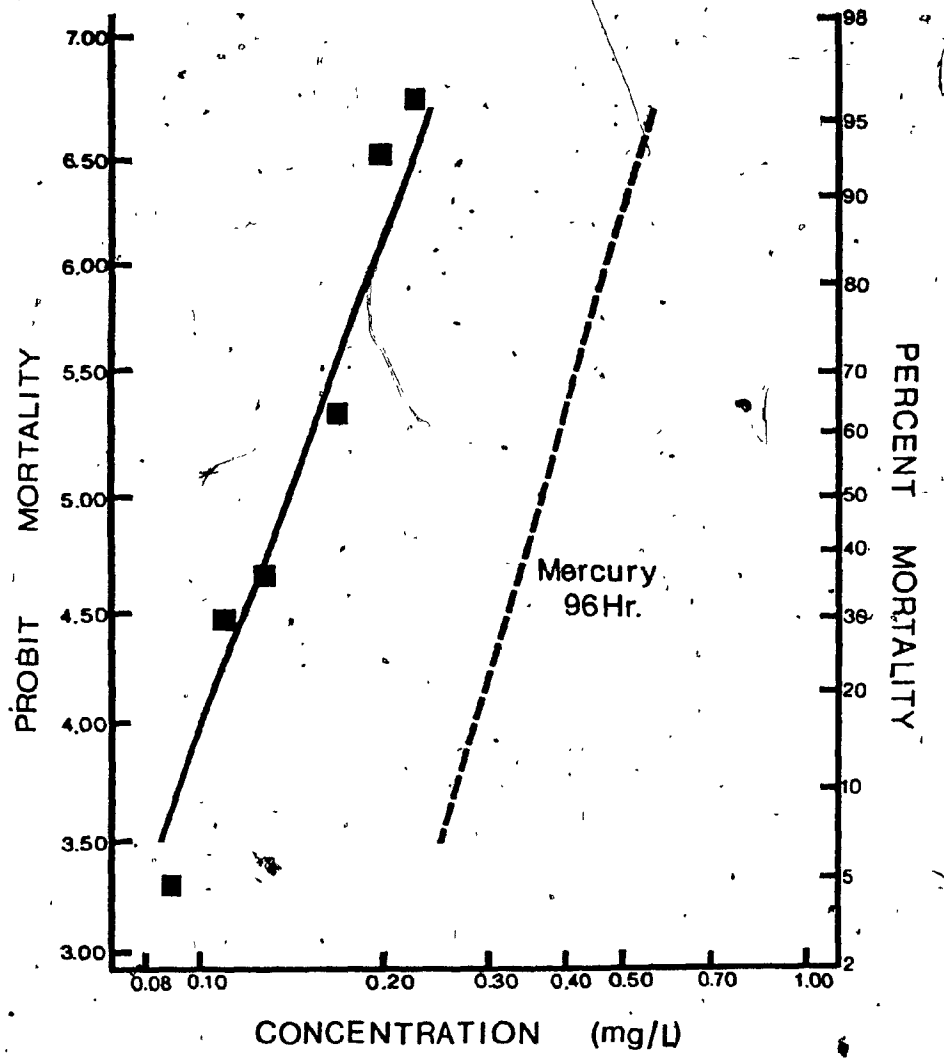

Figure 25. The lethal response curve for zebrafish, preexposed for 96 hours to cadmium, exposed to mercury. The response curve for zebrafish exposed to mercury for 96 hours is depicted for reference (—). . .

DISCUSSION

The evidence presented in this study suggests that the interactive effect on fish of mixtures containing accumulative and non-accumulative toxicants may vary through time. Furthermore, there was a high degree of agreement between the dose-response and time-response analysis in accounting for this variation in time-related toxicity. However, there were some exceptions with respect to the form of interaction implied by the toxic unit method of time response analysis.

Discrete Toxicants: Dose-Response

The following series represents the relative lethal potencies (LC_{50} values, Table 6) of the discrete metals at 96 hours:

Cu^{++} (0.26 mg/L) > Hg^{++} (0.36 mg/L) > Cd^{++} (5.82 mg/L)

and at 240 hours

Hg^{++} (0.15 mg/L) > Cu^{++} (0.26 mg/L) > Cd^{++} (5.82 mg/L).

It should be stressed that potency is a comparative term rather than an absolute expression of a toxicants' activity. Therefore, it is inappropriate to express the relative activities of two (or more) toxicants unless they produce their effects by the same mechanism. It is reasonable to assume that toxicants which possess similar chemical or physicochemical properties and initiate the same selective pharmacologic response do so by the same mechanism (Levine, 1973). From this it follows that toxicants which operate through a common mode of action will have parallel dose-response curves. Therefore, lacking complete mechanistic knowledge it is the statistical similarity of

dose-response curves which justifies the determination of relative potencies.

The regression coefficients for each of the discrete toxicants were not found to be significantly different ($P>0.05$). Thus it is considered correct to discuss copper, cadmium and mercury in terms of their relative potencies. The methodology of Finney (1971), in which dose-response curves are constrained to absolute parallelism, was employed to derive numerical values to represent the relative potencies of the toxicants. It was found that at 96 hours mercury was 17.71 times more potent than cadmium and 0.77 times as potent as copper. The same analysis revealed that at 240 hours mercury was 40.73 times more potent than cadmium and 1.78 times more potent than copper.

The relative toxicity of heavy metals is generally perceived to be a reflection of their different affinities for various ligands in a critical organ(s). If a sufficient number of "sites" are bound a functional change may occur, the consequence of which is dependent upon the function of the binding ligands. Therefore, it is considered that the binding of metals to ligands may constitute the common mode of toxic action at the biochemical level. With respect to copper, cadmium and mercury their affinity for various ligands has been documented in the literature (Shaw and Grushkin, 1957; Passow et al., 1960). In all cases the order of affinity was reported to be $Hg^{++}>Cu^{++}>Cd^{++}$ which corresponds with the relative potency distribution noted in the present study. This would not however be expected to be the order of potency in every situation because there has been no accounting

for different rates of absorption or tolerances between aquatic organisms. Thus the relative potencies of the heavy metals may not be accurately ranked strictly from a knowledge of their relative affinities for ligands. Chapman (1978) has attempted to rank the overall hazard of heavy metals which are common to the aquatic environment. He has listed nine metals in five categories as follows:

Metal	Hg,Ag,Cd	Cu	Ni,Pb,Zn	Sb	Sn
Toxic level	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M

As can be seen from Chapman's list all three metals included in this study are considered to be extremely toxic. Chapman (1978) also identified copper, cadmium and mercury to be of particular concern because they are mobilized within the environment in amounts which approach their toxic levels.

Discrete Toxicants: Time-Response

Dose-response data are generally considered to be the best standard of reference between toxic substances (Bliss, 1937). However, a comprehensive profile of relative toxicities must also include an analysis of time-response data. The toxicity response curves for cadmium and copper were found to be curvilinear with asymtote relative to the x axis at 5.83 mg/L and 0.26 mg/L respectively (Figure 11 and 12). The curvilinear shape of these toxicity curves has been previously reported in the literature: Cadmium-Schweiger, 1957; Lloyd, 1960; Roch, 1979: Copper-Lloyd, 1960; Spraque and Ramsay, 1965; Spear, 1977. Brown (1973) rationalized that the death of fish, as witnessed in toxicity tests is the summative consequence of the failure of a number of

physiological functions. The curvilinear shape of the toxicity response curve that leads to an asymtote parallel to the time axis is interpreted as the progressive achievement of homeostasis in the test fish as the concentration decreases.

The suitability of using a logarithmic scale for both time and concentration has been indicated by many researchers (Gardner et. al., 1977). The time-response data for cadmium and copper have been converted to straight line relationships by a log- log transformation (Figures 15,16 and Table 11). This representation has the advantage of allowing a clear determination of the exposure duration at which the toxicity response curve becomes parallel to the time axis. This was determined to be 5190 minutes and 3442 minutes for cadmium and copper respectively.

There was no evidence of a threshold to mercury toxicity within the 10 day exposure period. Furthermore, survivors of the 10 day exposure period (0.018 mg/L), continued to die after having been placed in a contaminant free environment. The toxicity response curve for mercury (Figures 14 and 17) was found to be rectilinear. The accuracy of this assessment is indicated by previous documentation in the literature by Macleod and Pessah (1973). It follows from Browns' (1973) rationale that the test fish were not acclimating to the presence of mercury even at the lowest concentration tested of 0.018 mg/L. Furthermore, it implies that the processes of accumulation and elimination have not reached equilibrium within the 10 day exposure period. Therefore, mercury is categorized as an accumulative toxicant whereas cadmium and copper are referred to as non-accumulative toxicants.

There are two general methods of expressing the relative potencies of toxic substances from time-response data. The first involves a comparison of lethal threshold concentrations irrespective of when the threshold is attained. For example according to the following scale recommended by a joint committee of experts from IMCO/FAO/UNESCO/WHO (1969), the present study rates mercury and copper as "very toxic" and cadmium as "toxic".

(1) "Very toxic" Threshold below 1 ppm;

(2) "Toxic" Threshold between 1 and 100 ppm;

(3) "Moderately toxic" Threshold between 100 and 1000 ppm;

(4) "Slightly toxic" Threshold between 1000 and 10,000 ppm;

The second method involves a comparison of doses which have lead to equivalent responses at the same point in time. According to this method the order of relative potencies at 96 hours was:

$Cu^{++} > Hg^{++} > Cd^{++}$

and at 240 hours was

$Hg^{++} > Cu^{++} > Cd^{++}$

These data confirm the order of relative potencies which were specified by the dose-response data. A more rigorous analysis of the time to response data indicated that at 96 hours mercury was 10.16 times more potent than cadmium and 0.45 times as potent as copper. At 240 hours mercury was 32.33 times more potent than cadmium and 1.44 times more potent than copper.

Comparison of dose-response and time-response data:

Discrete Toxicant

Table 30 lists the relative potency factors which have been derived from the two methods of analysis. It would appear that both representations of the toxicity data are in close agreement.

Table 30: Relative potency factors calculated from dose-response and time-response data.

	Relative Potency Factor			
	96 Hours		240 Hours	
	Time Response	Dose Response	Time Response	Dose Response
Hg/Cu	0.45	0.77	1.44	1.78
Hg/Cd	10.16	17.71	32.33	40.73

MIXTURES OF TOXICANTS

Cadmium and Copper: Dose-Response

The results of this study indicated that neither the concentration addition nor response addition model (Bliss, 1939; Anderson and Weber, 1975) adequately predicted the toxicity of mixtures containing copper and cadmium (Tables 12, 13 and Figure 18). These toxicants were initially assumed to be concentration additive because there were no significant differences between the slopes of their respective lethal tolerance distribution curves. However the empirically determined dose-response range indicated that copper and cadmium were supra-additive (2.01 x) to the toxicity predicted for concentration addition (Table 14). Eisler and Gardner (1973), working with Fundulus heteroclitus, reported that the lethal toxicity of copper and cadmium mixtures were supra-additive. These results supported the findings of La Roche (1972) who observed that the lethal effects of copper-cadmium mixtures were supra-additive.

We can perceive in a general way that the observed supra-additive response must be a result of interactions which alter the rate at which the toxicants reach their site of action and or the sequence of events which follow the arrival of the toxicant molecules at the site of action (Figure 7). A potential explanation for the interaction of cadmium and copper is offered later in the discussion.

Cadmium and Copper: Time-Response

Analysis of the toxicity response curve for the mixtures containing cadmium and copper supports the conclusion of supra-additivity. The basic curvilinear shape which characterized the discrete toxicity response curves appears to adequately represent the mixture (Figure 21). The graphical analysis of the curve suggests an asymtote relative to the x axis at approximately 2.8 ppm (total concentration as cadmium). A "predicted" toxicity response curve can be computed assuming concentration addition. This curve is intermediate between the two discrete response curves, taking into account the ratio of toxicants within the mixture (Figure 22). The enhanced potency of the mixture was computed to be approximately 2.08 times that predicted. The comparison of the dose-response (2.01 x) and time-response (2.08 x) enhancement factors indicates a strong correlation.

Another method for presenting toxicity response curves has been proposed by Lloyd (1962). The concentration of toxicants are expressed as fractions of their median lethal threshold concentrations.

$$Ms/Mt \qquad (8)$$

Where Ms= the concentration of toxicant

Mt= the median lethal threshold concentration.

The effective concentration of a mixture is computed from the sum of the fractions derived for each toxicant present in the mixture. The time to response data obtained for copper and cadmium mixtures were subjected to the preceding technique

Table 31 and Figure 26). While this representation clearly indicates a supra-additive response the method suggests an enhancement factor of approximately 4.0 x the predicted. The previous analysis of time-response data has indicated an enhancement of 2.08. Therefore, there is a difference in the estimation of potency between these methodologies.

Cadmium and Mercury: Dose Response.

The results of this study indicated that the 96 hour toxicity of cadmium-mercury mixtures could be predicted from a knowledge of the discrete dose-response curves. The slopes of the dose-response curves for 96 hours exposure to pure solutions of cadmium and mercury were not significantly different ($P < 0.05$). Therefore, mixtures of mercury and cadmium were composed on the assumption that they were concentration additive. It was subsequently found that observed and predicted responses were in close agreement. Thus it was concluded that concentration addition adequately described the toxicity of the mixtures at 96 hours.

The dose response curves for discrete solutions of cadmium and mercury were parallel at 240 hours; consequently concentration addition was predicted for the binary mixtures. The results of bioassays indicated the mixtures to be neither concentration nor response additive but supra-additive (2.03 x, Table 18). This is a particularly surprising result when it is considered that the concentration of cadmium in the mixture is significantly lower than its median lethal threshold level as a discrete agent. In practical terms what this means is that on the basis of time response data we suspect that the test organisms are able to

Table 31 Toxic unit analysis of cummulative time-mortality data for zebrafish exposed to mixtures of cadmium and copper.

Mean Assayed Concentration of Copper (mg/L)	Ms/Mt Value for Copper.	Mean Assayed Concentration of Cadmium (mg/L)	Ms/Mt Value for Cadmium	Total Ms/Mt Value	LT_{50} (Min)
0.016	0.06	1.310	0.23	0.29	2176
0.029	0.11	1.395	0.24	0.35	1930
0.033	0.13	1.437	0.25	0.38	2501
0.050	0.19	1.685	0.29	0.48	1711
0.065	0.25	1.623	0.28	0.53	1856
0.058	0.22	1.955	0.34	0.56	1177
0.097	0.37	1.395	0.24	0.61	2122
0.061	0.24	2.135	0.37	0.61	1193

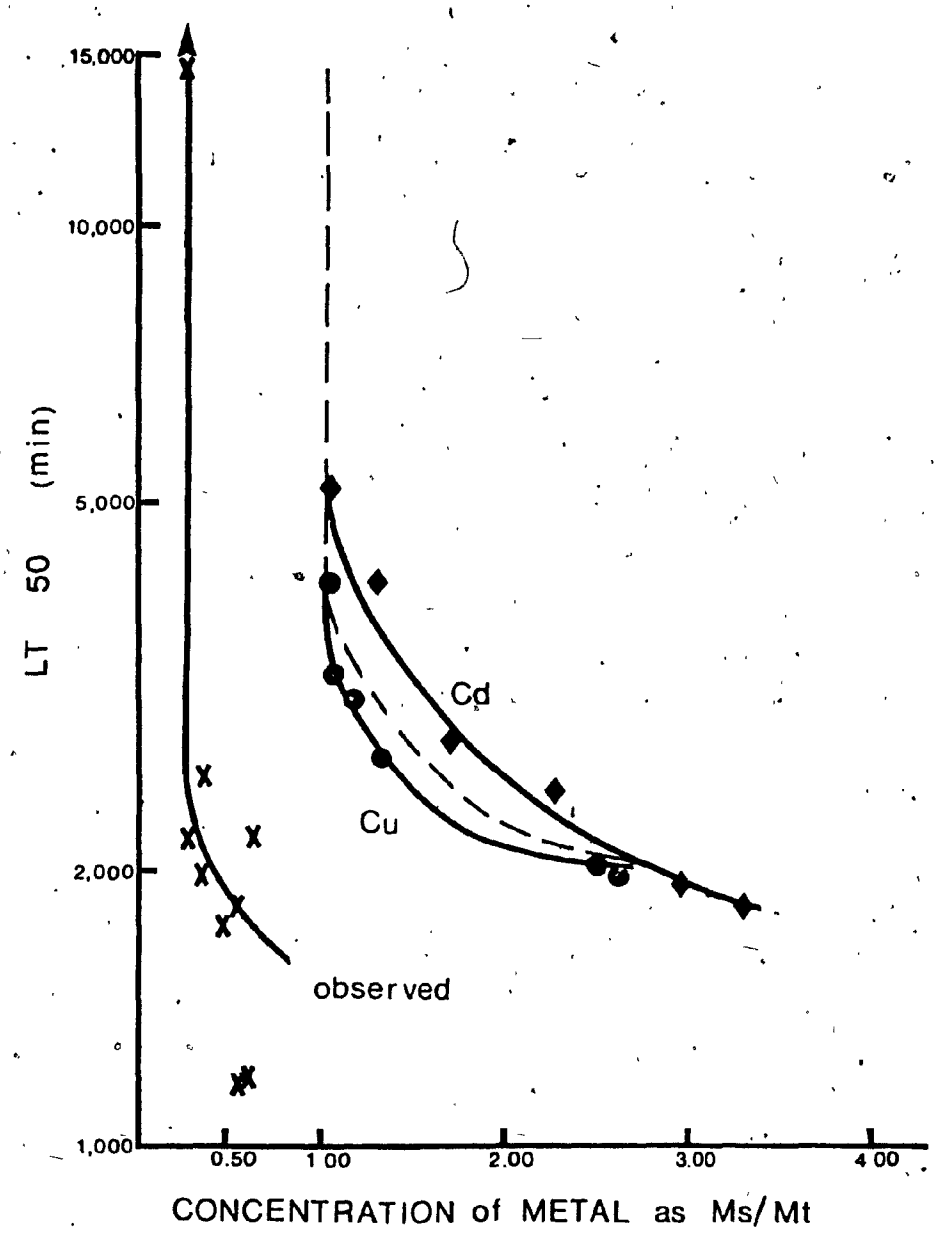

Figure 26. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and copper. For an explanation of Ms/Mt see text. The relationship predicted on the basis of strict additivity is depicted for reference (– – –).

achieve a state of absorption - elimination equilibrium with cadmium levels this low. Therefore, beyond 96 hours cadmium would not be expected to contribute directly to the toxicity of the mixture.

To our knowledge there are no lethal studies involving combinations of cadmium and mercury to which our results could be compared. However a study conducted by Weis and Weis (1978), utilizing the rate of tail regeneration in F. confluents as an indicator, observed an infra-additive response. Clearly the interactions of cadmium and mercury are complex and a potential explanation of the observed effects is offered later in the discussion.

Cadmium and Mercury: Time Response

The toxicity response curves, derived from the mercury and cadmium mixture data (Figure 24), support the dose-response findings i.e. concentration additive at 96 hours and supra-additive at 240 hours. A predicted toxicity curve could be computed, as previously described, for exposure periods not in excess of the median lethal threshold for cadmium (5190 minutes). For exposure periods in excess of the lethal threshold of cadmium it was deemed reasonable to use the discrete mercury toxicity curve as representative of the predicted response. The observed response curve intersects the "predicted" curve at 102 hours. This indicates that the response to mixtures may be accurately predicted by assuming concentration addition in the region of 102 hours. Analysis of the toxicity response curves at 240 hours indicates that mercury and cadmium are supra-additive (3.6x, Figure 24).

These data supports the dose-response data which indicated the mixtures to be concentration additive at 96 hours and supra-additive at 240 hours (2.03 x, Table 18).

An interesting aspect of the toxicity response curve was the indication of infra-addition for relatively short exposure periods. To confirm the accuracy of this observation a dose-response analysis was conducted for forty eight hours exposure (Figure 27).

While there are only two observed response points they both indicate an infra-additive response (both occur outside the 95% confidence limits). Analysis of the toxicity curves suggests that the mixtures will be 0.43 times as potent as predicted. The dose-response analysis indicates that the potency of the mixtures is 0.61 x that predicted. While the relative potency factors are not in close agreement the "short exposure" infra-additive response was confirmed.

The toxic unit method for presenting toxicity response data indicated the mixtures to be approximately concentration additive for all mixtures with effective concentrations in excess of unity (Table 32 and Figure 28). The reason why this representation of the data does not reveal the infra-additive response is not immediately clear.

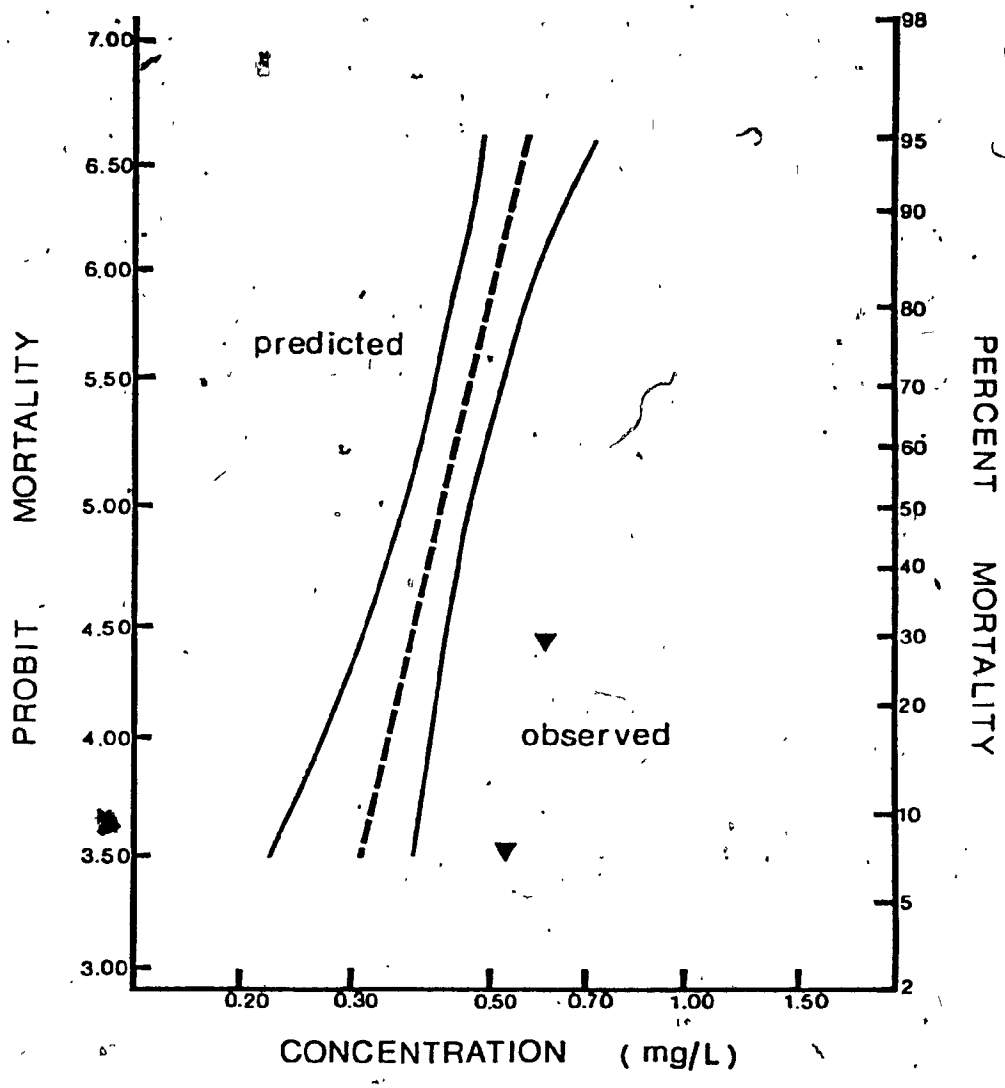

Figure 27. The lethal response of zebrafish exposed to two mixtures of cadmium and mercury expressed as equivalent units of mercury for 48 hours. The lethal response curve predicted on the basis of concentration addition plus the 95% confidence limits is depicted for reference.

Table 32 Toxic unit analysis of cummulative time-mortality data for zebrafish exposed to mixtures of cadmium and mercury.

Mean Assayed Concentration of Mercury (mg/L)	Ms/Mt Value for Mercury	Mean Assayed Concentration of Cadmium (mg/L)	Ms/Mt Value for Cadmium	Total Ms/Mt Value	LT_{50} (Min)
.059	0.17	1.28	0.22	0.39	15356
.062	0.18	1.36	0.23	0.41	10717
.069	0.20	1.45	0.25	0.45	11374
.077	0.21	1.60	0.28	0.49	12340
.085	0.23	1.75	0.30	0.53	10935
.125	0.33	1.85	0.32	0.65	9240
.235	0.85	1.71	0.29	1.15	7301
.306	1.29	2.09	0.36	1.65	6127
.431	1.43	2.09	0.36	1.79	5106
.452	1.51	2.49	0.43	1.93	4470
.626	1.97	2.71	0.47	2.44	3301

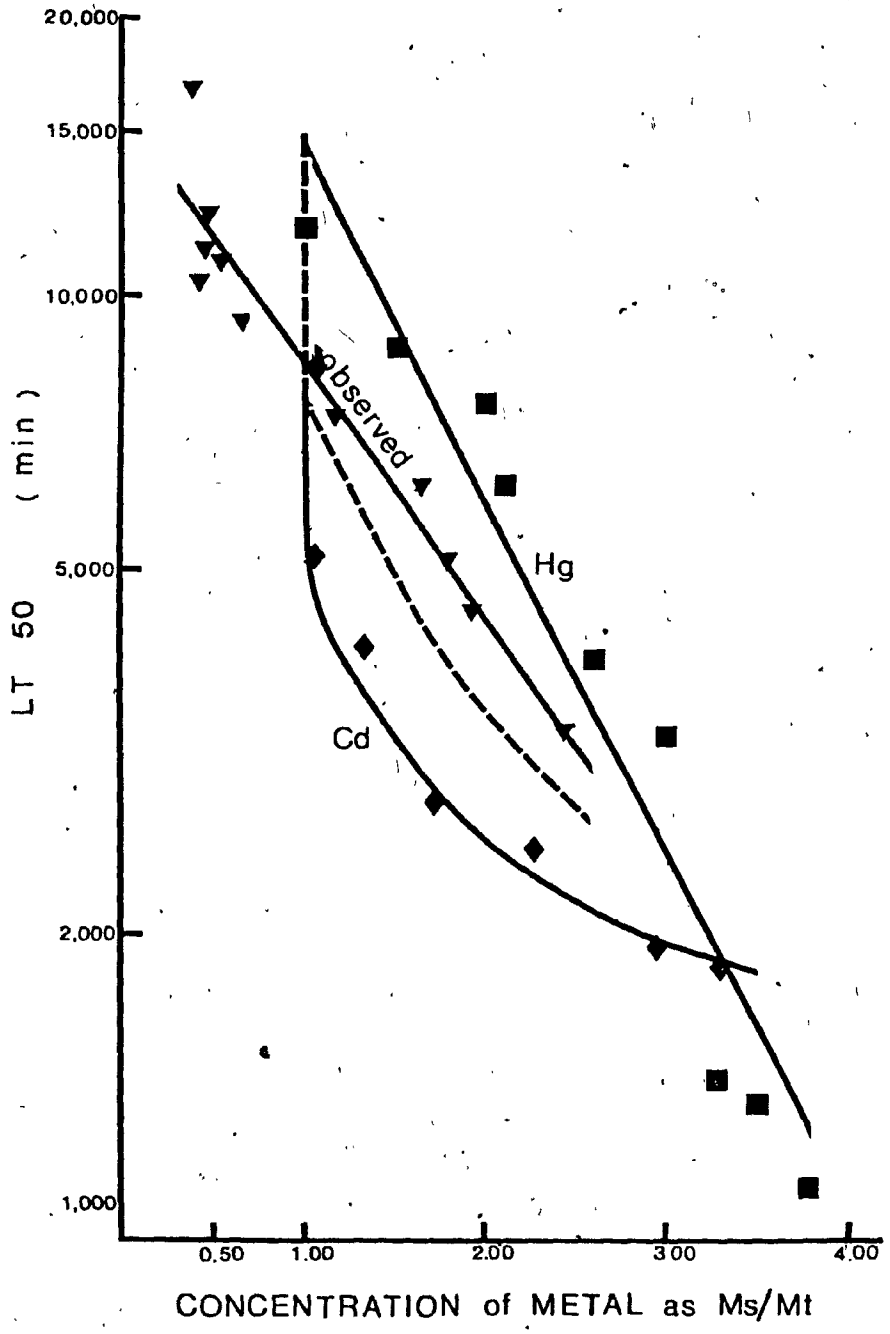

Figure 28. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and mercury. For an explanation of Ms/Mt see text. The relationship predicted on the basis of strict additivity is depicted for reference (———).

Cadmium and Copper: Supra-additive interaction

A general theory has been proposed in which the toxicity of heavy metals results from their interaction with gill tissues (Lloyd, 1962). Analysis of the tissues of rainbow trout exposed to a lethal solution of zinc, containing Zn^{65} as a tracer, revealed that the highest percentage of zinc was located in the gill tissues (Lloyd, 1960). Schweiger (1957) determined the body burden of cobalt and manganese in carp which had been killed by these metals. Subsequently he injected carp with three times the body burden of cobalt and three hundred times the amount of manganese and found the fish survived. These results suggest the toxic action of at least some metals is not internal but related to the epithelial cells of the gill lamellae. The body of current literature would tend to suggest that most heavy metals, at the acutely lethal level, have a common site of action in the gills. Furthermore it is the binding of the divalent cations to protein legands eg. OH, COOH, PO_3H_2, SH_2, NH_2 which leads to a breakdown in normal cellular metabolism (Passow et al., 1960). Although specific affinity for these ligands may vary between metal species the general effect may be identical or indistinguishable because of biological variability between test organisms. This may explain why the dose-response functions derived for copper, cadmium and mercury were not statistically different.

The supra-additive toxicity of cadmium and copper mixtures must result from some kinetic and or dynamic interaction (Ariens, 1972; Anderson and D'Appollonia, 1978). Two pieces of evidence in our studies have suggested that the physiological interaction is a kinetic phenomena (however they do not exclude the possibility of a dynamic interaction). Firstly, the regression coefficient of the observed dose - response function was not significantly different from the regression coefficient of the predicted dose-response function. This suggests (for reasons previously given) that the mode of action is unchanged and has merely been promoted in some fashion.

The second piece of evidence comes from a comparison of the toxicity response curves (Figure 22). The slopes of the observed and predicted toxicity curves are parallel. This suggests that there has been no change in the mode of action even though the toxicity has been enhanced.

Preliminary studies to determine if a kinetic interaction is involved indicate that cadmium and copper potentiate each others' uptake into gill tissue. Observation of tables 23, 24, 25 and 26 indicates that only the gills and not the bodies were sites of significant metal accumulation during the initial 20 hours of exposure. Tables 23, 24 and figures 29a to 30c indicate that a causal relationship appears to exist between increased ambient concentrations of discrete solutions and increased levels of metal in the gill tissue. Additional evidence for this hypothesis is gained from two areas. Firstly, for each concentration of discrete metal, the rate of metal accumulation is well described by a least squares regression equation (Table 27).

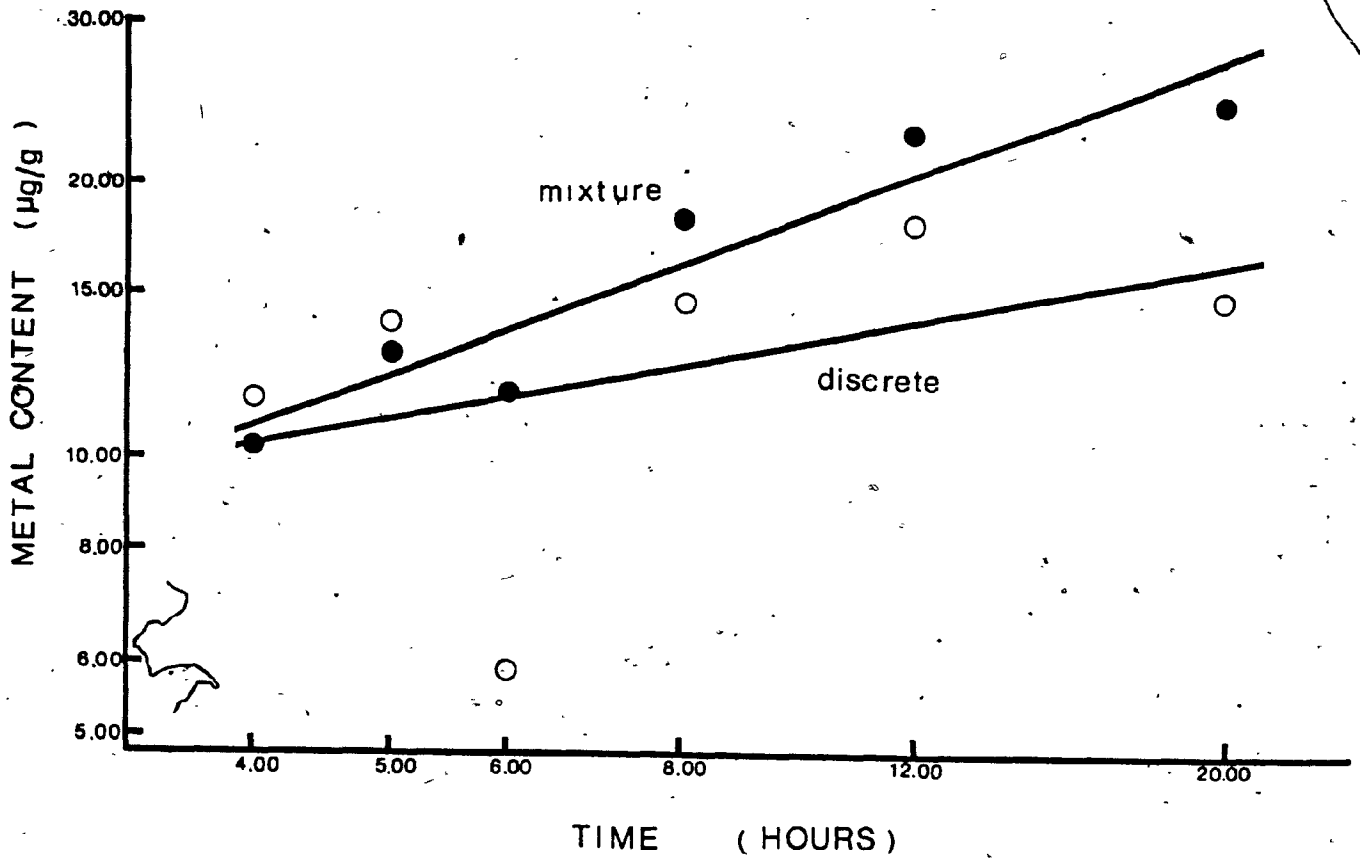

Figure 29a. Accumulation of copper in gill tissue during exposure to discrete copper (○ , 0.22 mg/L) and when concurrent with cadmium (● , 0.21 mg/L + 1.880 mg/L Cd).

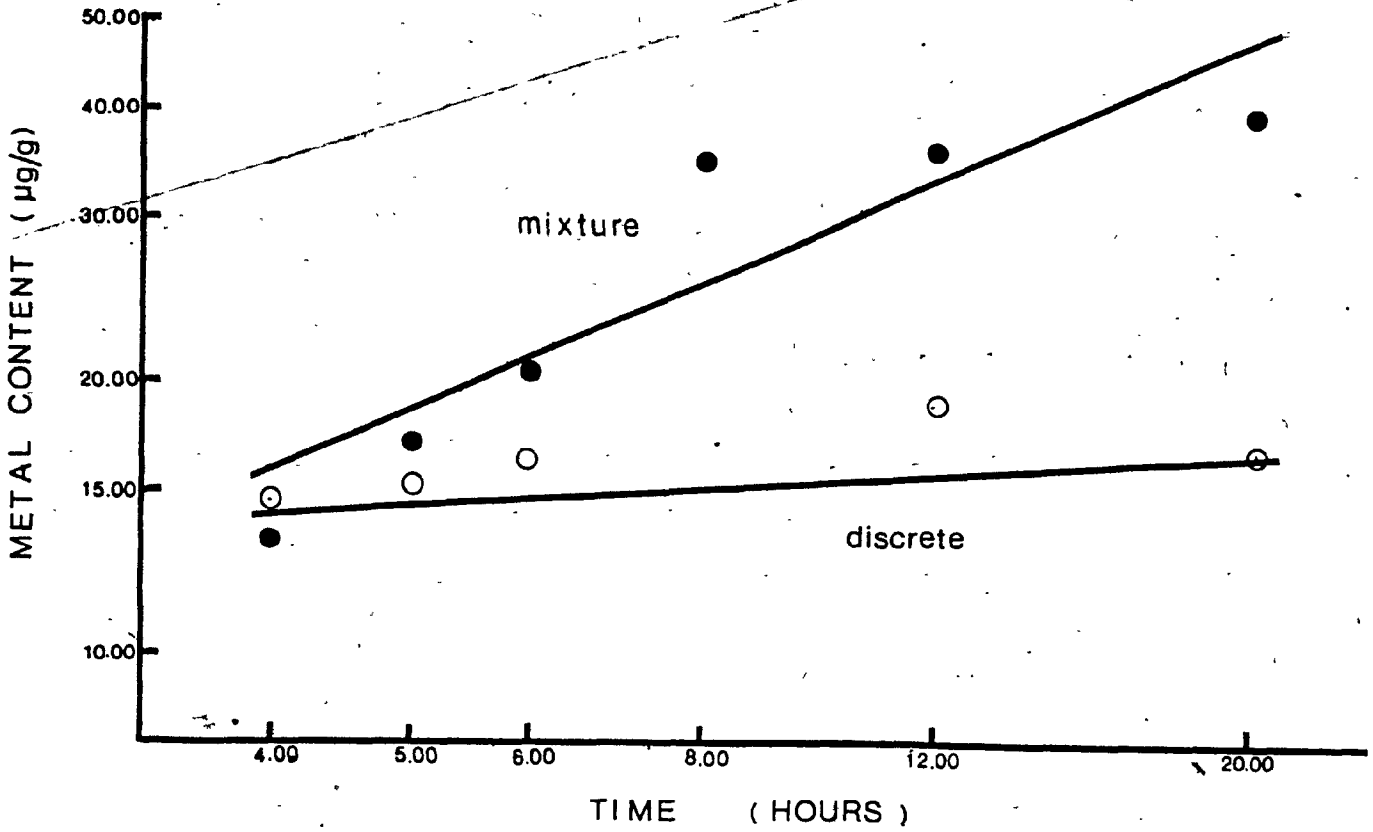

Figure 29 b. Accumulation of copper in gill tissue during exposure to discrete copper (○, 0.043 mg/L) and when concurrent with cadmium (●, 0.043 mg/L + 2.826 mg/L Cd).

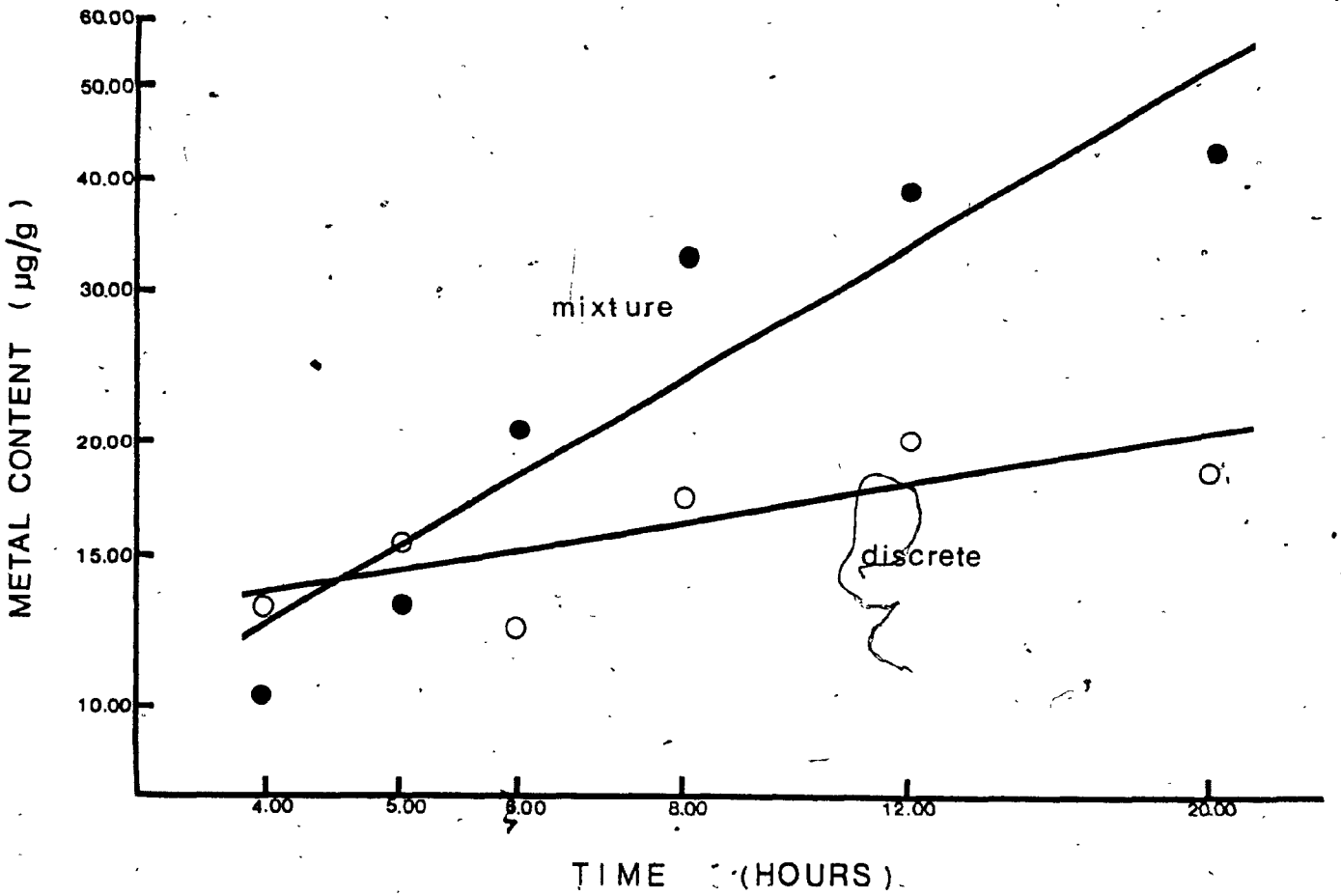

Figure 29. c. Accumulation of copper in gill tissue during exposure to discrete copper (○ , 0.083 mg/L) and when concurrent with cadmium (● , 0.088 mg/L + 3.565 mg/L Cd).

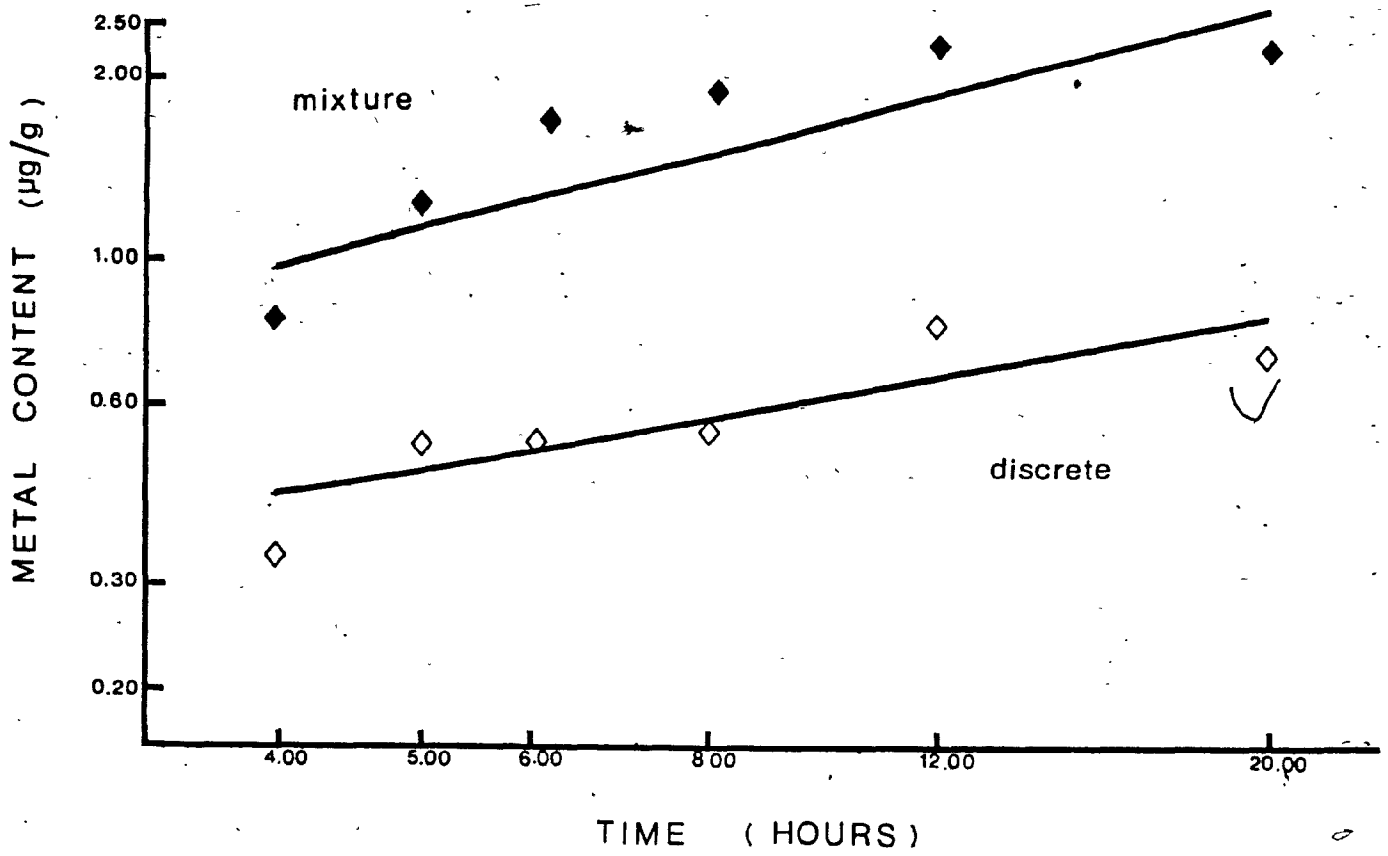

Figure 30 a. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 1.982 mg/L) and when concurrent with copper (◆ , 1.880 mg/L + 0.021 mg/L Cu)

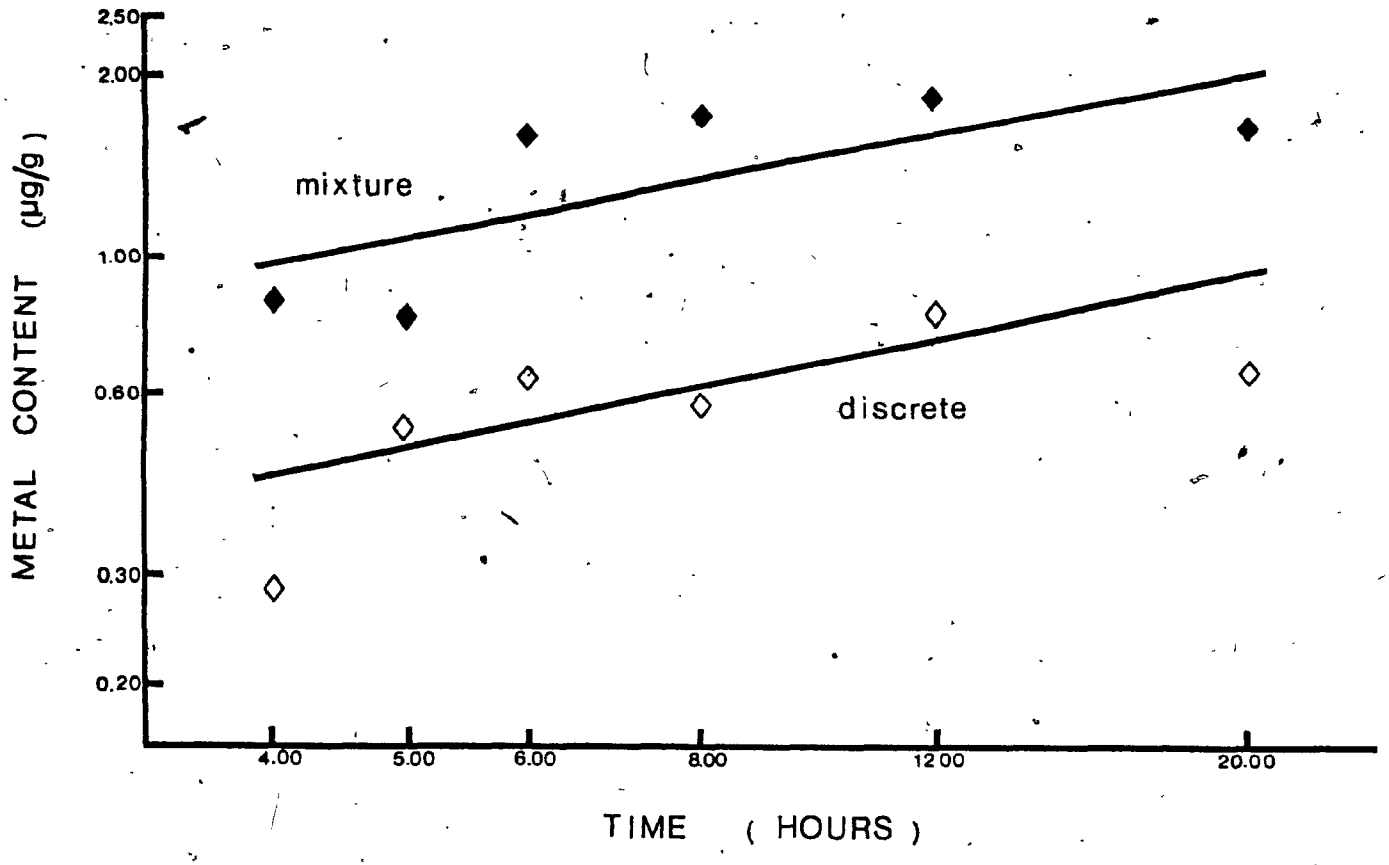

Figure 30 b. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 2.718 mg/L) and when concurrent with copper (◆ , 2.626 mg/L + 0.043 mg/L Cu).

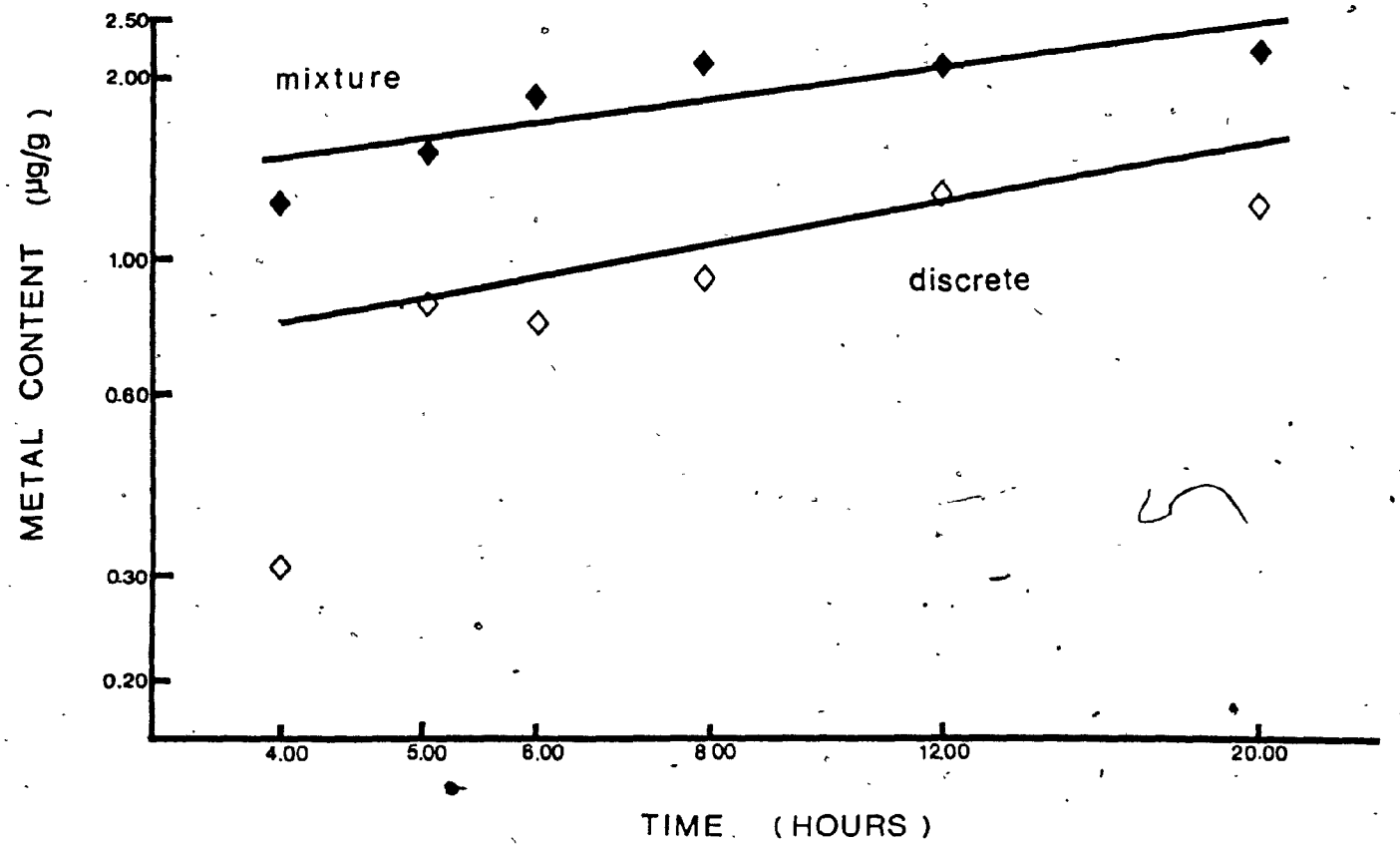

Figure 30 c. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 3.555 mg/L) and when concurrent with copper (◆ , 3.565 mg/L + 0.088 mg/L Cu).

This suggests a mechanistic relationship between metal accumulation and the ambient concentrations. Secondly, between concentrations, at twenty hours exposure, a least squares regression equation with positive slope and high correlation coefficient can be derived (Tables 23, 24 and Figures 31 and 32).

The most significant aspect of this study is that the relative bioconcentration of copper and cadmium is greater in gill tissues of fish exposed to mixtures than to discrete solutions. However, there are three further points of interest. Firstly, the stoichastic relationship noted earlier for each discrete metal ambient concentrations and the accumulation in the gills appears to be maintained for the mixtures. i.e. the rate of metal accumulation is well described by a least squares regression function. This tends to suggest that the mechanism of bioconcentration has not been altered but merely enhanced. Secondly, between concentration of copper, at twenty hours exposure, a least squares regression equation with positive slope and high correlation coefficient can be derived (Table 23 and Figure 31). This represents further evidence for the mechanistic relationship between accumulation and the ambient concentration which has been promoted through the concurrent presence of cadmium. Thirdly, there is no apparent relationship between cadmium content in gill tissues and the three different mixtures concentrations, at twenty hours exposure (Figure 32). The absolute amounts which have been accumulated in gill tissue are significantly greater for the mixtures relative to their respective discrete solutions but no dose related increase exists for the mixtures. This suggests that

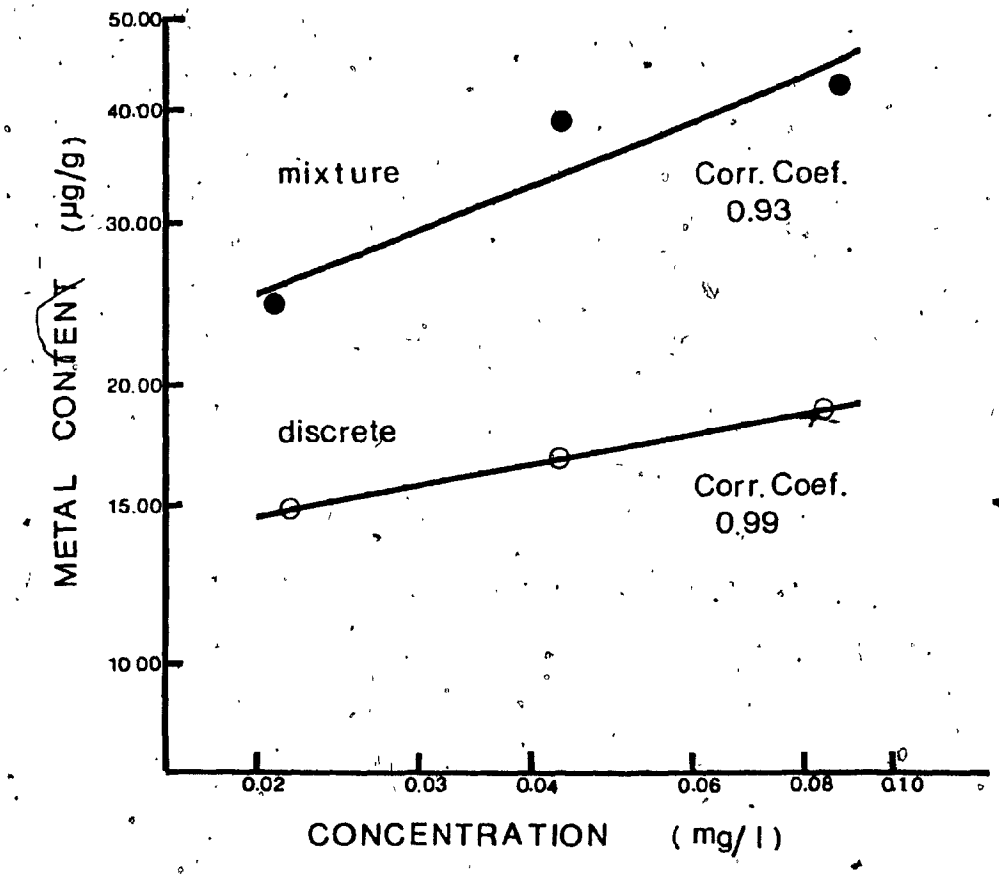

Figure 31. Copper content in fish gill tissue following 20 hours exposure to discrete (O) solutions and in combination (●) with cadmium. The ordinate value gives the copper content of the gills and the abscissal value gives the exposure concentration of copper. The binary combinations contained 1.880, 2.626, 3.565 mg/l cadmium in order left to right.

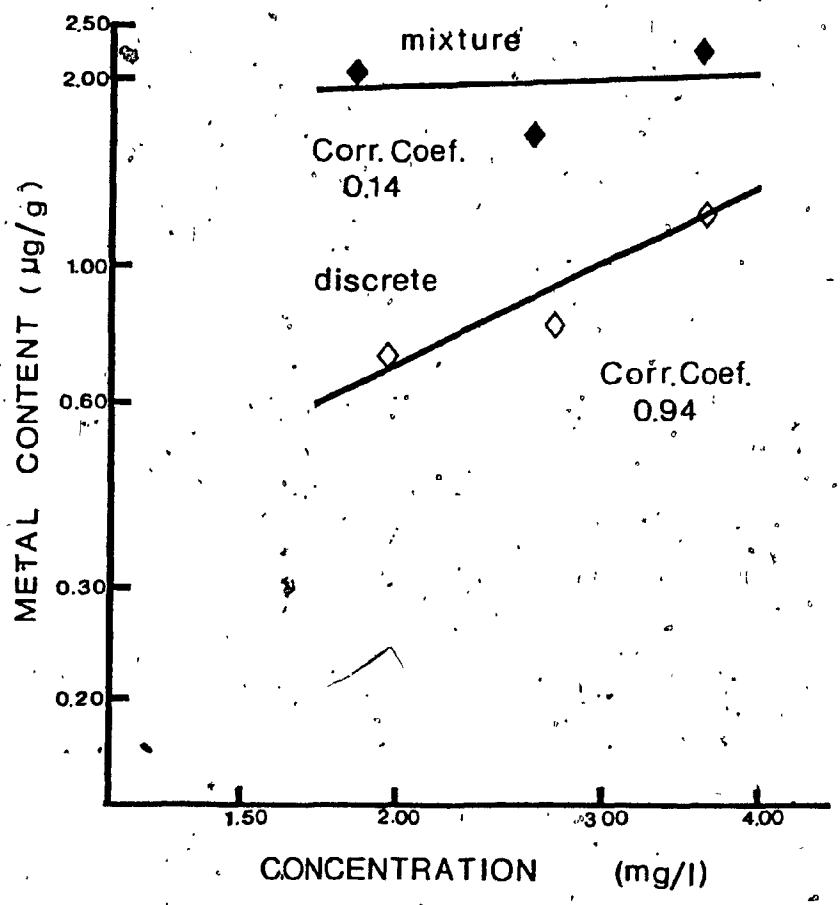

Figure 32. Cadmium content in fish gill tissue following 20 hours exposure to discrete (◇) solutions and in combination (◆) with copper. The ordinate value gives the cadmium content of the gills and the abscissal value gives the exposure concentration of cadmium. The binary combinations contained 0.021, 0.043, 0.088 mg/l copper in order left to right.

the mechanism for cadmium accumulation has been saturated for even the lowest mixture concentration.

Therefore there would appear to be a physiological interaction between copper and cadmium which results in a reciprocal potentiation of uptake rates. In as much as the magnitude of response is related to the concentration of toxicant at the site of action these results may explain the supra-additive response. Furthermore, the increased rate of uptake may explain the observed enhancement in the time-response data (Figure 22).

It is important to point out that while these results clearly indicate the presence of a kinetic interaction there is no reason to exclude the possibility of a dynamic interaction as well.

Cadmium and Mercury: Variable interactive effect.

The interactive effect of cadmium and mercury on fish would appear to change through time. Empirically the effect of the mixtures may be separated into three segments:

1. an infra-additive interaction observed at 48 hours exposure ,
2. a concentration additive interaction at 96 hours exposure and
3. a supra-additive interaction at 240 hours exposure.

The available data for the infra-additive effect of rapidly lethal mixtures of Cd and Hg consists of two response points (Figure 27). Without a knowledge of the response over the entire response range (0-100%) it is difficult to draw any inferences concerning the mechanism of this effect. A study which was conducted by Weis and Weis (1978) utilizing the rate of tail regeneration in F. confluents as an indicator also observed an infra-additive response to Cd and Hg mixtures. However the authors did not publish a mechanism for the infra-additive effect therefore it is difficult to assess to what degree the results of their sub-lethal experiment could apply to the effect noted in this study. A less than predicted response to lethal concentrations of metals has been reported for mammals pretreated with sub-lethal quantities of cadmium (Schnell, 1978). The mechanism of this effect is thought to be related to elevated levels of the hepatic protein, metallothionein. It has been shown that cadmium can induce the synthesis of this protein which has

the capacity to bind heavy metals thereby preventing their interaction with vital cellular constituents. Thus the underlying mechanism for this cadmium-induced tolerance is an increased detoxification capacity. There are, however, at least two reasons why this mechanism is unlikely to explain the infra-additive effect noted in this study. Firstly, the protective effect is noted only if the organism is pretreated in such a way that it has enough time to produce sufficient metallothionein to withstand the subsequent lethal challenge. This was not the case in the present study where the exposure was concurrent. Secondly, the results of a sequential experiment which was conducted (Table 29) did not indicate any protective capacity of cadmium pretreatment. Thus the explanation of the infra-additive effect seen in this study awaits further research, possibly in the area of bioaccumulation.

The response of test organisms exposed to mixtures of Cd and Hg for 96 hours would appear to be effectively described by the empiricale model of concentration addition (Table 15, Figure 19). Considering the overall pattern of the interactive effect (Infra-additive-Supra-additive) of these metals through time it is difficult to interpret exactly what this observation means. More clearly, does this represent a definite physiological event or is it a coincidental observation marking the mid point of the transition from one form of interaction to another. Of these two possibilities the second would seem to be the more appropriate.

The supra-additive response of the organisms exposed to mixtures of Cd-Hg is a particularly interesting occurance in light of the fact that Cd, as a discrete agent, is non-lethal following 96 hours exposure (Table 8). In theory there is no "a priori" reason why a non-lethal pollutant cannot promote the lethality of another toxic pollutant. Anderson and D'Apollonia (1978) suggest that it may be possible to separate, empirically, interactions in which the non-lethal component promotes binding properties (Sensitization; Ariens, 1972) from those which enhance the mechanism of toxic action (Potentiation; Ariens, 1972). This is possible on the assumption that in the former interaction the non-lethal agent acts prior to or concurrently with the toxic agent whereas in the latter the non-lethal agent can act only after binding has occurred (concurrent exposure). From the studies already discussed it is known that cadmium (the non-lethal agent) can promote the lethality of mercury if both are present simultaneously. Thus following the preceding rationale it may be possible to determine if this interaction involves sensitization or potentiation through a sequential exposure experiment.

Cadmium and Mercury: Sequential Exposure Experiment

The results of this study (Table 29) indicate that the toxicity of mercury can be enhanced through prior exposure to cadmium. Following the theory developed in the preceding section these data are consistent with a role of cadmium as a sensitizing agent. Two further facts support this assessment. Firstly, the slope of the dose-response line derived for the sequential

exposure experiment (7.843) is almost identical to that of exposure to discrete mercury (8.123). This would suggest that the mode of action has not been altered but merely promoted in some fashion. Secondly, the enhancement factor for this study is 2.62 ± 0.16, which is approximately the same as that calculated for concurrent exposure i.e. 2.03 ± 0.23 (Table 18).

The facts which have been presented would suggest that cadmium contributes to the toxicity of the mixture, post 96 hours, indirectly by promoting mercury toxicity.

In over view the interactive effects of Cd-Hg observed in this study have profound implications. In practical terms it is apparent that a standard 48 or 96 hours bioassay would not have identified the true nature of the hazard presented by these mixtures. Possibly of even greater importance however, is the observation that toxicants which on the basis of their incipient LC_{50}'s would be considered non-lethal may yet provide a significant effect through interactions with lethal contaminants.

Summary

With respect to the individual toxicants it was determined that for Brachydanio rerio the order of lethal potency was Hg(240 hr LC_{50}=0.15 mg/l)>Cu(240 hr LC_{50}= 0.26 mg/l)>Cd (240 hr LC_{50}=5.82 mg/l). The results of the present study are consistent with the hypothesis that a similar mode of toxic action exists for each of these metals. Furthermore the data support the definition of cadmium and copper as non-accumulative toxicants.

The response of B. rerio to mixtures containing two non-accumulative toxicants, cadmium and copper, was supra-additive. The magnitude of the supra-additive response was quantified through comparison of the observed data with that predicted for concentration addition. This analysis indicated the mixtures to be 2.011 $\pm$ 0.488 times more toxic (Table 14). This enhancement factor was corroborated by the time-response data as interpreted following the methods of Anderson and Weber (1975) but not by the toxic unit method.

Preliminary studies to identify the mechanistic basis of the supra-additive response have shown that a reciprocal potentiation of each metals' bioaccumulation into gill tissue occurs when in the presence of the other metal. This pharmacokinetic phenomena may, at least in part, explain the increased toxicity of these metals when in combination.

The response of B. rerio to mixtures containing an accumulative and a non accumulative toxicant, mercury and cadmium, was found to change through time. The observed data

suggested an infra-additive response at 48 hours, a concentration additive response at 96 hours and a supra-additive response at 240 hours (2.030 ± 0.227, Table 18). This dose-response pattern was closely paralleled by the time-response data as interpreted following the methods of Anderson and Weber (1975) however not by the toxic unit method.

Preliminary studies to further elucidate the nature of the interactive effect of Cd and Hg mixtures were conducted. The data suggest that the supra-additive response observed at 240 hours may result from a cadmium induced sensitization to the toxicity of mercury.

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APPENDIX 1

Formula For Calculating Comcentrations Of Metal 2 In Equivalent Units Of Metal 1.

$$\text{Log } X_1 = \text{Log } X_2 - (I - y) \left[(1/b_1) - (1/b_2) \right]$$

Where X_1 = Concentration of metal 1

X_2 = Concentration of metal 2

I = Probit value at the intersection of the dose - response functions for the individual metals.

y = Probit value corresponding to X .

b_1 = Regression coefficient of the dose - response curve for metal 1.

b_2 = Regression coefficient of the dose - response curve for metal 2.

Sample Calculation: 0.264 mg/L (96 Hr. LC_{50}) Copper in Equivalent units of Cadmium.

Dose - response functions. Copper: $Y = 7.325 + 4.022 (X)$
Cadmium: $Y = -1.989 + 9.137 (X)$

(i) Calculation of I.

(A) $7.325 + 4.022 (X) = -1.989 + 9.137 (X)$

$9.314 = 5.115 (X)$

$(X) = 1.821$

(B) $I = Y = 7.325 + 4.022 (1.821)$

$I = 14.64866$

(ii) $\text{Log } X_1 = - 0.578 - (14.64866 - 5.00) \left[(1/4.022) - (1/9.137) \right]$

$= 0.765$

$X_1 = 5.822$ mg/L = 96 Hr. LC_{50} Cadmium

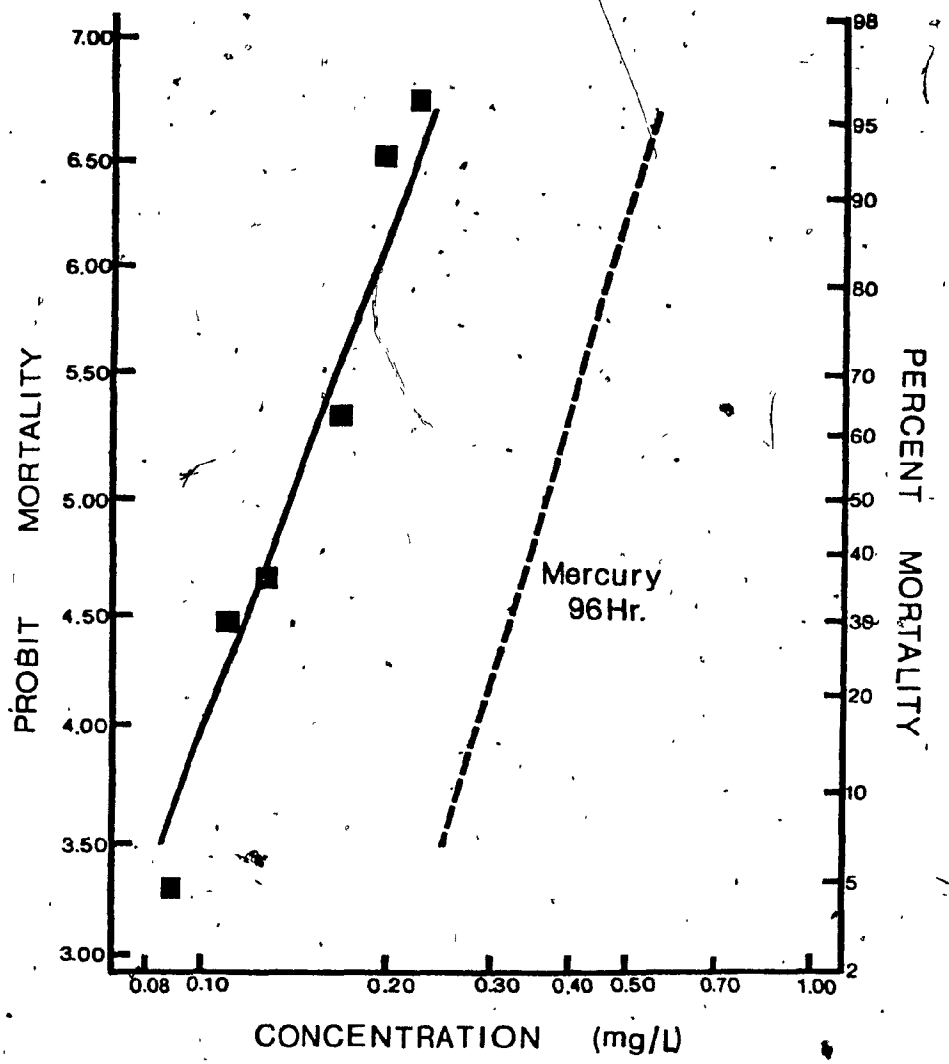

Figure 25. The lethal response curve for zebrafish, preexposed for 96 hours to cadmium, exposed to mercury. The response curve for zebrafish exposed to mercury for 96 hours is depicted for reference (—).

DISCUSSION

The evidence presented in this study suggests that the interactive effect on fish of mixtures containing accumulative and non-accumulative toxicants may vary through time. Furthermore, there was a high degree of agreement between the dose-response and time-response analysis in accounting for this variation in time-related toxicity. However, there were some exceptions with respect to the form of interaction implied by the toxic unit method of time response analysis.

Discrete Toxicants: Dose-Response

The following series represents the relative lethal potencies (LC_{50} values, Table 6) of the discrete metals at 96 hours:

Cu++ (0.26 mg/L)>Hg++ (0.36 mg/L)>Cd++ (5.82 mg/L)

and at 240 hours

Hg++ (0.15 mg/L)>Cu++ (0.26 mg/L)>Cd++ (5.82 mg/L).

It should be stressed that potency is a comparative term rather than an absolute expression of a toxicants' activity. Therefore, it is inappropriate to express the relative activities of two (or more) toxicants unless they produce their effects by the same mechanism. It is reasonable to assume that toxicants which possess similar chemical or physicochemical properties and initiate the same selective pharmacologic response do so by the same mechanism (Levine, 1973). From this it follows that toxicants which operate through a common mode of action will have parallel dose-response curves. Therefore, lacking complete mechanistic knowledge it is the statistical similarity of

dose-response curves which justifies the determination of relative potencies.

The regression coefficients for each of the discrete toxicants were not found to be significantly different ($P>0.05$). Thus it is considered correct to discuss copper, cadmium and mercury in terms of their relative potencies. The methodology of Finney (1971), in which dose-response curves are constrained to absolute parallelism, was employed to derive numerical values to represent the relative potencies of the toxicants. It was found that at 96 hours mercury was 17.71 times more potent than cadmium and 0.77 times as potent as copper. The same analysis revealed that at 240 hours mercury was 40.73 times more potent than cadmium and 1.78 times more potent than copper.

The relative toxicity of heavy metals is generally perceived to be a reflection of their different affinities for various ligands in a critical organ(s). If a sufficient number of "sites" are bound a functional change may occur, the consequence of which is dependent upon the function of the binding ligands. Therefore, it is considered that the binding of metals to ligands may constitute the common mode of toxic action at the biochemical level. With respect to copper, cadmium and mercury their affinity for various ligands has been documented in the literature (Shaw and Grushkin, 1957; Passow et al., 1960). In all cases the order of affinity was reported to be $Hg^{++}>Cu^{++}>Cd^{++}$ which corresponds with the relative potency distribution noted in the present study. This would not however be expected to be the order of potency in every situation because there has been no accounting

for different rates of absorption or tolerances between aquatic organisms. Thus the relative potencies of the heavy metals may not be accurately ranked strictly from a knowledge of their relative affinities for ligands. Chapman (1978) has attempted to rank the overall hazard of heavy metals which are common to the aquatic environment. He has listed nine metals in five categories as follows:

Metal	Hg,Ag,Cd	Cu	Ni,Pb,Zn	Sb	Sn
Toxic level	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M

As can be seen from Chapman's list all three metals included in this study are considered to be extremely toxic. Chapman (1978) also identified copper, cadmium and mercury to be of particular concern because they are mobilized within the environment in amounts which approach their toxic levels.

Discrete Toxicants: Time-Response

Dose-response data are generally considered to be the best standard of reference between toxic substances (Bliss, 1937). However, a comprehensive profile of relative toxicities must also include an analysis of time-response data. The toxicity response curves for cadmium and copper were found to be curvilinear with asymtote relative to the x axis at 5.83 mg/L and 0.26 mg/L respectively (Figure 11 and 12). The curvilinear shape of these toxicity curves has been previously reported in the literature: Cadmium-Schweiger, 1957; Lloyd, 1960; Roch, 1979: Copper-Lloyd, 1960; Spraque and Ramsay, 1965; Spear, 1977. Brown (1973) rationalized that the death of fish, as witnessed in toxicity tests is the summative consequence of the failure of a number of

physiological functions. The curvilinear shape of the toxicity response curve that leads to an asymtote parallel to the time axis is interpreted as the progressive achievement of homeostasis in the test fish as the concentration decreases.

The suitability of using a logarithmic scale for both time and concentration has been indicated by many researchers (Gardner et. al., 1977). The time-response data for cadmium and copper have been converted to straight line relationships by a log- log transformation (Figures 15,16 and Table 11). This representation has the advantage of allowing a clear determination of the exposure duration at which the toxicity response curve becomes parallel to the time axis. This was determined to be 5190 minutes and 3442 minutes for cadmium and copper respectively.

There was no evidence of a threshold to mercury toxicity within the 10 day exposure period. Furthermore, survivors of the 10 day exposure period (0.018 mg/L), continued to die after having been placed in a contaminant free environment. The toxicity response curve for mercury (Figures 14 and 17) was found to be rectilinear. The accuracy of this assessment is indicated by previous documentation in the literature by Macleod and Pessah (1973). It follows from Browns' (1973) rationale that the test fish were not acclimating to the presence of mercury even at the lowest concentration tested of 0.018 mg/L. Furthermore, it implies that the processes of accumulation and elimination have not reached equilibrium within the 10 day exposure period. Therefore, mercury is categorized as an accumulative toxicant whereas cadmium and copper are referred to as non-accumulative toxicants.

There are two general methods of expressing the relative potencies of toxic substances from time-response data. The first involves a comparison of lethal threshold concentrations irrespective of when the threshold is attained. For example according to the following scale recommended by a joint committee of experts from IMCO/FAO/UNESCO/WHO (1969), the present study rates mercury and copper as "very toxic" and cadmium as "toxic".

(1) "Very toxic" Threshold below 1 ppm;

(2) "Toxic" Threshold between 1 and 100 ppm;

(3) "Moderately toxic" Threshold between 100 and 1000 ppm;

(4) "Slightly toxic" Threshold between 1000 and 10,000 ppm;

The second method involves a comparison of doses which have lead to equivalent responses at the same point in time. According to this method the order of relative potencies at 96 hours was:

$Cu^{++} > Hg^{++} > Cd^{++}$

and at 240 hours was

$Hg^{++} > Cu^{++} > Cd^{++}$

These data confirm the order of relative potencies which were specified by the dose-response data. A more rigorous analysis of the time to response data indicated that at 96 hours mercury was 10.16 times more potent than cadmium and 0.45 times as potent as copper. At 240 hours mercury was 32.33 times more potent than cadmium and 1.44 times more potent than copper.

Comparison of dose-response and time-response data:

Discrete Toxicant

Table 30 lists the relative potency factors which have been derived from the two methods of analysis. It would appear that both representations of the toxicity data are in close agreement.

Table 30: Relative potency factors calculated from dose-response and time-response data.

	Relative Potency Factor			
	96 Hours		240 Hours	
	Time Response	Dose Response	Time Response	Dose Response
Hg/Cu	0.45	0.77	1.44	1.78
Hg/Cd	10.16	17.71	32.33	40.73

MIXTURES OF TOXICANTS

Cadmium and Copper: Dose-Response

The results of this study indicated that neither the concentration addition nor response addition model (Bliss, 1939; Anderson and Weber, 1975) adequately predicted the toxicity of mixtures containing copper and cadmium (Tables 12, 13 and Figure 18). These toxicants were initially assumed to be concentration additive because there were no significant differences between the slopes of their respective lethal tolerance distribution curves. However the empirically determined dose-response range indicated that copper and cadmium were supra-additive (2.01 x) to the toxicity predicted for concentration addition (Table 14). Eisler and Gardner (1973), working with Fundulus heteroclitus, reported that the lethal toxicity of copper and cadmium mixtures were supra-additive. These results supported the findings of La Roche (1972) who observed that the lethal effects of copper-cadmium mixtures were supra-additive.

We can perceive in a general way that the observed supra-additive response must be a result of interactions which alter the rate at which the toxicants reach their site of action and or the sequence of events which follow the arrival of the toxicant molecules at the site of action (Figure 7). A potential explanation for the interaction of cadmium and copper is offered later in the discussion.

Cadmium and Copper: Time-Response

Analysis of the toxicity response curve for the mixtures containing cadmium and copper supports the conclusion of supra-additivity. The basic curvilinear shape which characterized the discrete toxicity response curves appears to adequately represent the mixture (Figure 21). The graphical analysis of the curve suggests an asymtote relative to the x axis at approximately 2.8 ppm (total concentration as cadmium). A "predicted" toxicity response curve can be computed assuming concentration addition. This curve is intermediate between the two discrete response curves, taking into account the ratio of toxicants within the mixture (Figure 22). The enhanced potency of the mixture was computed to be approximately 2.08 times that predicted. The comparison of the dose-response (2.01 x) and time-response (2.08 x) enhancement factors indicates a strong correlation.

Another method for presenting toxicity response curves has been proposed by Lloyd (1962). The concentration of toxicants are expressed as fractions of their median lethal threshold concentrations.

$$Ms/Mt \qquad (8)$$

Where Ms= the concentration of toxicant

Mt= the median lethal threshold concentration.

The effective concentration of a mixture is computed from the sum of the fractions derived for each toxicant present in the mixture. The time to response data obtained for copper and cadmium mixtures were subjected to the preceding technique

(Table 31 and Figure 26). While this representation clearly indicates a supra-additive response the method suggests an enhancement factor of approximately 4.0 x the predicted. The previous analysis of time-response data has indicated an enhancement of 2.08. Therefore, there is a difference in the estimation of potency between these methodologies.

Cadmium and Mercury: Dose Response.

The results of this study indicated that the 96 hour toxicity of cadmium-mercury mixtures could be predicted from a knowledge of the discrete dose-response curves. The slopes of the dose-response curves for 96 hours exposure to pure solutions of cadmium and mercury were not significantly different ($P < 0.05$). Therefore, mixtures of mercury and cadmium were composed on the assumption that they were concentration additive. It was subsequently found that observed and predicted responses were in close agreement. Thus it was concluded that concentration addition adequately described the toxicity of the mixtures at 96 hours.

The dose response curves for discrete solutions of cadmium and mercury were parallel at 240 hours; consequently concentration addition was predicted for the binary mixtures. The results of bioassays indicated the mixtures to be neither concentration nor response additive but supra-additive (2.03 x, Table 18). This is a particularly surprising result when it is considered that the concentration of cadmium in the mixture is significantly lower than its median lethal threshold level as a discrete agent. In practical terms what this means is that on the basis of time response data we suspect that the test organisms are able to

Table 31 Toxic unit analysis of cummulative time-mortality data for zebrafish exposed to mixtures of cadmium and copper.

Mean Assayed Concentration of Copper (mg/L)	Ms/Mt Value for Copper.	Mean Assayed Concentration of Cadmium (mg/L)	Ms/Mt Value for Cadmium	Total Ms/Mt Value	LT_{50} (Min)
0.016	0.06	1.310	0.23	0.29	2176
0.029	0.11	1.395	0.24	0.35	1930
0.033	0.13	1.437	0.25	0.38	2501
0.050	0.19	1.685	0.29	0.48	1711
0.065	0.25	1.623	0.28	0.53	1856
0.058	0.22	1.955	0.34	0.56	1177
0.097	0.37	1.395	0.24	0.61	2122
0.061	0.24	2.135	0.37	0.61	1193

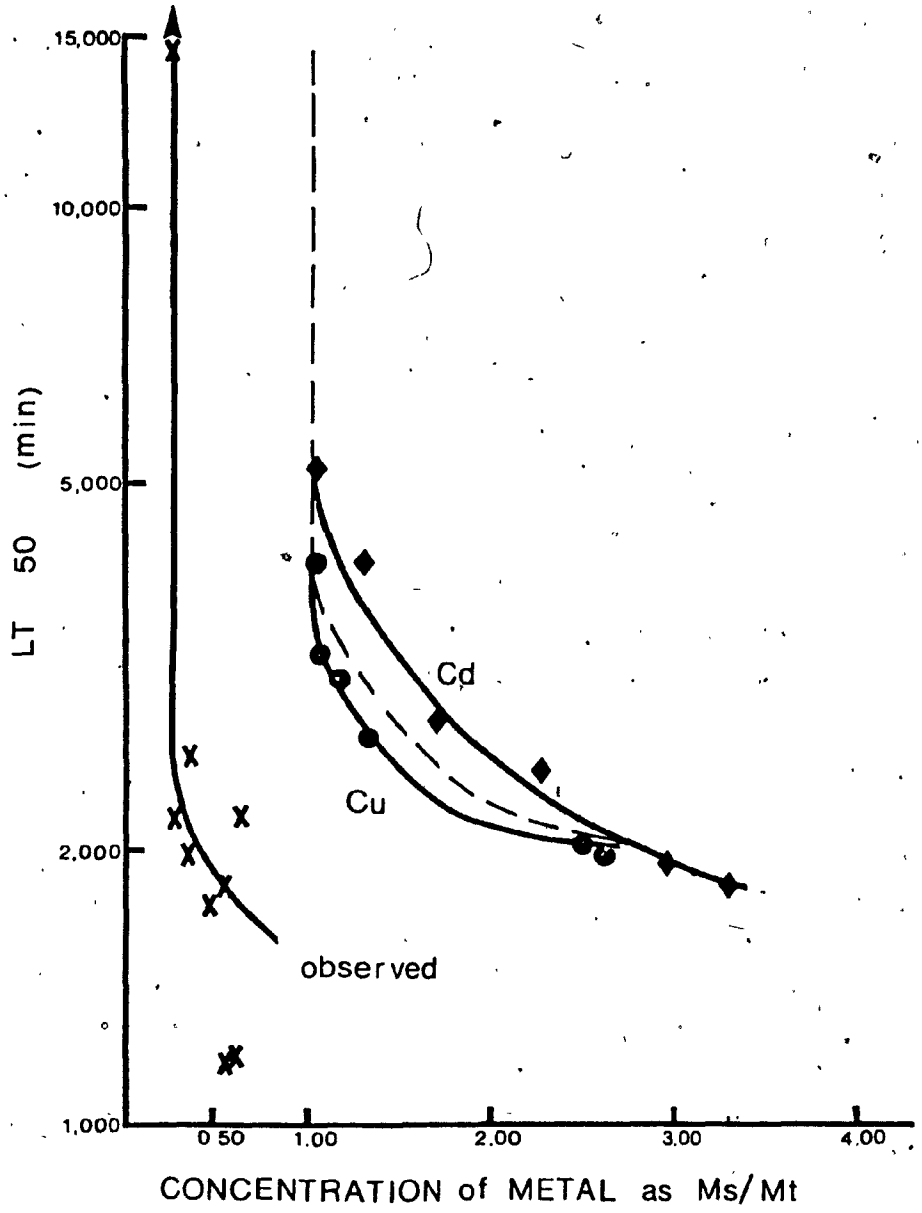

Figure 26. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and copper. For an explanation of Ms/Mt see text. The relationship predicted on the basis of strict additivity is depicted for reference (— — —).

achieve a state of absorption - elimination equilibrium with cadmium levels this low. Therefore, beyond 96 hours cadmium would not be expected to contribute directly to the toxicity of the mixture.

To our knowledge there are no lethal studies involving combinations of cadmium and mercury to which our results could be compared. However a study conducted by Weis and Weis (1978), utilizing the rate of tail regeneration in F. confluents as an indicator, observed an infra-additive response. Clearly the interactions of cadmium and mercury are complex and a potential explanation of the observed effects is offered later in the discussion.

Cadmium and Mercury: Time Response

The toxicity response curves, derived from the mercury and cadmium mixture data (Figure 24), support the dose-response findings i.e. concentration additive at 96 hours and supra-additive at 240 hours. A predicted toxicity curve could be computed, as previously described, for exposure periods not in excess of the median lethal threshold for cadmium (5190 minutes). For exposure periods in excess of the lethal threshold of cadmium it was deemed reasonable to use the discrete mercury toxicity curve as representative of the predicted response. The observed response curve intersects the "predicted" curve at 102 hours. This indicates that the response to mixtures may be accurately predicted by assuming concentration addition in the region of 102 hours. Analysis of the toxicity response curves at 240 hours indicates that mercury and cadmium are supra-additive (3.6x, Figure 24).

These data supports the dose-response data which indicated the mixtures to be concentration additive at 96 hours and supra-additive at 240 hours (2.03 x, Table 18).

An interesting aspect of the toxicity response curve was the indication of infra-addition for relatively short exposure periods. To confirm the accuracy of this observation a dose-response analysis was conducted for forty eight hours exposure (Figure 27).

While there are only two observed response points they both indicate an infra-additive response (both occur outside the 95% confidence limits). Analysis of the toxicity curves suggests that the mixtures will be 0.43 times as potent as predicted. The dose-response analysis indicates that the potency of the mixtures is 0.61 x that predicted. While the relative potency factors are not in close agreement the "short exposure" infra-additive response was confirmed.

The toxic unit method for presenting toxicity response data indicated the mixtures to be approximately concentration additive for all mixtures with effective concentrations in excess of unity (Table 32 and Figure 28). The reason why this representation of the data does not reveal the infra-additive response is not immediately clear.

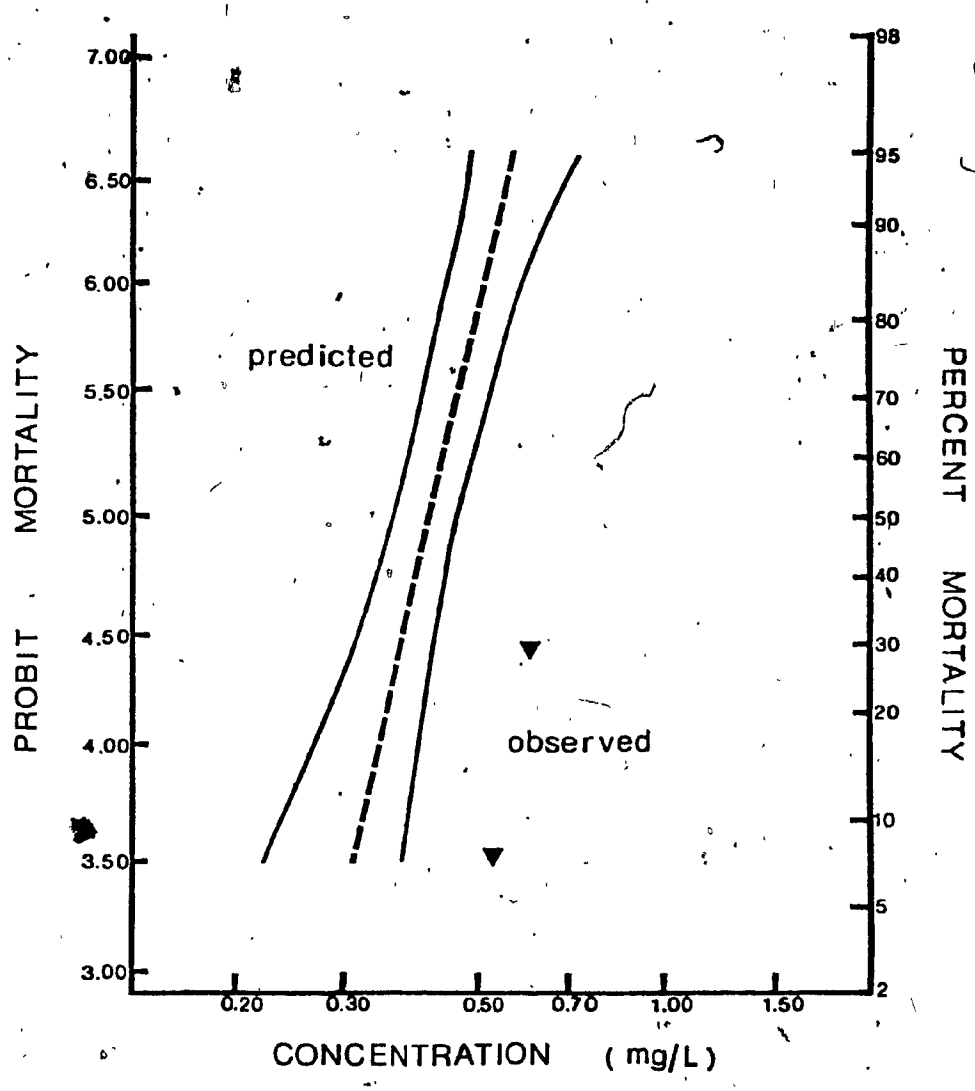

Figure 27. The lethal response of zebrafish exposed to two mixtures of cadmium and mercury expressed as equivalent units of mercury for 48 hours. The lethal response curve predicted on the basis of concentration addition plus the 95% confidence limits is depicted for reference.

Table 32 Toxic unit analysis of cummulative time-mortality data for zebrafish exposed to mixtures of cadmium and mercury.

Mean Assayed Concentration of Mercury (mg/L)	Ms/Mt Value for Mercury	Mean Assayed Concentration of Cadmium (mg/L)	Ms/Mt Value for Cadmium	Total Ms/Mt Value	LT_{50} (Min)
.059	0.17	1.28	0.22	0.39	15356
.062	0.18	1.36	0.23	0.41	10717
.069	0.20	1.45	0.25	0.45	11374
.077	0.21	1.60	0.28	0.49	12340
.085	0.23	1.75	0.30	0.53	10935
.125	0.33	1.85	0.32	0.65	9240
.235	0.85	1.71	0.29	1.15	7301
.306	1.29	2.09	0.36	1.65	6127
.431	1.43	2.09	0.36	1.79	5106
.452	1.51	2.49	0.43	1.93	4470
.626	1.97	2.71	0.47	2.44	3301

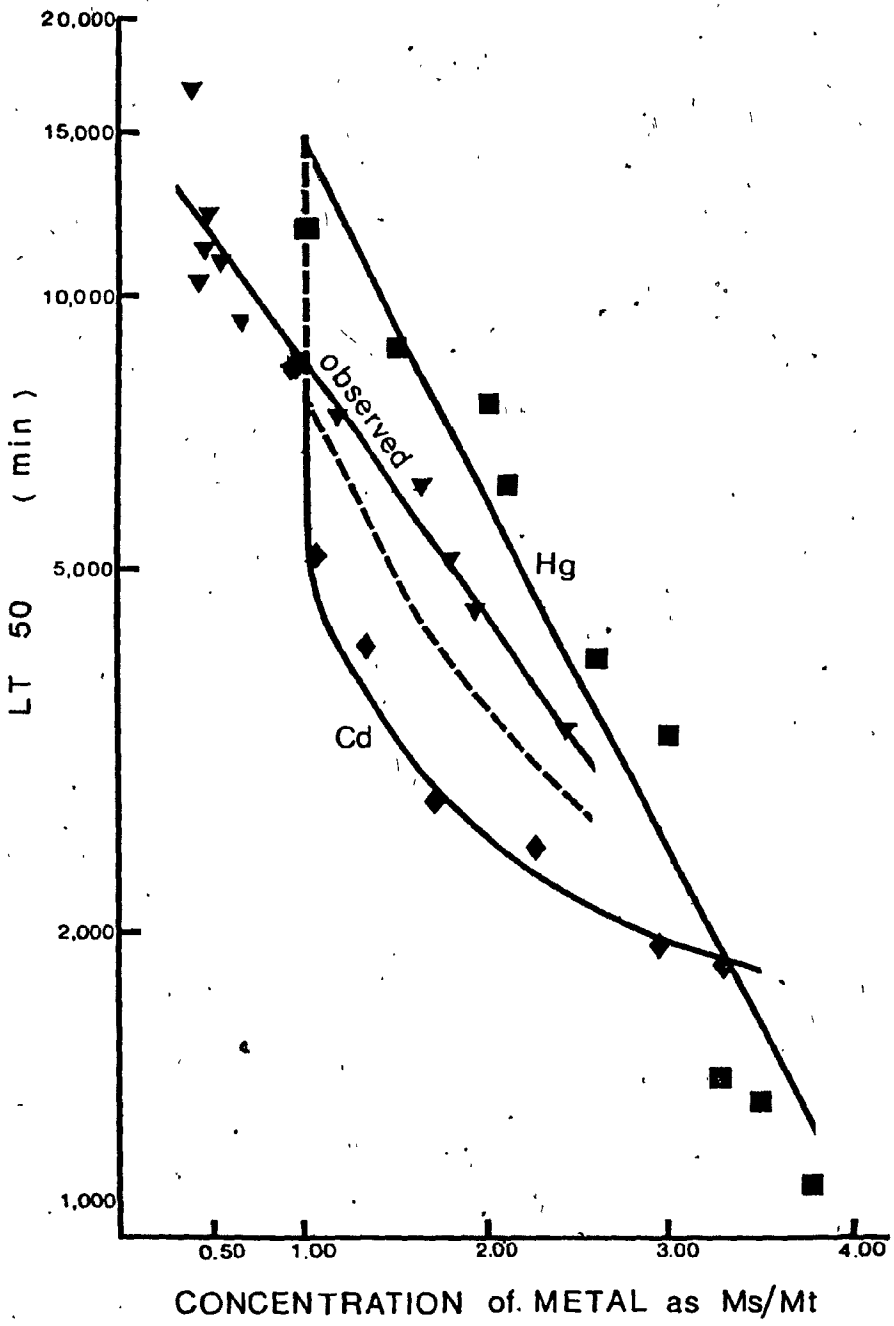

Figure 28. Median mortality-time curve (LT_{50}) for zebrafish exposed to mixtures of cadmium and mercury. For an explanation of Ms/Mt see text. The relationship predicted on the basis of strict additivity is depicted for reference (———).

Cadmium and Copper: Supra-additive interaction

A general theory has been proposed in which the toxicity of heavy metals results from their interaction with gill tissues (Lloyd, 1962). Analysis of the tissues of rainbow trout exposed to a lethal solution of zinc, containing Zn^{65} as a tracer, revealed that the highest percentage of zinc was located in the gill tissues (Lloyd, 1960). Schweiger (1957) determined the body burden of cobalt and manganese in carp which had been killed by these metals. Subsequently he injected carp with three times the body burden of cobalt and three hundred times the amount of manganese and found the fish survived. These results suggest the toxic action of at least some metals is not internal but related to the epithelial cells of the gill lamellae. The body of current literature would tend to suggest that most heavy metals, at the acutely lethal level, have a common site of action in the gills. Furthermore it is the binding of the divalent cations to protein legands eg. OH, COOH, PO_3H_2, SH_2, NH_2 which leads to a breakdown in normal cellular metabolism (Passow et al., 1960). Although specific affinity for these ligands may vary between metal species the general effect may be identical or indistinguishable because of biological variability between test organisms. This may explain why the dose-response functions derived for copper, cadmium and mercury were not statistically different.

The supra-additive toxicity of cadmium and copper mixtures must result from some kinetic and or dynamic interaction (Ariens, 1972; Anderson and D'Appollonia, 1978). Two pieces of evidence in our studies have suggested that the physiological interaction is a kinetic phenomena (however they do not exclude the possibility of a dynamic interaction). Firstly, the regression coefficient of the observed dose - response function was not significantly different from the regression coefficient of the predicted dose-response function. This suggests (for reasons previously given) that the mode of action is unchanged and has merely been promoted in some fashion.

The second piece of evidence comes from a comparison of the toxicity response curves (Figure 22). The slopes of the observed and predicted toxicity curves are parallel. This suggests that there has been no change in the mode of action even though the toxicity has been enhanced.

Preliminary studies to determine if a kinetic interaction is involved indicate that cadmium and copper potentiate each others' uptake into gill tissue. Observation of tables 23, 24, 25 and 26 indicates that only the gills and not the bodies were sites of significant metal accumulation during the initial 20 hours of exposure. Tables 23, 24 and figures 29a to 30c indicate that a causal relationship appears to exist between increased ambient concentrations of discrete solutions and increased levels of metal in the gill tissue. Additional evidence for this hypothesis is gained from two areas. Firstly, for each concentration of discrete metal, the rate of metal accumulation is well described by a least squares regression equation (Table 27).

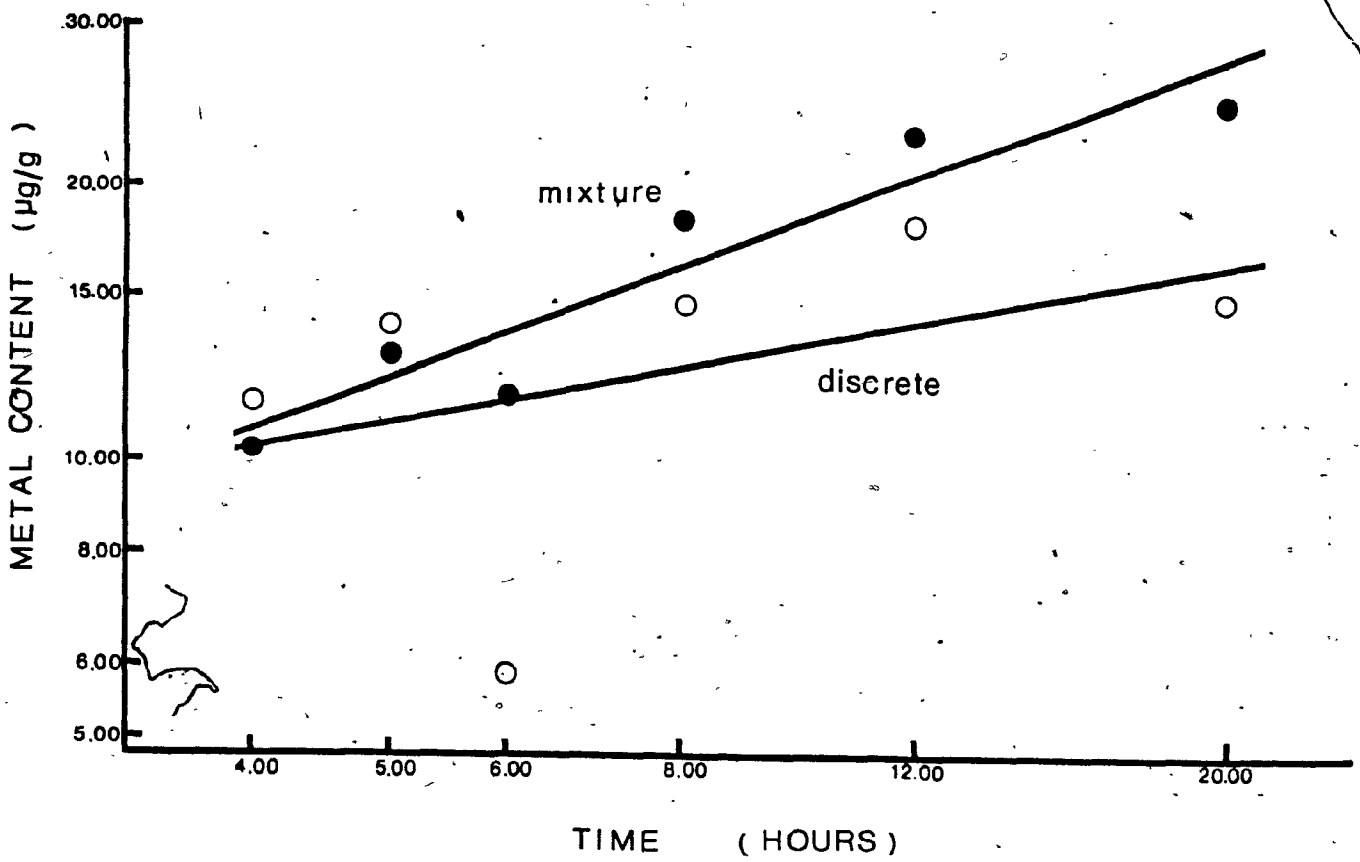

Figure 29a. Accumulation of copper in gill tissue during exposure to discrete copper (O , 0.22 mg/L) and when concurrent with cadmium (● , 0.21 mg/L + 1.880 mg/L Cd).

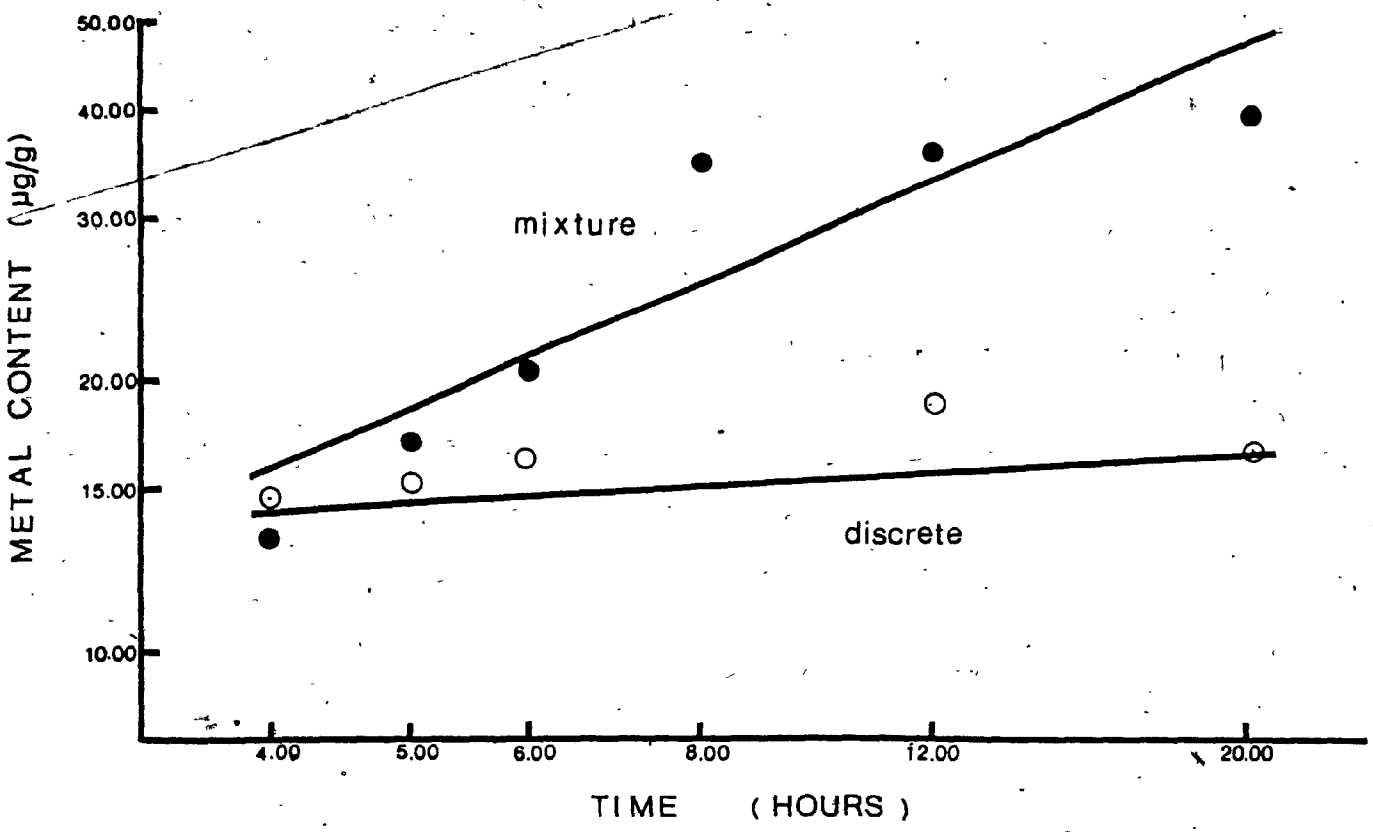

Figure 29 b. Accumulation of copper in gill tissue during exposure to discrete copper (○, 0.043 mg/L) and when concurrent with cadmium (●, 0.043 mg/L + 2.826 mg/L Cd).

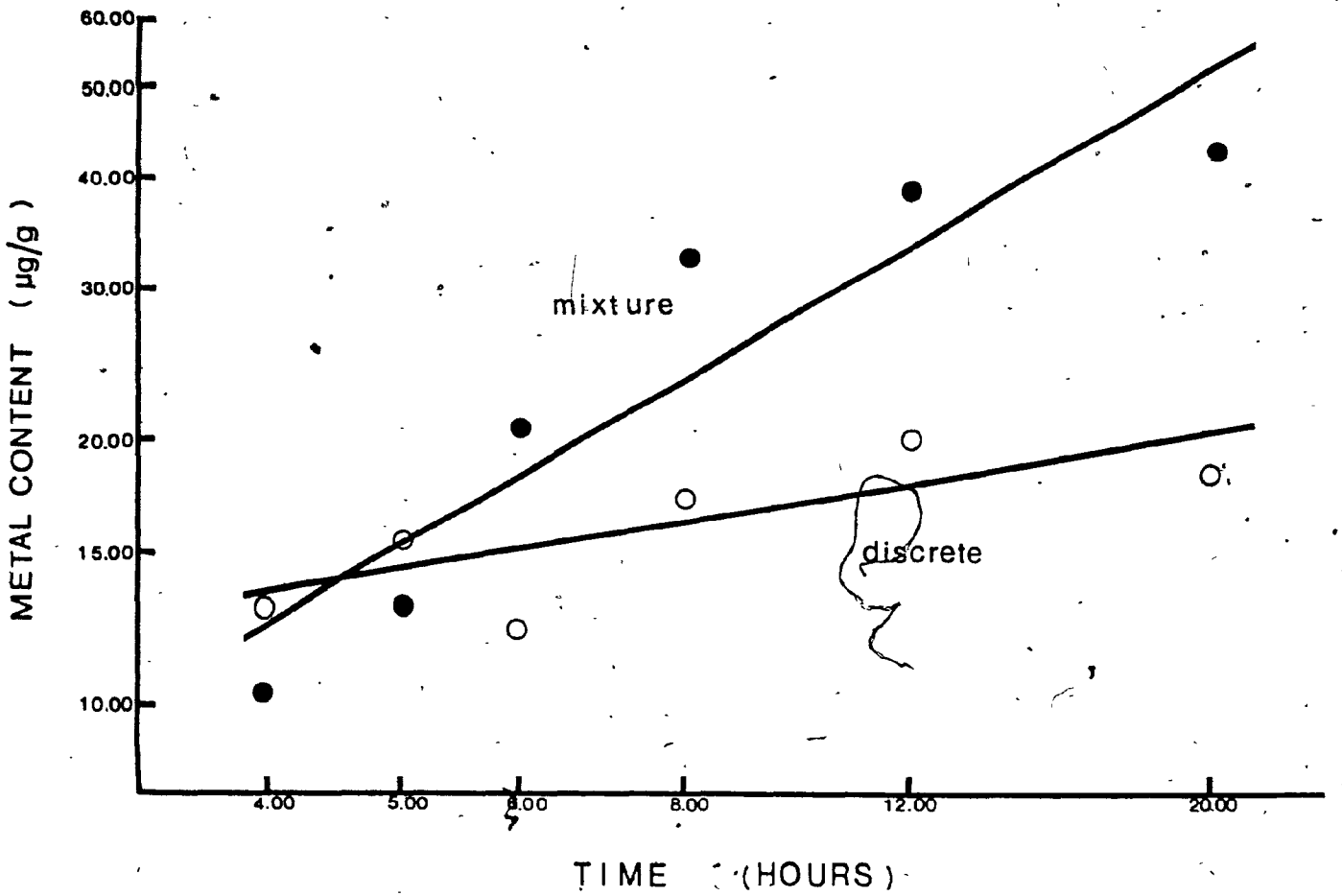

Figure 29. c. Accumulation of copper in gill tissue during exposure to discrete copper (○ , 0.083 mg/L) and when concurrent with cadmium (● , 0.088 mg/L + 3.565 mg/L Cd).

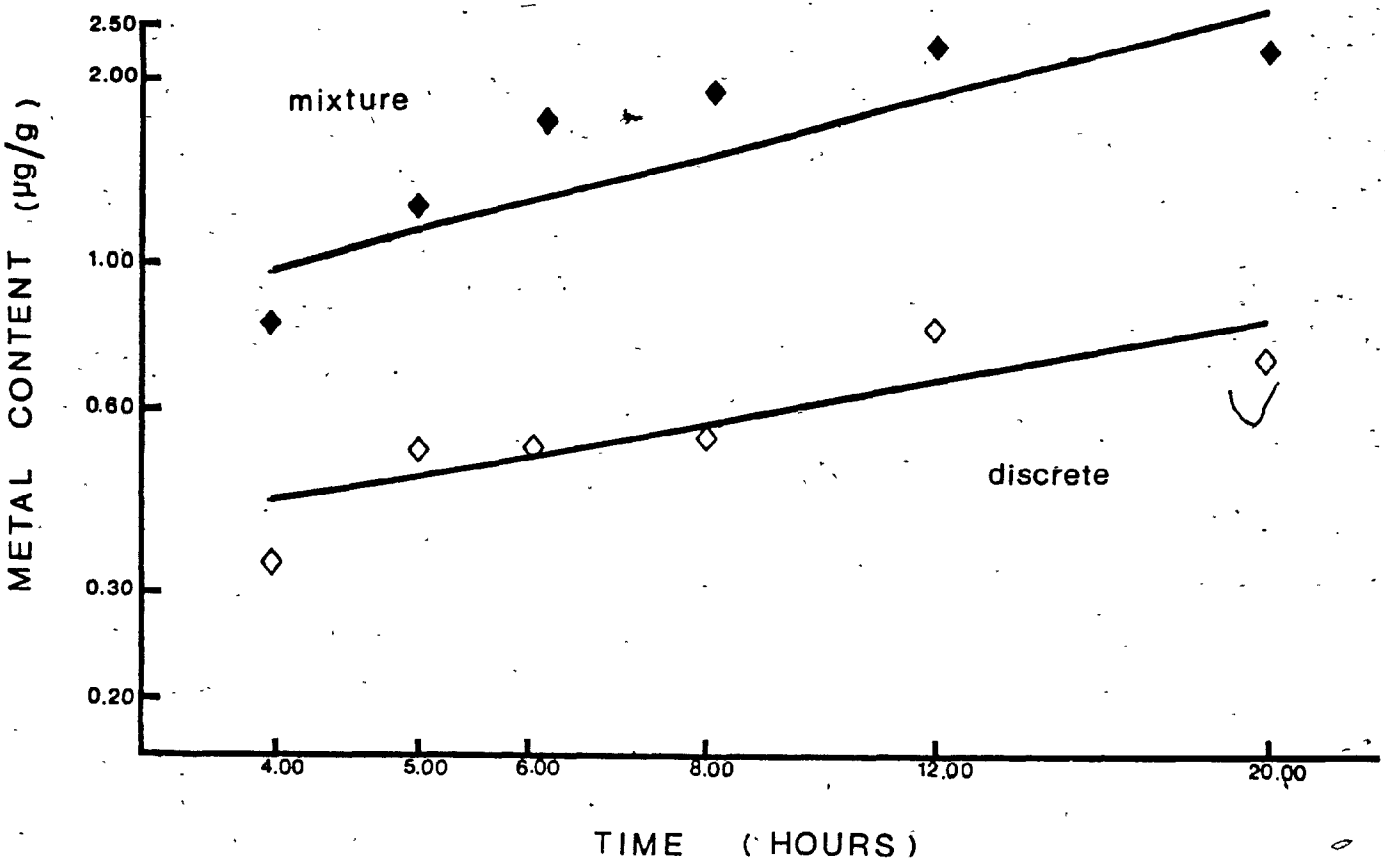

Figure 30 a. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 1.982 mg/L) and when concurrent with copper (◆ , 1.880 mg/L + 0.021 mg/L Cu)

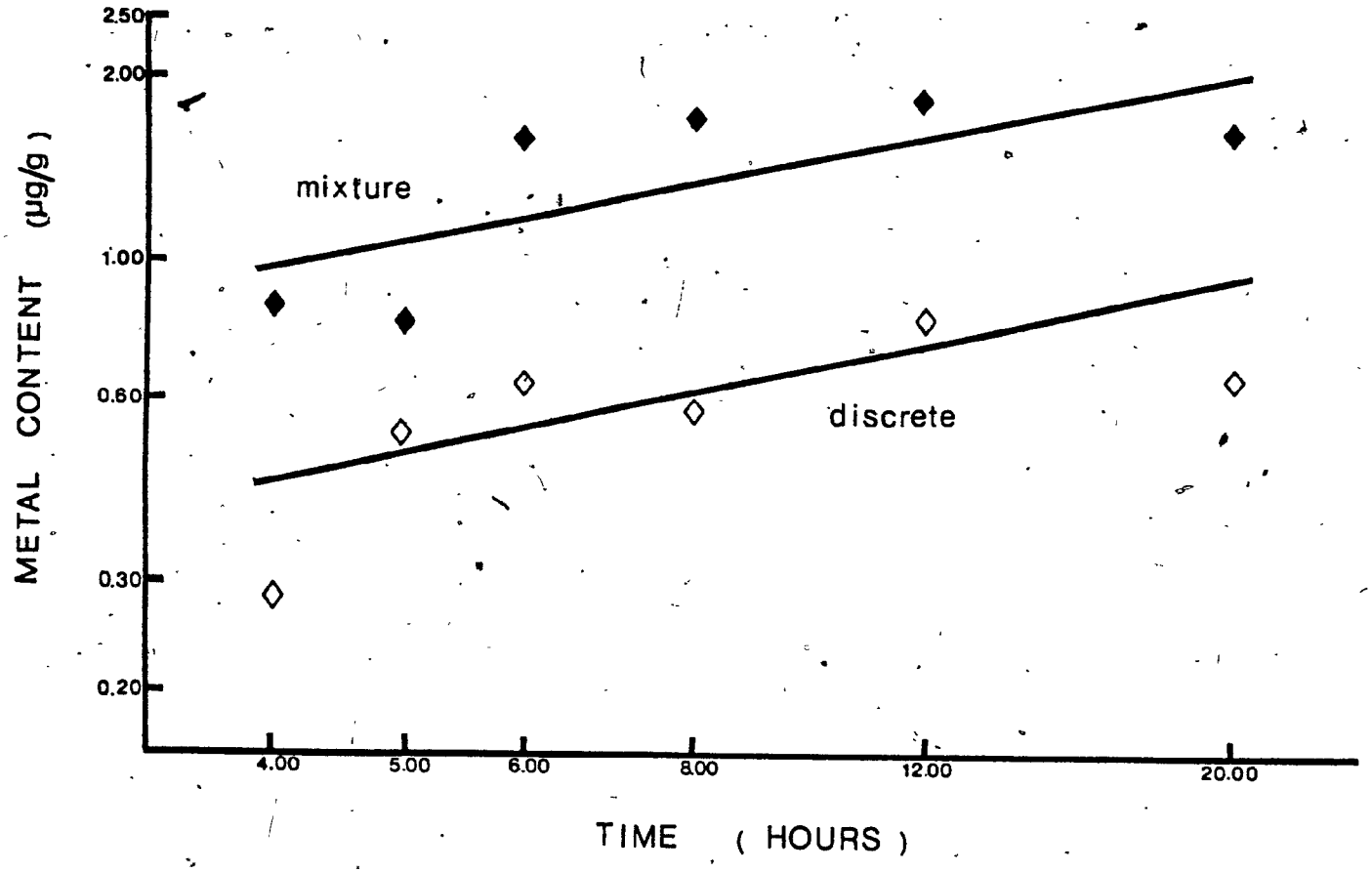

Figure 30 b. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 2.718 mg/L) and when concurrent with copper (◆ , 2.626 mg/L + 0.043 mg/L Cu).

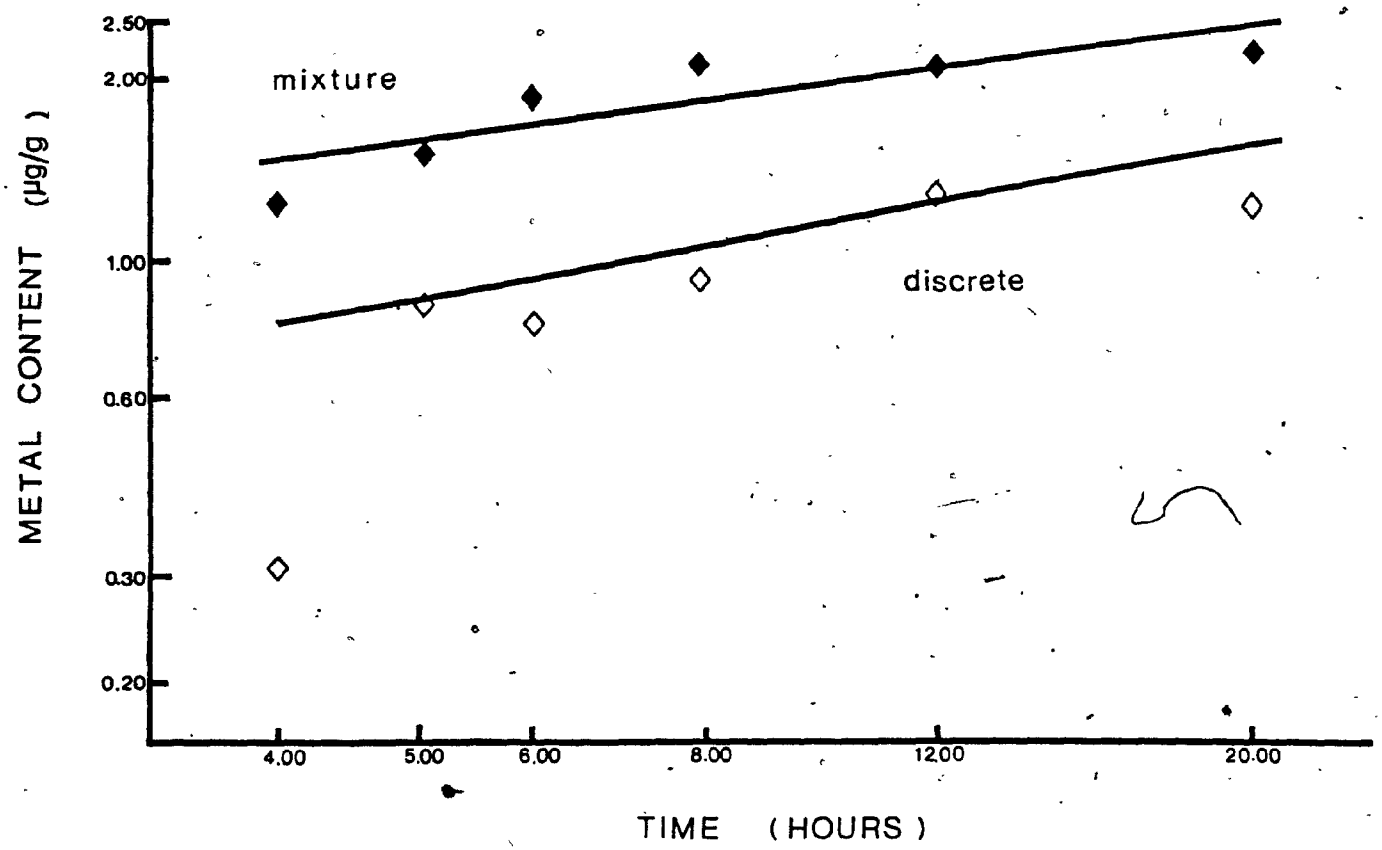

Figure 30 c. Accumulation of cadmium in gill tissue during exposure to discrete cadmium (◇ , 3.555 mg/L) and when concurrent with copper (◆ , 3.565 mg/L + 0.088 mg/L Cu).

This suggests a mechanistic relationship between metal accumulation and the ambient concentrations. Secondly, between concentrations, at twenty hours exposure, a least squares regression equation with positive slope and high correlation coefficient can be derived (Tables 23, 24 and Figures 31 and 32).

The most significant aspect of this study is that the relative bioconcentration of copper and cadmium is greater in gill tissues of fish exposed to mixtures than to discrete solutions. However, there are three further points of interest. Firstly, the stoichastic relationship noted earlier for each discrete metal ambient concentrations and the accumulation in the gills appears to be maintained for the mixtures. i.e. the rate of metal accumulation is well described by a least squares regression function. This tends to suggest that the mechanism of bioconcentration has not been altered but merely enhanced. Secondly, between concentration of copper, at twenty hours exposure, a least squares regression equation with positive slope and high correlation coefficient can be derived (Table 23 and Figure 31). This represents further evidence for the mechanistic relationship between accumulation and the ambient concentration which has been promoted through the concurrent presence of cadmium. Thirdly, there is no apparent relationship between cadmium content in gill tissues and the three different mixtures concentrations, at twenty hours exposure (Figure 32). The absolute amounts which have been accumulated in gill tissue are significantly greater for the mixtures relative to their respective discrete solutions but no dose related increase exists for the mixtures. This suggests that

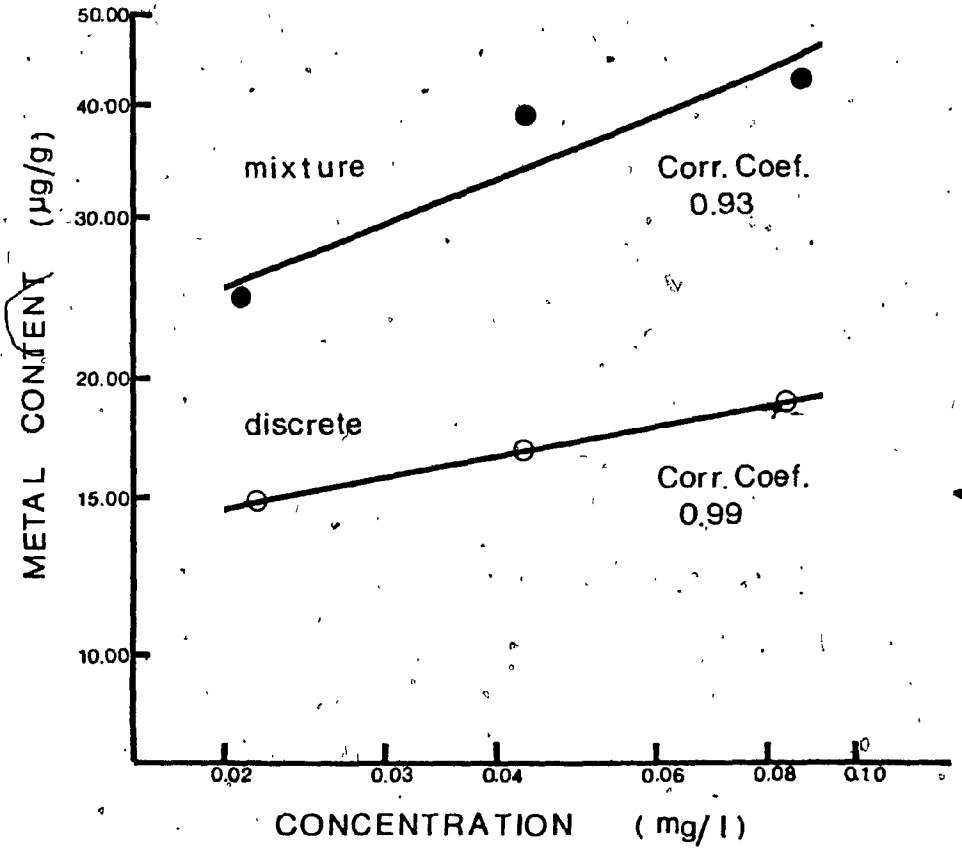

Figure 31. Copper content in fish gill tissue following 20 hours exposure to discrete (O) solutions and in combination (●) with cadmium. The ordinate value gives the copper content of the gills and the abscissal value gives the exposure concentration of copper. The binary combinations contained 1.880, 2.626, 3.565 mg/l cadmium in order left to right.

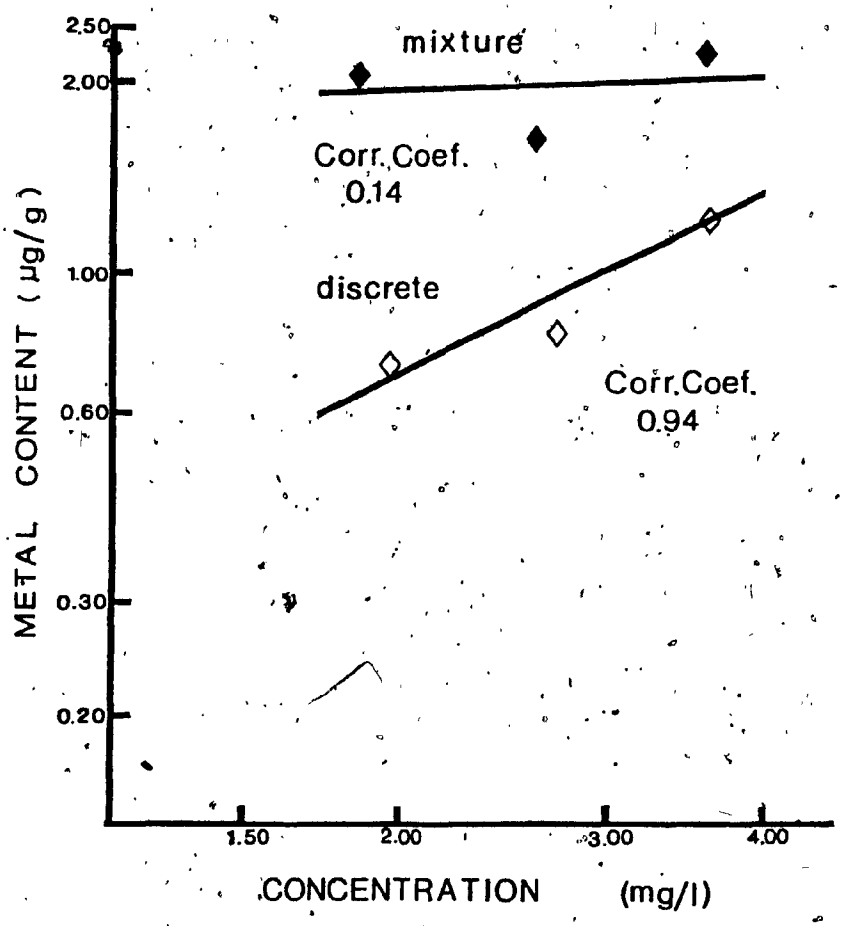

Figure 32. Cadmium content in fish gill tissue following 20 hours exposure to discrete (◇) solutions and in combination (◆) with copper. The ordinate value gives the cadmium content of the gills and the abscissal value gives the exposure concentration of cadmium. The binary combinations contained 0.021, 0.043, 0.088 mg/l copper in order left to right.

the mechanism for cadmium accumulation has been saturated for even the lowest mixture concentration.

Therefore there would appear to be a physiological interaction between copper and cadmium which results in a reciprocal potentiation of uptake rates. In as much as the magnitude of response is related to the concentration of toxicant at the site of action these results may explain the supra-additive response. Furthermore, the increased rate of uptake may explain the observed enhancement in the time-response data (Figure 22).

It is important to point out that while these results clearly indicate the presence of a kinetic interaction there is no reason to exclude the possibility of a dynamic interaction as well.

Cadmium and Mercury: Variable interactive effect.

The interactive effect of cadmium and mercury on fish would appear to change through time. Empirically the effect of the mixtures may be separated into three segments:

1. an infra-additive interaction observed at 48 hours exposure ,
2. a concentration additive interaction at 96 hours exposure and
3. a supra-additive interaction at 240 hours exposure.

The available data for the infra-additive effect of rapidly lethal mixtures of Cd and Hg consists of two response points (Figure 27). Without a knowledge of the response over the entire response range (0-100%) it is difficult to draw any inferences concerning the mechanism of this effect. A study which was conducted by Weis and Weis (1978) utilizing the rate of tail regeneration in F. confluents as an indicator also observed an infra-additive response to Cd and Hg mixtures. However the authors did not publish a mechanism for the infra-additive effect therefore it is difficult to assess to what degree the results of their sub-lethal experiment could apply to the effect noted in this study. A less than predicted response to lethal concentrations of metals has been reported for mammals pretreated with sub-lethal quantities of cadmium (Schnell, 1978). The mechanism of this effect is thought to be related to elevated levels of the hepatic protein, metallothionein. It has been shown that cadmium can induce the synthesis of this protein which has

the capacity to bind heavy metals thereby preventing their interaction with vital cellular constituents. Thus the underlying mechanism for this cadmium-induced tolerance is an increased detoxification capacity. There are, however, at least two reasons why this mechanism is unlikely to explain the infra-additive effect noted in this study. Firstly, the protective effect is noted only if the organism is pretreated in such a way that it has enough time to produce sufficient metallothionein to withstand the subsequent lethal challenge. This was not the case in the present study where the exposure was concurrent. Secondly, the results of a sequential experiment which was conducted (Table 29) did not indicate any protective capacity of cadmium pretreatment. Thus the explanation of the infra-additive effect seen in this study awaits further research, possibly in the area of bioaccumulation.

The response of test organisms exposed to mixtures of Cd and Hg for 96 hours would appear to be effectively described by the empiricale model of concentration addition (Table 15, Figure 19). Considering the overall pattern of the interactive effect (Infra-additive-Supra-additive) of these metals through time it is difficult to interpret exactly what this observation means. More clearly, does this represent a definite physiological event or is it a coincidental observation marking the mid point of the transition from one form of interaction to another. Of these two possibilities the second would seem to be the more appropriate.

The supra-additive response of the organisms exposed to mixtures of Cd-Hg is a particularly interesting occurance in light of the fact that Cd, as a discrete agent, is non-lethal following 96 hours exposure (Table 8). In theory there is no "a priori" reason why a non-lethal pollutant cannot promote the lethality of another toxic pollutant. Anderson and D'Apollonia (1978) suggest that it may be possible to separate, empirically, interactions in which the non-lethal component promotes binding properties (Sensitization; Ariens, 1972) from those which enhance the mechanism of toxic action (Potentiation; Ariens, 1972). This is possible on the assumption that in the former interaction the non-lethal agent acts prior to or concurrently with the toxic agent whereas in the latter the non-lethal agent can act only after binding has occurred (concurrent exposure). From the studies already discussed it is known that cadmium (the non-lethal agent) can promote the lethality of mercury if both are present simultaneously. Thus following the preceding rationale it may be possible to determine if this interaction involves sensitization or potentiation through a sequential exposure experiment.

Cadmium and Mercury: Sequential Exposure Experiment

The results of this study (Table 29) indicate that the toxicity of mercury can be enhanced through prior exposure to cadmium. Following the theory developed in the preceding section these data are consistent with a role of cadmium as a sensitizing agent. Two further facts support this assessment. Firstly, the slope of the dose-response line derived for the sequential

exposure experiment (7.843) is almost identical to that of exposure to discrete mercury (8.123). This would suggest that the mode of action has not been altered but merely promoted in some fashion. Secondly, the enhancement factor for this study is 2.62 ± 0.16, which is approximately the same as that calculated for concurrent exposure i.e. 2.03 ± 0.23 (Table 18).

The facts which have been presented would suggest that cadmium contributes to the toxicity of the mixture, post 96 hours, indirectly by promoting mercury toxicity.

In over view the interactive effects of Cd-Hg observed in this study have profound implications. In practical terms it is apparent that a standard 48 or 96 hours bioassay would not have identified the true nature of the hazard presented by these mixtures. Possibly of even greater importance however, is the observation that toxicants which on the basis of their incipient LC_{50}'s would be considered non-lethal may yet provide a significant effect through interactions with lethal contaminants.

Summary

With respect to the individual toxicants it was determined that for Brachydanio rerio the order of lethal potency was Hg(240 hr LC_{50}=0.15 mg/l)>Cu(240 hr LC_{50}= 0.26 mg/l)>Cd (240 hr LC_{50}=5.82 mg/l). The results of the present study are consistent with the hypothesis that a similar mode of toxic action exists for each of these metals. Furthermore the data support the definition of cadmium and copper as non-accumulative toxicants.

The response of B. rerio to mixtures containing two non-accumulative toxicants, cadmium and copper, was supra-additive. The magnitude of the supra-additive response was quantified through comparison of the observed data with that predicted for concentration addition. This analysis indicated the mixtures to be 2.011 $\pm$ 0.488 times more toxic (Table 14). This enhancement factor was corroborated by the time-response data as interpreted following the methods of Anderson and Weber (1975) but not by the toxic unit method.

Preliminary studies to identify the mechanistic basis of the supra-additive response have shown that a reciprocal potentiation of each metals' bioaccumulation into gill tissue occurs when in the presence of the other metal. This pharmacokinetic phenomena may, at least in part, explain the increased toxicity of these metals when in combination.

The response of B. rerio to mixtures containing an accumulative and a non accumulative toxicant, mercury and cadmium, was found to change through time. The observed data

suggested an infra-additive response at 48 hours, a concentration additive response at 96 hours and a supra-additive response at 240 hours (2.030 ± 0.227, Table 18). This dose-response pattern was closely paralleled by the time-response data as interpreted following the methods of Anderson and Weber (1975) however not by the toxic unit method.

Preliminary studies to further elucidate the nature of the interactive effect of Cd and Hg mixtures were conducted. The data suggest that the supra-additive response observed at 240 hours may result from a cadmium induced sensitization to the toxicity of mercury.

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APPENDIX 1

Formula For Calculating Comcentrations Of Metal 2 In Equivalent Units Of Metal 1.

$$\text{Log } X_1 = \text{Log } X_2 - (I - y) \left[(1/b_1) - (1/b_2) \right].$$

Where X_1 = Concentration of metal 1

X_2 = Concentration of metal 2

I = Probit value at the intersection of the dose - response functions for the individual metals.

y = Probit value corresponding to X .

b_1 = Regression coefficient of the dose - response curve for metal 1.

b_2 = Regression coefficient of the dose - response curve for metal 2.

Sample Calculation: 0.264 mg/L (96 Hr. LC_{50}) Copper in Equivalent units of Cadmium.

Dose - response functions. Copper: $Y = 7.325 + 4.022 (X)$
Cadmium: $Y = -1.989 + 9.137 (X)$

(i) Calculation of I.

(A) $7.325 + 4.022 (X) = -1.989 + 9.137 (X)$

$9.314 = 5.115 (X)$

$(X) = 1.821$

(B) $I = Y = 7.325 + 4.022 (1.821)$

$I = 14.64866$

(ii) $\text{Log } X_1 = - 0.578 - (14.64866 - 5.00) \left[(1/4.022) - (1/9.137) \right]$

$= 0.765$

$X_1 = 5.822$ mg/L = 96 Hr. LC_{50} Cadmium