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Toxicity and Toxicokinetics of Copper, Cadmium and Their Mixtures on Rainbow Trout (*Salmo gairdneri*).

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A Thesis

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ABSTRACT

Rainbow trout were exposed to copper and cadmium as individual metals and as mixtures for exposure periods of 96 hours to determine their mode of acute toxicity. Copper and cadmium showed a supra-additive (greater than expected) toxic action when combined as mixtures. Kinetic studies to compare metal residues in fish exposed to each individual metal or to the metal mixtures suggests that the uptake of copper in gills may not be influenced by the presence of cadmium. Inversely, cadmium uptake in gills appears to be potentiated by the presence of copper.

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Finally, I wish to dedicate this thesis to members of my family and closest friends for their understanding, tolerance and support, which made the work more enjoyable.

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INTRODUCTION

The majority of toxicological research relating to aquatic pollutants has involved single chemicals acting alone upon aquatic organisms. It follows that proposed criteria for safeguarding aquatic organisms against the adverse effects of toxicants relates primarily to the risk posed by single substances (Sprague, 1970; Spehar, 1986). In reality however, it is the exception to find only a single substance polluting surface waters while it is the rule to find mixtures of pollutants.

Exposure to two or more contaminants creates a likelihood of coexisting substances interacting physiologically to evoke effects greater than that expected for each agent if encountered alone (Anderson and D'Appollonia, 1978). Safety criteria based on single agent risk assessment may then be inadequate as safeguards against the effects of multi-contaminant exposure.

A simple and quantitative approach of assessing the joint toxicity of two or more substances was developed by Anderson and Weber (1975). They formulated two empirical models, named Concentration Addition and Response Addition respectively, based on the theoretical concepts of similarly and independently acting constituents of toxic mixtures originally presented by Bliss (1939). The models have been adapted to the traditional format for assessing the toxicity of aquatic pollutants (Sprague, 1970). Probit analyses are applied to data sets representing exposure concentration-response correlates. Typically, this analysis leads to straight line formulations which may

then be subject to further analyses and statistical tests (Hewlett & Plackard, 1952).

Various binary combinations of heavy metals have been tested in accordance with the Concentration or Response Addition models in the Concordia University Ecotoxicology Laboratory (Horovitch, 1979, Weinstein and Anderson, 1978; Hewitt, 1980). More specifically, these studies showed metal mixtures having copper as one of the constituents to be typically supra-additive, both at the lethal and sublethal level (Table 1).

Other investigators have supported a similar pattern for copper-containing metal mixtures (Sprague and Ramsay, 1965; Eisler and Gardner, 1973). Sprague and Ramsay (1965), observed that lethal concentrations of copper-zinc mixtures act two and three times faster on the Atlantic salmon (Salmo salar L.) than similar concentrations of the single metal. Similarly, the observed mortality in mummichogs (Fundulus heterochitus) was significantly greater under conditions of combined copper-cadmium exposure than with either of the metals individually (Eisler and Gardner, 1973). More recently, Spehar and Fiandt (1986) found many standard water quality criteria to be ineffective for metal mixtures containing as one of six constituents, copper. The reasons for such supra-additive toxicity for metal mixtures involving copper are not clear (Weinstein and Anderson, 1978; Hewitt, 1980).

The main objective of this research was to explore the synergistic properties of metal mixtures containing copper on the hypothesis that copper interacts kinetically with other coexisting

Table 1

Toxicity patterns resulting from metal mixture exposure with copper or cadmium as one of the constituents.

Metal mixture	Time test	Species	Multiple toxicity patterns	Research team
Cu-Zn	96 h lethal	guppy*	supra-additive	Anderson & Weber (1977)
Cu-Zn	96 h lethal	zebra+	supra-additive	Horovitch & Anderson (1979)
Cu-Zn	96 h lethal	zebra+	supra-additive	Horovitch & Anderson (1979)
Cu-Zn	96 h lethal	zebra+	supra-additive	Horovitch & Anderson (1979)
Cu-Cd	96 h lethal	zebra+	supra-additive	Hewitt & Anderson (1980)
Cu-Cd	96 h lethal	trout**	supra-additive	Carrillo & Anderson (1987)
Cu-Ni	96 h lethal	zebra+	supra-additive	Weinstein & Anderson (1978)
Cu-Ni	10 day sublethal	zebra+	supra-additive	Weinstein & Anderson (1978)
Cd-Zn	96 h lethal	zebra+	additive	Gallimore & Anderson (1980)
Cd-Zn	48 h lethal	daphnia++	infra-additive	Attar & Maly (1982)
Cd-Hg	48 h lethal	zebra+	infra-additive	Hewitt & Anderson (1980)
Cd-Hg	96 h lethal	zebra+	additive	Hewitt & Anderson (1980)
Cd-Hg	10 day lethal	zebra+	supra-additive	Hewitt & Anderson (1980)

* guppy (Poecilia reticulatus)
\+ zebra (Brachydanio rerio)
** rainbow trout (Salmo gairdneri)
++ daphnia (Daphnia magna)

metals. The first phase of this study was designed to gain insight into the synergistic mode of action of copper. It was decided to test this metal in a mixture format with cadmium using the conventional dose-response format with exposure concentrations in the lethal range and decide on appropriateness of a multiple toxicity model. Dose-response experiments consisted of exposure to copper as a single metal, exposure to cadmium as a single metal, and exposure to a binary mixture of copper and cadmium.

The second phase of the study dealt with kinetic studies of the two metals singly and in a mixture. This was done to see if there was any evidence of kinetic interaction between the two metals, in the gill and serum, that would explain the joint mode of action of the mixture. The gill compartment was chosen since studies performed by Spear and Anderson (1978) suggested the membrane of gill epithelia to be the target of heavy metal toxic action.

The serum was selected for accumulation testing, on the assumption that any heavy metal absorbed by the gills and translocated internally would be transported in the blood. In this way, serum evaluation could perhaps provide some index of translocation within the organism, hereby enhancing our appreciation of the toxicokinetics of the mixture.

The second phase of this study also comprised studies of the Na^+-K^+ ATPase enzyme. This enzyme was selected for assay to serve as an end-point response parameter within the gill, which would reflect copper or cadmium loading and protein denaturation action in gill tissue. This particular enzyme was chosen on the basis of its vital

importance in cellular water balance and osmoregulation of the animal (Pfeiler and Kirschner, 1972; Johnson et al., 1977).

The mixture containing copper as one of its constituents is of great concern since copper is one of the most prevalent heavy metals pollutants arising from man's industrial and agricultural activities (Spear and Pierce, 1979). Naturally occurring copper is often mobilized within water ways in amounts which approach toxic levels (Chapman, 1978)(Table 2). Although the toxicity of copper as a single agent is attributed to its free ion form (EIFAC, 1978); Sunda et al., 1978), its exact mode of action is not known. Many researchers have suggested that its critical target organ in fish, at least, is the gill where it has been shown to cause extensive damage to the epithelial cells (Loyd, 1962; Baker, 1969).

Cadmium was selected in this study as the metal to be present in a binary mixture with copper because of its relatively great toxicity, coupled with the fact that its toxic levels are about equal to cadmium concentrations frequently found in the environment (Chapman, 1978) (Table 2). Cadmium sources of above natural level contamination include volatilization in smelters, coal and oil burning furnaces, and metal processing plants, which contribute to atmospheric washout of air-borne cadmium (Peterson et al., 1983).

The toxicity of cadmium to aquatic organisms is determined by the free cadmium ion concentrations in solution, which at the pH range of 5 - 7 was found to be 99% in fresh water (Florence, 1982). Relatively little is known regarding the mechanism of the toxic action of cadmium; this is surprising due to the marked sensitivity that

Table 2

General ranking of toxicity of heavy metals in the aquatic environment.

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

(taken from Chapman, 1978)

* Toxic levels of these heavy metals may be closest to environmental levels.

salmonids have towards this metal. Cardiovascular-respiratory impairment (Bishop et al., 1981), blood electrolyte changes (McCarty et al., 1978), enzymatic and hormonal changes (Christensen, 1975), increased locomotor activity (Benoit et al., 1976) and retardation of growth (Benoit et al., 1976) have been reported for different fish species exposed to sublethal concentrations of cadmium. Whereas the diverse effects or signs of cadmium intoxication have been described, the physiological mechanism whereby these effects arise are largely unknown (Giles, 1984).

Rainbow trout (Salmo gairdneri) was chosen as our test animal because of its importance and prevalence as a game fish species in our ecosystem. The size of their gills facilitated dissection and made collation of samples quick and simple.

MATERIALS AND METHODS

1.0 Experimental Fish and Holding Facilities

Rainbow trout, Salmo gairdneri, were purchased from Mount Sutton Hatchery, Sutton, Quebec. The test fish were divided into lots of 100, and acclimated to laboratory conditions for a minimum of two weeks. During this holding period, they were kept in 240 L blue fiberglass tanks (51 cm width, 114 cm length, 41 cm depth). Holding tanks were supplied with a continuous flow of dechlorinated, activated charcoal-treated water with an exchange rate of 1.8 L/min.

During the acclimation phase of the experiment, the fish were fed once daily at 17:00 hours on an ad libitum basis using a commercial dry-food pellet, size #4 (Trout Chow-Martin Feed Mill, Elmira-Tavistock, Ontario). Any excess of food in the tanks was regularly siphoned. Forty-eight hours prior to and during the experimental period, test fish were not fed. Two weeks prior to the beginning of the experimental period the wet weight of the experimental fish was determined, using a Sartorius (1364 MP) digital balance. To facilitate the weighing procedure fish were anesthesized using a 100 ug/L solution of tricaine methane sulfonate (MS222). The weight ranges for the test lots of rainbow trout in the lethal response and kinetic studies are listed in Table 3.

Table 3

Weight of experimental rainbow trout in lethal response studies and kinetic studies.

			Fish Weight (g)
Lethal Response Studies	Copper	July 1982	14 ± 3.2
		November 1982	19 ± 4.6
	Cadmium	February 1985	25 ± 4.4
	Copper-Cadmium	February 1985	25 ± 4.4
Kinetic and Enzyme Studies	Copper	May 1984	50 ± 5.6
	Cadmium	February 1984	49 ± 5.1
	Copper-Cadmium	April 1985	60 ± 5.3

2.0 Experimental Conditions

The photoperiod consisted of a 12:12 hour light/dark regime, with the photophase commencing at 08:00 hours. The water temperature was maintained at 12 ± 1°C. Information on source water characteristics was obtained on request from the public works division of the City of Montreal. Their readings yielded the following parameters: alkalinity = 85 ± 2 mg/L as $CaCO_3$, hardness = 125 ± 5 mg/L as $CaCO_3$, dissolved oxygen 80-90% saturated, and pH = 7.6 ± 0.5 for the duration of the experiments. Measurements performed in the Concordia University Ecotoxicology lab in October 1981 corresponded closely with these values, where alkalinity was 81 ± 1 mg/L as $CaCO_3$ measured by acid titration, hardness was 130 ± 2 mg/L as $CaCO_3$ determined by the EDTA method, oxygen saturation was 85 ± 1%, and pH was 7.2 ± 0.5.

3.0 Test Substances

The metal salts used in the experiments were reagent grade cupric chloride dihydrate ($CuCl_2.2H_2O$)(American Chemicals, Ltd.), and reagent grade cadmium chloride dihydrate ($CdCl_2.2\frac{1}{2}$)(Fisher).

4.0 Experimental Apparatus

Acidified HNO_3 stock solutions of copper chloride and cadmium chloride were prepared three days in advance of experiments and held in 55 liter polyethylene containers. These stock solutions were dripped into a modified Chadwick-type serial dilutor (Anderson and Weber, 1975). The serial dilutor provided independently operating channels with the potential of delivering a toxicant either individually or in

combination with another to a similar number of test tanks (Figure 1). The hydraulic principles of adjustable glass faucets and vertical arrangements of each dilutor stage provided precise control over the various concentrations delivered on a continuous flow basis to 12 test chambers.

Intramedic tubing was used to slowly release the toxicants from the Mariotte bottle at the rate of 2 mL/min into the serial dilution apparatus. Solutions of the toxicants flowed through the experimental tanks 48 hours before commencing experiments to ensure an equilibrium interphase of the metal ions with the surface of the tanks (Florence, 1982).

Each of the twelve exposure tanks received a flow-rate of 600 mL per minute which provided a minimum of 90% replacement within 5 hours (Sprague, 1973).

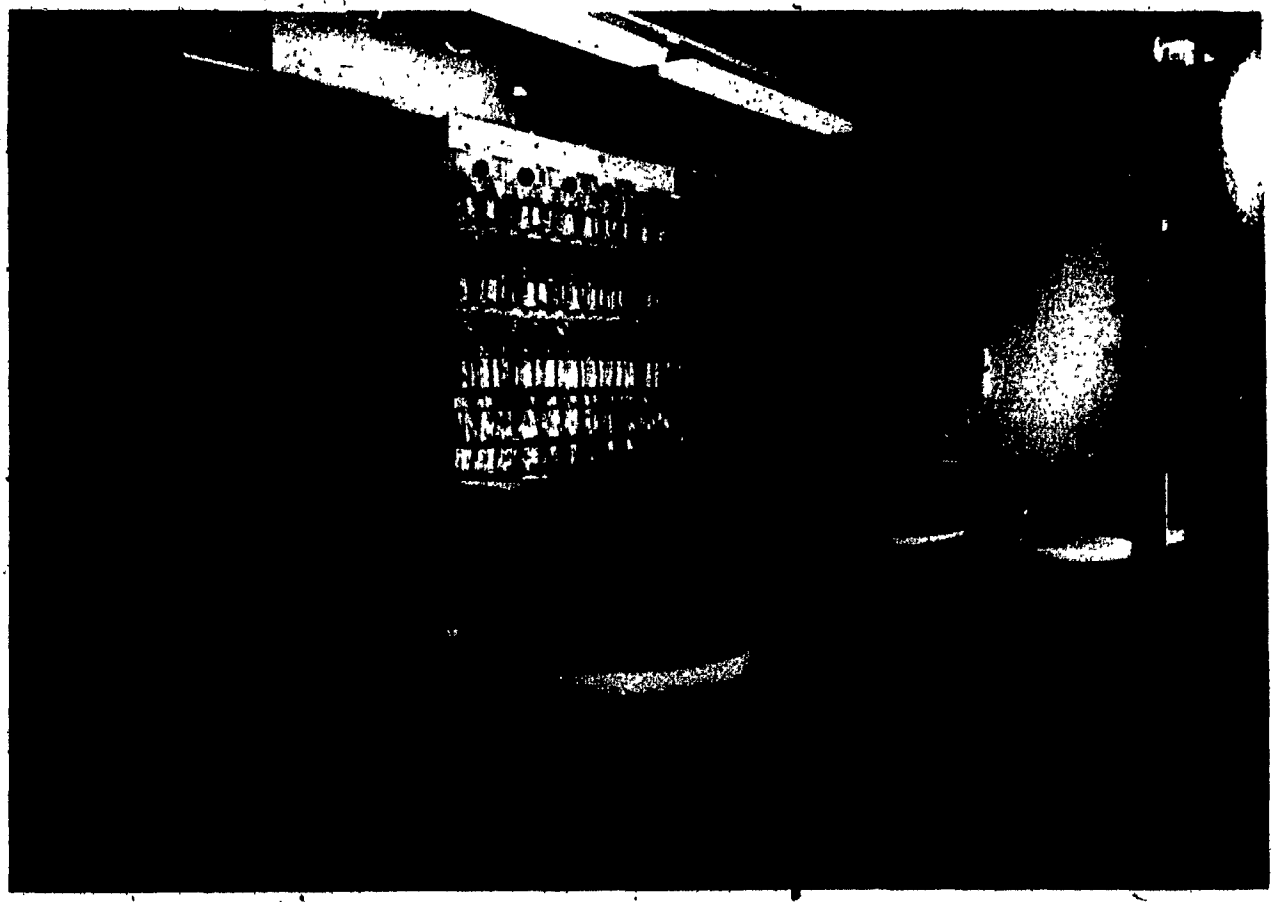
5.0 Water Samples Analysis

Polyethylene vials were used to collect ten mL samples of water from control and experimental tanks for determination of copper and/or cadmium content. Two drops of ultra pure nitric acid (Ultrex-Fisher Scientific Company) were added to each water sample to ensure that metals remained in solution. Water samples were stored at 4°C and were immediately analyzed following experiments. Florence (1982) reported no changes in the concentration of Cu, Pb, Cd or Zn in freshwater samples stored at 4°C for several weeks.

Total copper concentration was determined using a Perkin Elmer Atomic Absorption Sepctrophotometer 503 equipped with a graphite

Figure 1. Photograph of toxicant testing apparatus, front view, showing multi-stage arrangement of serial dilutor and arrangement of experimental tanks

a. Serial diluter
b. Experimental tanks
c. Rotatory glass faucets
d. Mariotte bottle
e. Water pump



furnace. Total cadmium concentration was analyzed using a similar instrument equipped with a flame atomizer. Three determinations of each sample were made and the average value computed. The mean daily ambient concentration of metals in the respective test tanks were calculated on the basis of four average daily determinations in mortality studies and three average daily determinations in kinetic studies.

Concentrations of the metals in test tanks during the experimental period for mortality and kinetic studies were found to vary by no more than 10%. Background levels of cadmium and copper were monitored at 0 ug/L and 7 ± 3 ug/L respectively.

Water temperature and pH were recorded daily, oxygen saturation readings were taken every two days during experiments. All readings were within the limits of those established during the acclimation period.

All glassware and polyethylene bottles used throughout experiments for analytical determinations were immersed in a full strength nitric acid reagent bath for a minimum of two weeks and rinsed five times with deionized distilled water to prevent heavy metal contamination (Florence, 1982).

6.0 Lethal Response Studies

Lethal response studies were conducted for periods of 96 hours to investigate the toxic response of rainbow trout to pure solutions of either copper or cadmium and to their mixtures. All mortality studies started at 12:00 hours. Fish were considered to be dead when they showed no opercular movement and no response to mechanical stimulus. Dead fish were immediately removed from the experimental tanks.

6.1 Statistical Analyses - Lethal Response Studies

Where data sets were adequate, a computerized Litchfield-Wilcoxon analysis was performed to determine the LC_{50} values for the respective exposure periods (Litchfield and Wilcoxon, 1959). Where data sets were restricted in numbers of data points and where more than one 0% and 100% response were included in data sets, the trimmed Spearman-Karber method was employed to estimate the LC_{50} value and its confidence interval (Hamilton et al. 1977).

The linear equation for the distribution of coordinates in the probit-fitted line for a 64 hour or 96 hour period was computed as:

$$Y = a + b\,(X) \qquad \text{eq. 1}$$

where Y = % cumulative mortality in probit units

X = mean daily exposure concentrations

The "goodness-of-fit" of data distributions to a straight line relationship was evaluated by a chi-square test. Using a computerized program of Finney's (1971) method of probit analysis, straight line functions with 95% fiducial limits were fitted to response-exposure concentration coordinates.

6.2 Rationale to the Study of Multiple Toxicity

Results of joint toxicity experiments generally are presented and evaluated with the help of various terminologies and models. The predictive capacity of these models is based on a knowledge of the concentration response relationships existing for the discrete constituents in the mixture. In comparing the empirical multiple toxicity data to the theoretical predictions, the model most likely to represent the actual mechanism of joint action may be indicated.

6.2.1 Concentration Addition Model

This model assumes that each constituent contributes to the toxicity of the mixture in proportion to its relative potency (Anderson and Weber, 1975). However, prediction of the magnitude of effect evoked by mixtures is possible only if toxic constituents act in a similar manner. Concentration addition of constituents in a mixture occurs when the toxicants have the same receptor site within the affected organism. In such a mixture one constituent may be replaced by an equipotent amount of the other constituent without altering the potency of the mixture (Anderson and D'Apollonia, 1978). Similarly acting substances when tested singly and in mixtures will have parallel concentration response probit lines with similar slopes (Anderson and Weber, 1975). Although it is difficult, if not impossible in many instances to prove compliance with these assumptions, the model is useful empirically because it provides a quantitative measure of the joint toxicity of co-existing toxicants. Hermens and Leeuwangh (1982), applied the model to mixtures of organic

substances whose structures were closely inter-related and as such representative of organic series. The model was shown to be highly effective in predicting the joint toxicity supporting the assumption that members of these organic series may be similarly acting.

6.2.2 Response Addition Model

When present as constituents in a mixture, toxicants having different receptor sites or critical targets within the test organism act independently (Anderson and D'Apollonia, 1978). The Response Addition Model permits a quantitative measure of the joint effect of mixtures where constituents are not similarly acting. Parallelism between the respective concentration response curves of independent-acting constituents is not a requisite for this model. Rather, individual toxic constituent contributions to a mixture effect are deemed equivalent to the magnitude of response each would evoke if acting singly at the level present within the mixture (Anderson & Weber, 1975). Thus, when the level of individual and independently acting constituents are below their respective threshold to no-effect, they do not contribute to the effect of a mixture (Anderson and Weber, 1975). When there is no correlation of the susceptibilities to each of the discrete constituents, then the coefficient of association, r, is equal to zero and:

$$Pc = Pa + Pb - PaPb \qquad \text{eq. 2}$$

where, Pc is the proportion responding to the mixture; Pa is the proportion responding to "A" alone; and Pb is the proportion responding to "B" alone.

These interactions may lead to either an unexpectably greater or lesser response than predicted. When the response is greater than that predicted for Concentration Addition, the pattern is called supra-addition, a form of synergism. When the response is less than predicted for Concentration Addition, the pattern is designated infra-additive. If the degree of reduction is less than that expected for Response Addition then the infra-additive effect is called antagonism (Anderson and Weber, 1975). Substances, which in mixtures are supra-additive are of particular concern because they jointly defy no-effect or threshold criteria.

6.2.3 Toxic Unit Analysis

This approach computes the toxicity of a mixture by adding the toxicity of each component. Each contaminant's contribution to the toxicity of a mixture is expressed as a fraction of its LC_{50}.

$$\text{Toxic Unit} = \frac{\text{actual concentration in solution}}{\text{64 hour } LC_{50}} \qquad \text{eq. 3}$$

where the 64 hour LC_{50} is the concentration which will result in 50% mortality in a test population at 64 hours of exposure.

A mixture of chemicals is expected to be strictly additive when the sum of the respective Toxic Units (TU) of its constituents equals unity i.e. ($\Sigma TU = 1$). This model predicts that the joint effect of toxicants will be more than additive when ($\Sigma TU < 1$), and less than additive when ($\Sigma TU > 1$) (Sprague, 1970).

6.2.4 Mixture Toxicity Index

A more theoretical approach that uses simple similar and independent action as two reference points for comparing toxicity of mixtures of more than two chemicals is the Mixture Toxicity Index (MTI) (Konemann, 1981). 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 (Konemann, 1981). The Mixture Toxicity Index is conventionally represented by:

$$MTI = 1 - \frac{\log M}{\log n} \qquad \text{eq. 4}$$

for equitoxic mixtures, where $M = \sum fi$, $fi = Ci/LC_{50i}$ or toxic unit (TU); n is the number of compounds in a mixture; and C_i is the concentration of constituent i.

The MTI is evaluated through comparison with the following scale for classifying the toxicity of mixtures (from Konemann, 1981):

MTI	Type of joint action
≤ 0	Antagonism
0	No addition (response addition, r = 1)
$0 < MTI < 1$	Partial addition
1	Concentration addition
> 1	Supra addition (potentiation of the toxic actions of one or more of the compounds in the mixture)

7.0 Kinetic Studies

Kinetic studies measuring the uptake of copper and cadmium in the gill and the serum compartments were conducted over a period of 72 hours. Kinetic studies consisted of experiments which were divided temporally into two phases, exposure and clearance.

7.1 Exposure Phase Methodology

The uptake phase of the kinetic study involved a maximum 64 hour exposure to one of the three metals conditions i.e. copper, cadmium or a Cu-Cd mixture. The sampling schedule was set at 2, 4, 8, 16, 32 and 64 hours of exposure. Experimental lots consisted of 18 fish. Each lot was replicated. The total of 36 fish per experimental lot permitted four fish (2 from each replicate) to be sampled at each of the six assigned times during the exposure period. The remaining 12 fish were used for the clearance studies (Methods, 7.2). At the time of sampling fish were transferred to a control-water tank where they were allowed to swim for five minutes in order to rinse non-absorbed cadmium and/or copper from the exterior body surfaces and buccal cavities. Following this rinsing period, fish were quickly sacrificed, their left gills were excised and a sample of blood was collected from each fish. Each individual gill was placed in a porcelain crucible in a drying oven at 70°C for 6 days. The serum was preserved using liquid nitrogen and stored at -12°C. All samples, both gill and blood were subsequently analyzed by atomic absorption spectrophotometry for their copper and cadmium content (Methods, 7.3 and 7.4).

7.2 Clearance Phase Methodology

The four sets of 12 fish (one set per concentration plus one control) that remained following the 64 hour uptake study were transferred to three respective ~~tanks~~ containing control-quality water. They were sampled in groups of four at intervals of 2, 4 and 8 hours or 4, 8 and 16 hours. At each sampling time, fish were sacrificed and gills and blood were collected for analyses of heavy metal content in accordance with sections 7.3 and 7.4, Methods.

7.3 Determination of Copper and Cadmium in Gill Tissue

A dry ashing procedure modified from that of Anderson (1972) and DeLuca (1979) was followed for metal determinations in gill tissue.

Immediately following the collection of blood from a fish (Methods, 7.4) the gills were excised. The right gill was preserved for enzyme analyses (Methods, 8.0) and the left gill was used for copper and/or cadmium determinations. Upon being excised, left gills were blotted and their respective wet weights were recorded. Subsequently each gill was placed in a labeled 10 mL porcelain crucible which was then covered by a loosely fitting lid. Each gill sample was placed in a drying oven at 70°C for a period of 6 days. The dry weight of each sample was then determined. All weighing was carried out using a Sartorius digital balance (1364 MP). Samples and blanks were transferred to a muffle furnace after which the temperature was raised to 100°C. After an initial 30 minute period at 100°C, the furnace temperature was raised an additional 50°C. Similar increments of 50°C were made every 30 minutes until an oven

temperature of 500°C was attained. This latter temperature was maintained for a period of 14 hours to reduce the samples to ashes.

Following cooling, 10 mL of 1:1 concentrated HNO_3 were added to each crucible to digest the ash and to oxidize carbon residues. The acid digestion procedure was enhanced by heating the sample close to the boiling point. Digestion was continued under these conditions until almost all of the acid had evaporated. Complete evaporation was not permitted in order to avoid charring of the samples. The metal was eluted from each container by washing the interior of the crucible five times with 10 mL (total) 0.2% HNO_3 (trace metal analysis reagent grade). The concentration of metal was measured by directly injecting 10 uL of sample into the graphite furnace of the atomic absorption spectrophotometer (Perkin Elmer 503). Each sample was analyzed three times and the average reading was recorded. The % recovery was 97% for copper and 98% for cadmium.

7.4 Determination of Copper and Cadmium in Blood Serum

Fish were sacrificed with a blow to the head and the caudal penduncle was quickly severed. The tip of a 1 cc tuberculin syringe, rinsed in sodium citrate, was pressed against the open end of the caudal artery and blood was withdrawn by suction. Samples of blood were then centrifuged in 1.5 mL Eppendorf microtest tubes for 1 minute in a Brinkman Eppendorf centrifuge (12,000 g). The serum was decanted from blood cells, frozen in liquid nitrogen and stored at -12°C until metal analyses were performed.

For cadmium analyses, 50 ul of each serum sample were diluted to

5 ml with metal-free, ammonium phosphate (0.5%), and 20 uL of this mixture was injected into the graphite furnace of the atomic absorption spectrophotometer (Wright, 1975). Each sample was analyzed three times and the average reading recorded. The % recovery of spiked samples was 98%.

For copper determinations, 50 uL of each serum sample were diluted to 10 mL with 10% HNO_3 (trace metal analysis grade) of which 20 uL were then injected into the graphite furnace (Evenson, 1975). Each sample was analyzed three times and the average reading recorded. The % recovery of spiked samples was 97%.

7.5 Statistical Analysis - Kinetic Studies

Geometric means and standard deviations were computed for the data since a cursory examination suggested that standard deviations of tissue metal levels were directly related to the size of the arithmetic mean. Hence, variance was non homogeneous.

A t-test ($p = 0.05$) was performed in comparing single metal control levels with a corresponding copper-cadmium mixture level at 64 hours of exposure.

8.0 Enzyme Studies.

The effect *in vivo* to ambient solutions of copper and cadmium on the Na^+-K^+ ATPase in the gill was investigated. Gill tissue for this study was taken from the right pharyngeal chamber of fish sampled at 4, 8, 16, 32 and 64 hours exposure periods in accordance with procedures outlined in 8.4. Four gills were prepared for each control

and experimental regime. The right gills in these samples were each weighed, quick-frozen in liquid nitrogen and stored at -12°C for subsequent enzyme analyses.

8.1 Na^+-K^+ ATPase Assays

A Tris-ATP colorimetric method modified from that of Johnson et al. (1977) and Ernstter et al. (1950) was used for analyses of the Na^+-K^+ ATPase enzyme.

Upon being excised, gills were blotted on Kimwipe tissue to remove excess water and blood, and then wet weighed. Each gill was placed in a 10 mL vial containing the following homogenate: 2mM EDTA, 30 mM sucrose, 100 mM Imidazole, 0.1% deoxycholate and a Na^+-K^+ ATPase stabilizer, 14 mM mercaptoethanol. Vials were then immersed in liquid nitrogen for fast freezing and stored at -12°C for no more than 21 days. According to reference tissue prepared and stored in this manner, gill tissue may be held for three weeks without significant loss of ATPase activity. Johnson et al.(1977) have shown that mercaptoethanol preserved ATPase activity in stored samples at levels comparable to untreated fresh preparations.

At the time of enzyme analyses, frozen experimental samples were thawed in an ice bath. Following, the contents of each vial were blended using a cooled Potter-Elvehjem homogenizer.

To isolate microsomes from other cellular fractions, whole homogenates were centrifuged at 8000 rpm for 10 min at 2°C. The supernatant containing the microsomal fraction was separated from the mitochondria - cell debris fraction and centrifuged at 37,000 rpm

at 4°C for 1 h to isolate the microsomes as a precipitate. Upon discarding the supernatant, the microsomal pellet was resuspended in 10 ml of the same homogenate medium previously described but with no deoxycholate. The microsomal preparation was then divided into two 0.1 mL lots. A solution of 0.2 mL containing 200 mM Na^+ and 50 mM K^+ in a 100 mM Tris buffer was added to one lot and a similar volume of the original homogenate medium was added to the other. The Na^+-K^+ ATPase activity was determined from the difference between the orthophosphate released in tubes having the Na^+-K^+ salts and that released in identical samples containing no Na^+-K^+ salts since ouabain was not used in the assays (Pfeiler and Kirschner, 1972).

Magnesium salts activate both the Na^+-K^+ and the Mg^{++} ATPase (Lehningher, 1975). Thus, colorimetric analysis of this preparation provides a measure of the combined activity of the Na^+-K^+ ATPase and that of the Mg^{++} ATPase enzyme. In order to isolate the Na^+-K^+ ATPase activity, all enzyme assays were run at 37°C. This high temperature inactivates the Mg^{++} ATPase, which is thermolabile, without affecting the activity of the Na^+-K^+ ATPase (Pfeiler and Kirschner, 1972).

At the end of the incubation period, the enzymatic reaction was started by adding 0.2 ml of 5 mM Tris ATP (in a 100 mM Tris buffer and 12 mM MgCl) to 0.3 ml of enzyme preparation. Addition of ATP was staggered by a 15 second interval between samples. The reaction was allowed to proceed for precisely 20 minutes at 37°C. Johnson et al. (1977) have shown that phosphate hydrolysis by rainbow trout microsomes continues at a linear rate over this time interval.

The reaction was stopped by adding 3 ml of 1.67 N H_2SO_4 to each

tube. The acidic condition promotes color development to a degree depending on the amount of inorganic phosphate hydrolyzed (Ernsttér et al., 1950). Samples were then analyzed for phosphate (Perkin Elmer Spectrophotometer 510) using a light beam at a wavelength of 730 nanometers.

The Bio Rad procedure using bovine serum albumin as a reference was used for determinations of protein per gram of gill tissue (Bradford, 1976). ATPase activity was expressed as u mole PO_4/mg protein/h.

RESULTS AND DISCUSSION

1.0 Lethal Response Studies

1.1 Copper Lethal Response Study

Two experiments, one in July, the other in November, were conducted to determine lethal concentrations parameters for test populations of juvenile rainbow trout exposed to copper. The average wet weight of fish in the July test was 14 g ± 3.2 g, while that of fish in the November test was 19 g ± 4.6 g. The 64 h LC_{50} values for both the July and November tests were determined to be 220 ug Cu/L and 200 ug Cu/L respectively, as shown in Tables 4 and 6, Figures 2 and 4. The 96 h LC_{50} values were 200 and 190 ug Cu/L for the same two tests respectively as shown in Tables 5 and 7, Figures 3 and 5. No difference in response parameters between the two test lots could be attributed to the size variable. This finding was in agreement with that of Spear and Anderson (1975) who found that size did not significantly modify the lethal toxicity of copper for juvenile rainbow trout over a size range from 3.9 g to 29 g. Response data for both experiments were therefore pooled.

Linear functions were fitted through probit analysis to the combined lethal response - mean daily exposure concentration data for the exposure periods of 64 and 96 hours (Figures 6 and 7). The 64 and 96 h LC_{50} values were calculated to be 210 ug Cu/L and 195 ug Cu/L respectively as shown in Tables 8 and 11.

Table 4

Dose response data for rainbow trout, mean wet weight 14 g, following exposure for 64 hours to lethal concentrations of copper, July 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

Nominal copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 64 hours	64 hour LC_{50} (ug/L)
80	82	17	6	
100	96	17	5*	
200	192	17	12	220
250	246	17	41	
300	295	17	76	
control	6	17	0	

* corrected value for a 0% response (Litchfield and Wilcoxon, 1959)

Figure 2 Dose response data for rainbow trout, mean wet weight 14 g, following exposure for 64 hours to lethal concentrations of copper, July 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

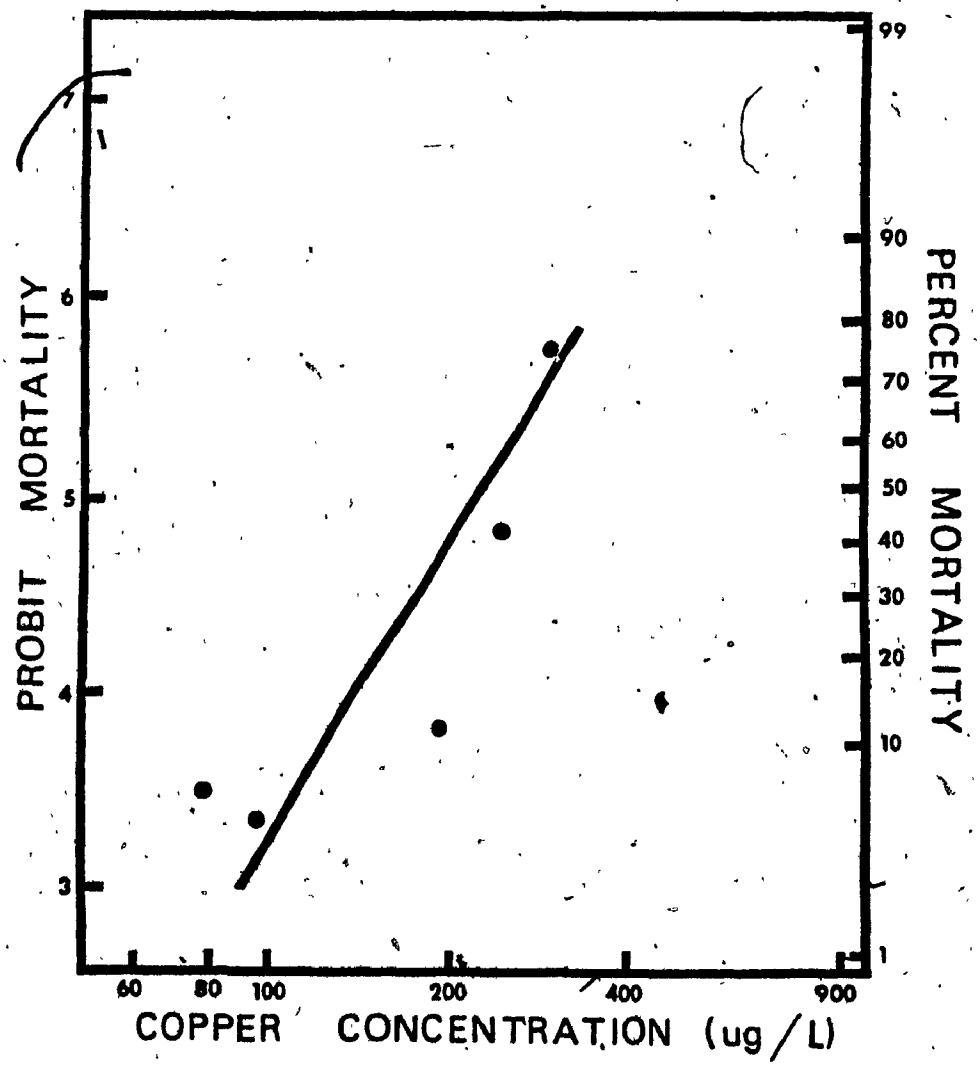

PROBIT MORTALITY
PERCENT MORTALITY
COPPER CONCENTRATION (ug/L)
3
4
5
6
60
80
100
200
400
900
1
10
20
30
40
50
60
70
80
90
99

Table 5

Dose response data for rainbow trout, mean wet weight 14 g, following exposure for 96 hours to lethal concentrations of copper, July 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

Nominal copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 96 hours	96 hour LC_{50} (ug/L)
80	79	17	6	
100	100	17	6	
200	196	17	29	
250	247	17	47	200
300	290	17	76	
control	7	17	0	

Figure 3 Dose response data for rainbow trout, mean wet weight 14 g, following exposure for 96 hours to lethal concentrations of copper, July 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

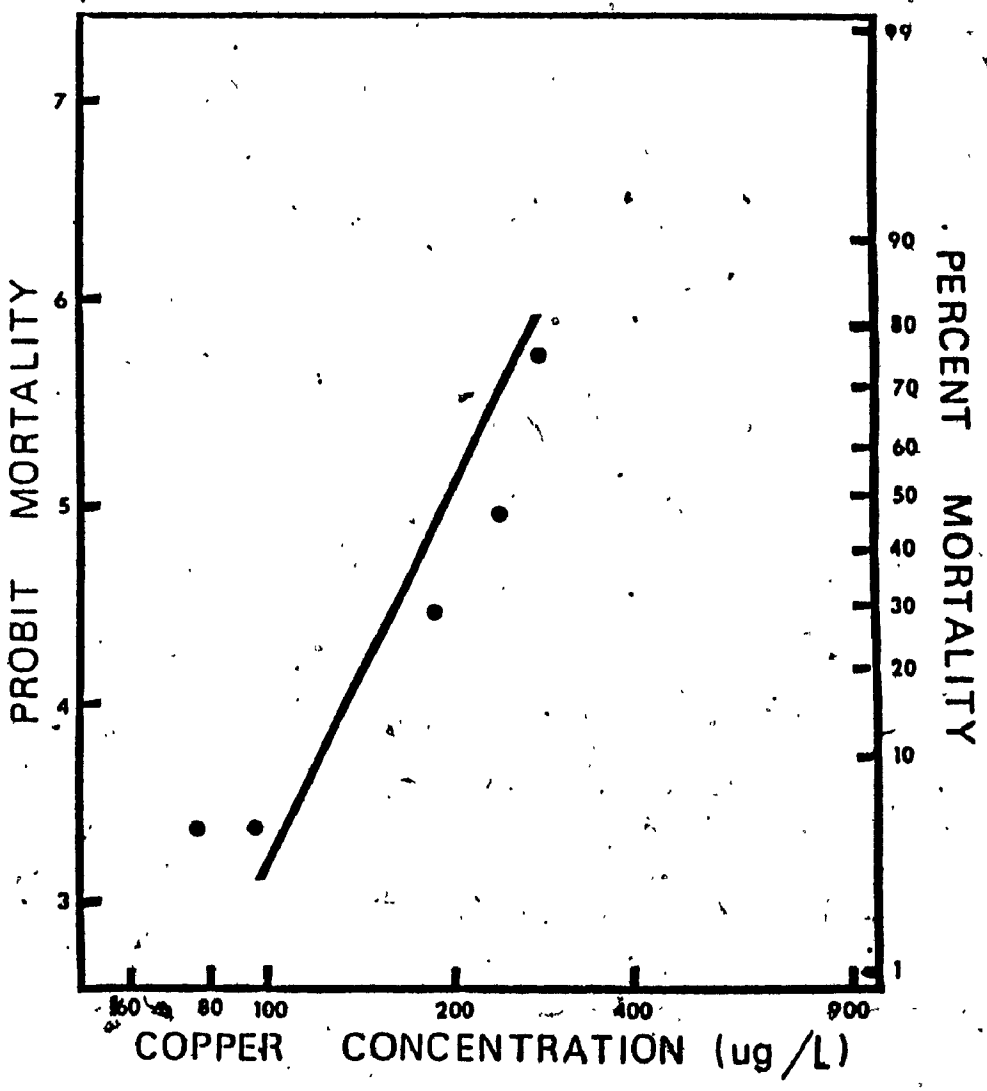

PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
1
60
80
100
200
400
900
COPPER CONCENTRATION (ug/L)

Table 6

Dose response data for rainbow trout, mean wet weight 19 g, following exposure for 64 hours to lethal concentrations of copper, November 1982 experiment. Derivation of the LC_{50} value followed that of the 10% trim Spearman-Karber method (Hamilton et al., 1977).

Nominal copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 64 hours	64 hour LC_{50} (ug/L)	95% C.I.
80	76	17	6		
100	98	17	4		192
200	174	17	6	200	210
250	205	17	53		
300	247	17	90		
control	7	17	0		

Figure 4 Dose response data for rainbow trout, mean wet weight 19 g, following exposure for 64 hours to lethal concentrations of copper, November 1982 experiment. Derivation of the LC_{50} value followed that of the 10% trim Spearman-Karber method (Hamilton et al., 1977).

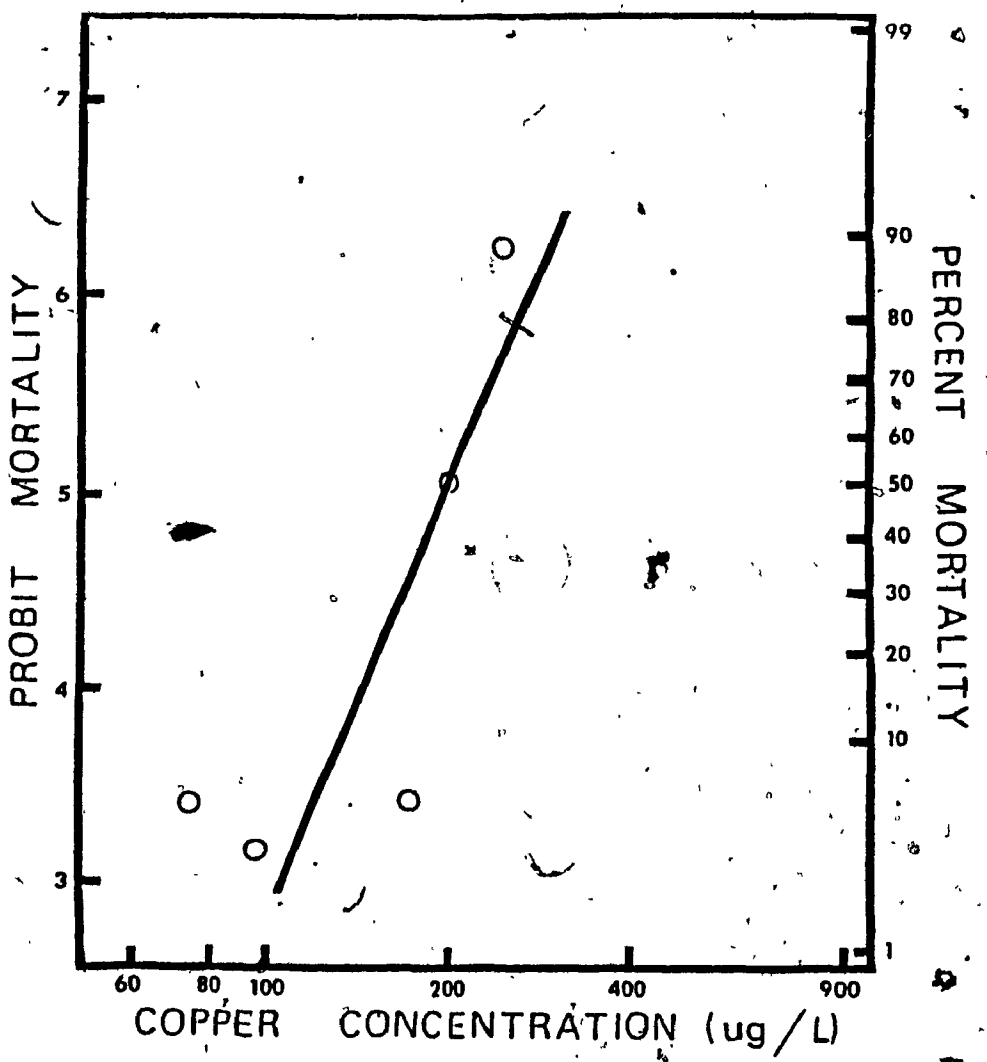

PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
1
60
80
100
200
400
900
COPPER CONCENTRATION (ug/L)

Table 7

Dose response data for rainbow trout, mean wet weight 19 g, following exposure for 96 hours to lethal concentrations of copper, November 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

Nominal copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 96 hours	96 hour LC_{50} (ug/L)
80	78	17	6	
100	103	17	3*	190
200	177	17	12	
250	200	17	65	
300	240	17	90*	
control	7	17	0	

* corrected value for a 0% response (Litchfield and Wilcoxon, 1959)

Figure 5 Dose response data for rainbow trout, mean wet weight 19 g, following exposure for 96 hours to lethal concentrations of copper, November 1982 experiment. Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

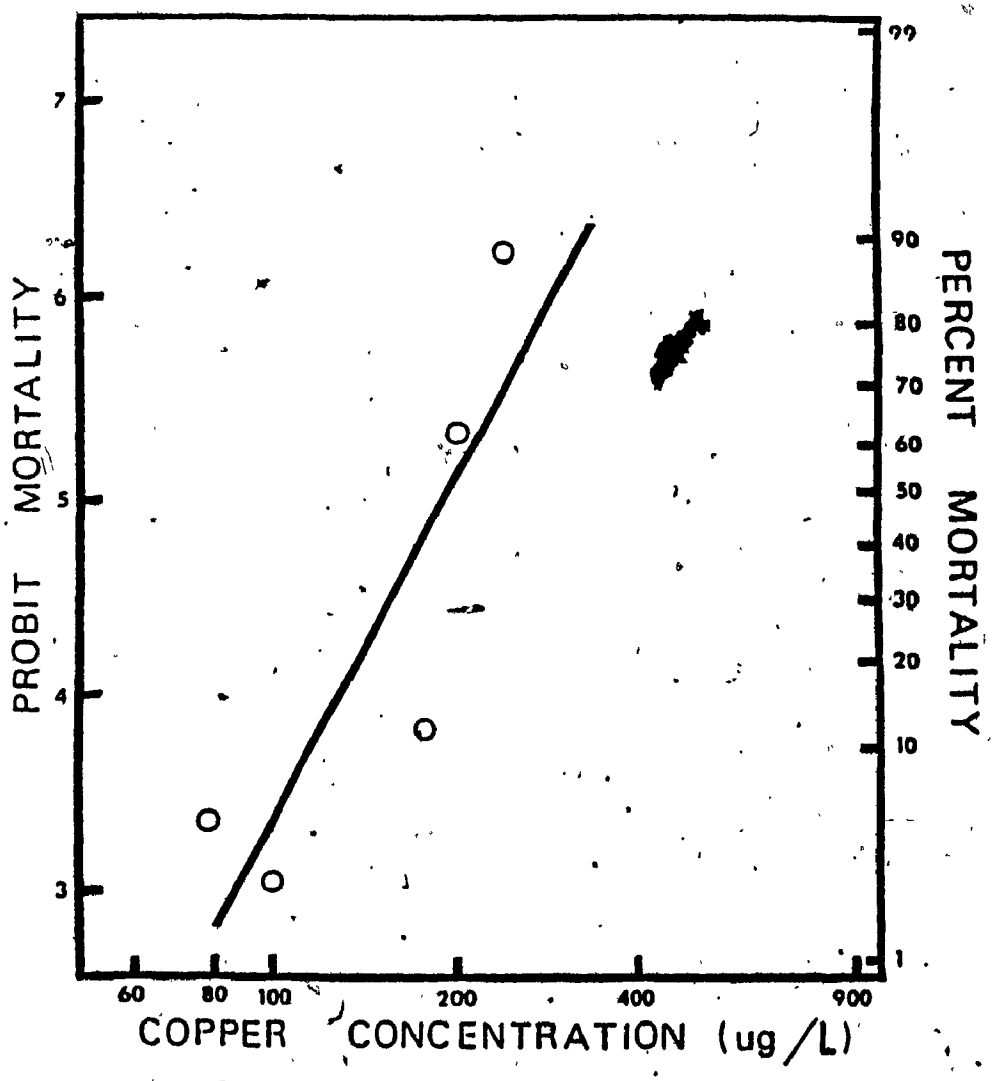

PROBIT MORTALITY
7
6
5
4
3
99
90
80
70
60
50
40
30
20
10
1
PERCENT MORTALITY
60
80
100
200
400
900
COPPER CONCENTRATION (ug/L)

Table 8

Combined dose response data for rainbow trout, mean wet weight 14 g and 19 g, following exposure for 64 hours to lethal concentrations of copper (combined data of the July and November 1982 experiment). Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

Nominal Copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 64 hours	64 hour LC_{50} (ug/L)
80	76	17	6	
80	82	17	6	
100	96	17	5*	
100	98	17	4	
200	174	17	6	
200	192	17	12	210
250	205	17	53	
250	246	17	41	
300	247	17	90	
300	295	17	76	
control	7	17	0	

* corrected value for a 0% response (Litchfield and Wilcoxon, 1959)

Table 9

Regression analysis and chi-square analysis of the lethal response data (presented in Table 8) for rainbow trout following exposure of 64 hours to copper solutions (combined data July-November 1982 experiments).

Regression equation	Slope function	Chi-square value	Chi-square 8 df 95%
y = -4.4 + 4 (x)	1.8	24	16

Table 10

Probit analysis with 95% fiducial limits of the lethal response data (presented in Table 8) for rainbow trout following exposure of 64 hours to copper solutions (combined data July-November 1982 experiments). Fiducial limits corrected for heterogeneity of data.

Log Dose	Predicted Probit	Lower limit	Upper limit
1.89207	3.0685	1.8883	4.2487
1.89760	3.0904	1.9246	4.2562
1.99997	3.4965	2.5889	4.4041
2.01281	3.5474	2.6708	4.4240
2.24795	4.4801	4.0105	4.9497
2.29223	4.6558	4.1909	5.1247
2.30100	4.6906	4.2227	5.1585
2.38018	5.0047	4.4568	5.5526
2.39267	5.0542	4.4866	5.6218
2.46237	5.3307	4.6304	6.0310

Figure 6 Combined dose response data for rainbow trout, mean wet weight 14 g and 19 g, following exposure for 64 hours to lethal concentrations of copper. (Combined data of the July and November 1982 experiments.) Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).)

● data for 14 g fish

○ data for 19 g fish

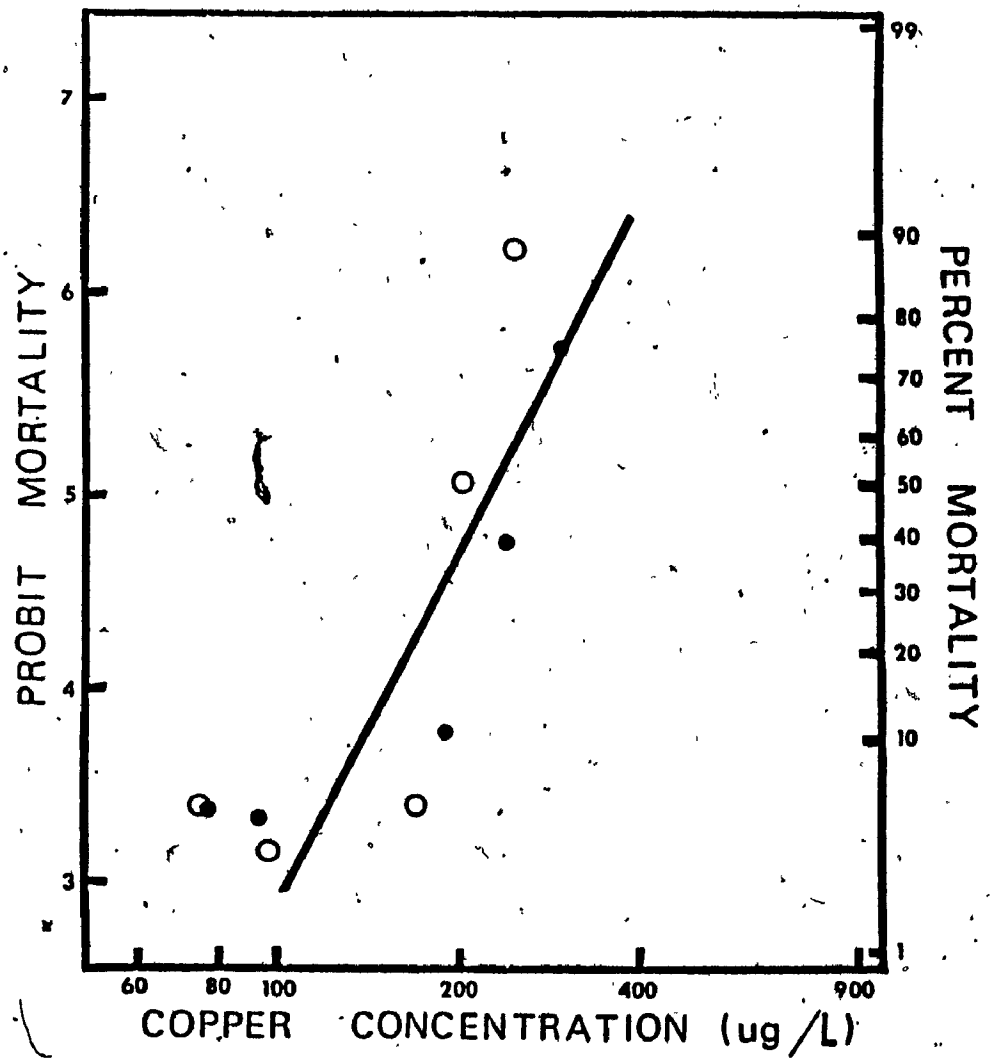

PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
1
60
80
100
200
400
900
COPPER CONCENTRATION (ug/L)

Table 11

Combined dose response data for rainbow trout, mean wet weight 14 g and 19 g, following exposure for 96 hours to lethal concentrations of copper (combined data of the July and November 1982 experiment). Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

Nominal copper concentration (ug/L)	Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality in 96 hours	96 hour LC_{50} (ug/L)
80	78	17	6	
80	79	17	6	
100	100	17	6	
100	103	17	3*	
200	177	17	12	
200	196	17	29	195
250	200	17	65	
250	247	17	47	
300	240	17	90*	
300	290	17	76	
control	7	17	0	

* corrected values for a 0% and 100% response (Litchfield and Wilcoxon, 1959)

Table 12

Regression analysis and chi-square of the lethal response data (presented in Table 11) for rainbow trout following exposure of 96 hours to copper solutions (combined data July-November 1982 experiments).

Regression equation	Slope function	Chi-square value	Chi-square 8 df 95%
$y = -5.4 + 4.5\ (x)$	1.7	19	16

Table 13

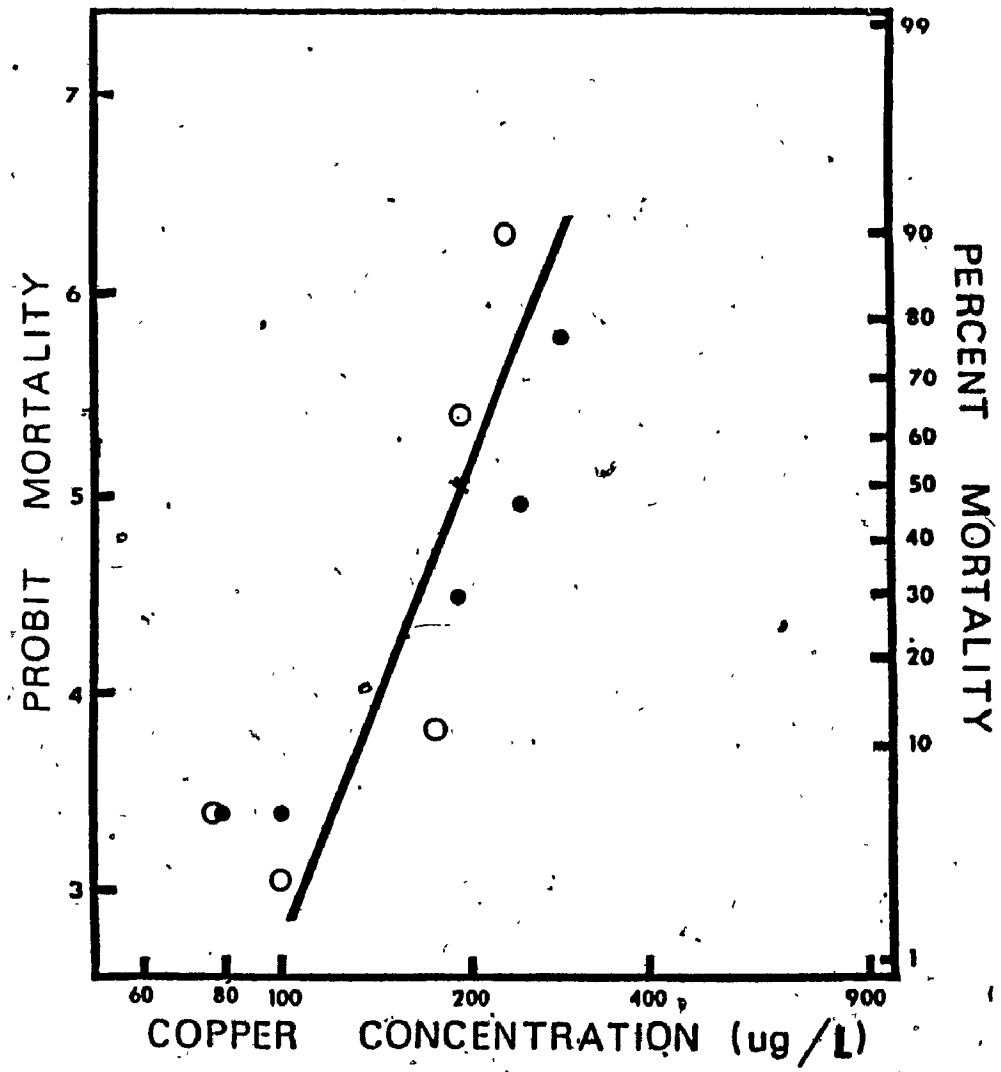
Probit analysis with 95% fiducial limits of the lethal response data (presented in Table 11) for rainbow trout following exposure of 96 hours to copper solutions (combined data July-November 1982 experiments). Fiducial limits corrected for heterogeneity of data.

Log Dose	Predicted Probit	Lower limit	Upper limit
1.89207	3.0685	1.8883	4.2487
1.89760	3.0904	1.9246	4.2562
1.99990	3.4965	2.5889	4.4041
2.01281	3.5474	2.6708	4.4240
2.24795	4.4801	4.0105	4.9497
2.29223	4.6558	4.1909	5.1207
2.30100	4.6906	4.2227	5.1685
2.38018	5.0047	4.4568	5.5526
2.39267	5.0542	4.4866	5.6218
2.46237	5.3307	4.6304	6.0310

Figure 7 Combined dose response data for rainbow trout, mean wet weight 14 g and 19 g, following exposure for 96 hours to lethal concentrations of copper (combined data of the July and November 1982 experiment). Derivation of the LC_{50} value followed that of Litchfield and Wilcoxon (1959).

● data for 14 g fish

○ data for 19 g fish


PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
1
60
80
100
200
400
900
COPPER CONCENTRATION (ug/L)

Test fish exposed to pure solutions of copper were observed to show distinct behavioral signs of toxicity. During the onset of toxicity, there was heightened locomotor activity, often near the water's surface but interspersed with sudden plunges to the depths of the tanks. Movement was often uncoordinated and jerky. These signs were apparent by 15 hours of exposure in test fish subject to the higher ambient concentrations of copper ($\geq$ 200 ug/L) while at lower copper concentrations in which fish died, the latency period to symptom onset was as much as 45 hours. A similar, time-related progression in mortality was recorded over the other concentration ranges used in tests (Table A, Appendix). Median mortality times were computed from LC_{50} data at 48, 64, 72, and 96 hours of exposure and are displayed in Table 14 and Figure 8.

Discussion

The dose response distributions for copper at 64 hours and 96 hours, as shown in Figs. 6 and 7 respectively, indicate a curvilinear pattern due to a systematic variation of response coordinates at the lower end of the lethality range. It was apparent that up to 6% the test population were hypersensitive to waterborne copper between concentrations of 80 to 100 ug/L. Since only about 6% of the population respond in this way it was estimated that the Litchfield and Wilcoxon methodology could be used to calculate the LC_{50}. The concentrations of cadmium. The mean size of fish between the two

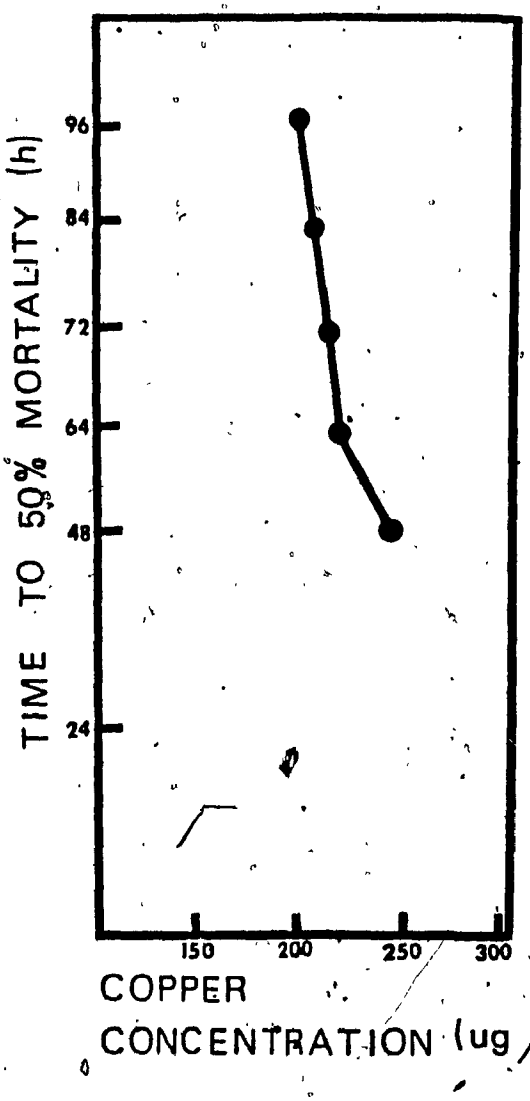
Table 14

Median mortality times of 14 g rainbow trout exposed to copper solutions, July 1982 experiment. Derivation of the LC_{50} values followed that of Litchfield and Wilcoxon (1959).

Mean assayed concentration of copper (ug/L)	# of fish tested	Observed % mortality	Time point (h)	LC_{50} (ug Cu/L)
80	17	6		
100	17	2*	48	245
190	17	12		
250	17	29		
294	17	76		
82	17	6		
96	17	5*	64	220
192	17	12		
246	17	41		
295	17	76		
82	17	6		
96	17	2*	72	217
192	17	24		
246	17	41		
295	17	76		
79	17	6		
100	17	2*	84	204
196	17	29		
247	17	47		
290	17	76		
79	17	6		
100	17	6	96	200
196	17	29		
247	17	47		
290	17	76		

* corrected values (Litchfield and Wilcoxon, 1959).

Figure 8 Time-mortality curve for 14 g rainbow trout exposed to copper solutions, July 1982 experiment. Derivation of the LC_{50} values followed that of Litchfield and Wilcoxon (1959).


TIME TO 50% MORTALITY (h)
96
84
72
64
48
24
150
200
250
300
COPPER
CONCENTRATION (ug/L)

Litchfield and Wilcoxon methodology places emphasis, to determine a LC_{50}, on data points found between 10% and 90% of the response. This provided LC_{50} estimates that were virtually the same as those determined with the model-free method of Spearman-Karber. Furthermore, the Litchfield and Wilcoxon method generated probit analysis and confidence intervals for the data to give a better estimate of toxicity for the test population.

As noted in Table 11 and Figure 7 the average 96 h LC_{50} resulting from the two copper mortality tests was 195 ug/L. The slope function of this line (1.7) is relatively steep indicating that variance in tolerance within the test population was not great. Spear found a very similar copper 96 h LC_{50} value of 190 ug cu/L for rainbow trout of approximately 29 g in weight (Spear and Anderson, 1975). The water hardness in both Spear's test and ours was 125 mg/L as $CaCO_3$, with an alkalinity of 85 mg/L as $CaCO_3$, both sets of experiments having been conducted in the same laboratory. Interestingly, Spear conducted the same tests under identical conditions with rainbow trout averaging only 3.9 g (Spear and Anderson, 1975). He obtained a copper 96 h LC_{50} value of 200 ug/L, very similar to the LC_{50} value of 190 ug Cu/L for the much larger 29 g trout mentioned above. Consequently, Spear concluded from his studies that copper toxicity in rainbow trout weighing 3.9 g to 29 g was apparently independent of weight (Spear and Anderson, 1975). Chakoumakos et al. (1979) working in a different laboratory with water conditions of 12.8°C, pH 7.8, alkalinity 174 mg/L as $CaCO_3$, found a 96 h LC_{50} value of 243 ug Cu/L for rainbow trout with a mean weight of 26 g. Brown et al. (1974) found a much

higher LC_{50} value of 550 ug Cu/L in a 96 hour study with 13-15 cm rainbow trout. This decreased toxicity was probably due primarily to the water hardness which was a relatively high 250 mg/L as $CaCO_3$ (Brown et al., 1974). That total copper toxicity is inversely proportional to water hardness has been well demonstrated by Chakoumakos et al. (1979). In fact their work has demonstrated that both hardness and alkalinity control the degree of copper toxicity. Estimated partial correlations between the copper LC_{50} value and either hardness or alkalinity (with the alternate factor being fixed) were calculated by Chakoumakos to be 0.882 (90% C.I. = 0.938 - 0.246) respectively (Chakoumakos et al., 1979).

The time-mortality curve (Fig. 8) appears to be asymptotic with the time-axis by 96 hours, suggesting that the incipient lethal level may approximate the 96 h LC_{50} of 195 ug/L. The copper concentration range of the time-mortality curve in my studies was from 200 to 245 ug Cu/L for the time interval between 48 and 96 hours (Table 14). A similar steep copper time-mortality curve for rainbow trout was reported by Brown et al. (1974) with a copper concentration range of 550 to 600 ug Cu/L over the time interval between 48 and 96 hours. As their water hardness was 250 mg/L as $CaCO_3$ it is not surprising that their time-mortality curve indicates a much lower copper toxicity than the one obtained by the author.

1.2 Cadmium Lethal Response Study

Two experiments, one in February, the other one in May, were conducted to determine the tolerance of rainbow trout to lethal

Table 15

Dose response data for rainbow trout, mean wet weight 25 g, following exposure for 64 hours to lethal concentrations of cadmium, February 1985 experiment. Derivation of the LC_{50} value was by graphical interpolation.

Nominal cadmium concentration (ug/L)	Mean assayed concentration of cadmium (ug/L)	# of fish tested	Observed % mortality in 64 hours	64 hour LC_{50} (ug/L)
1,000	905	16	19	
2,000	1,900	16	25	
3,000	2,975	16	31	9,730
4,000	3,977	16	31	
control	0	16	0	

Figure 9 Dose response data for rainbow trout, mean wet weight 25 g, following exposure for 64 hours to lethal concentrations of cadmium, February 1985 experiment. Derivation of the LC_{50} value was by graphical interpolation.

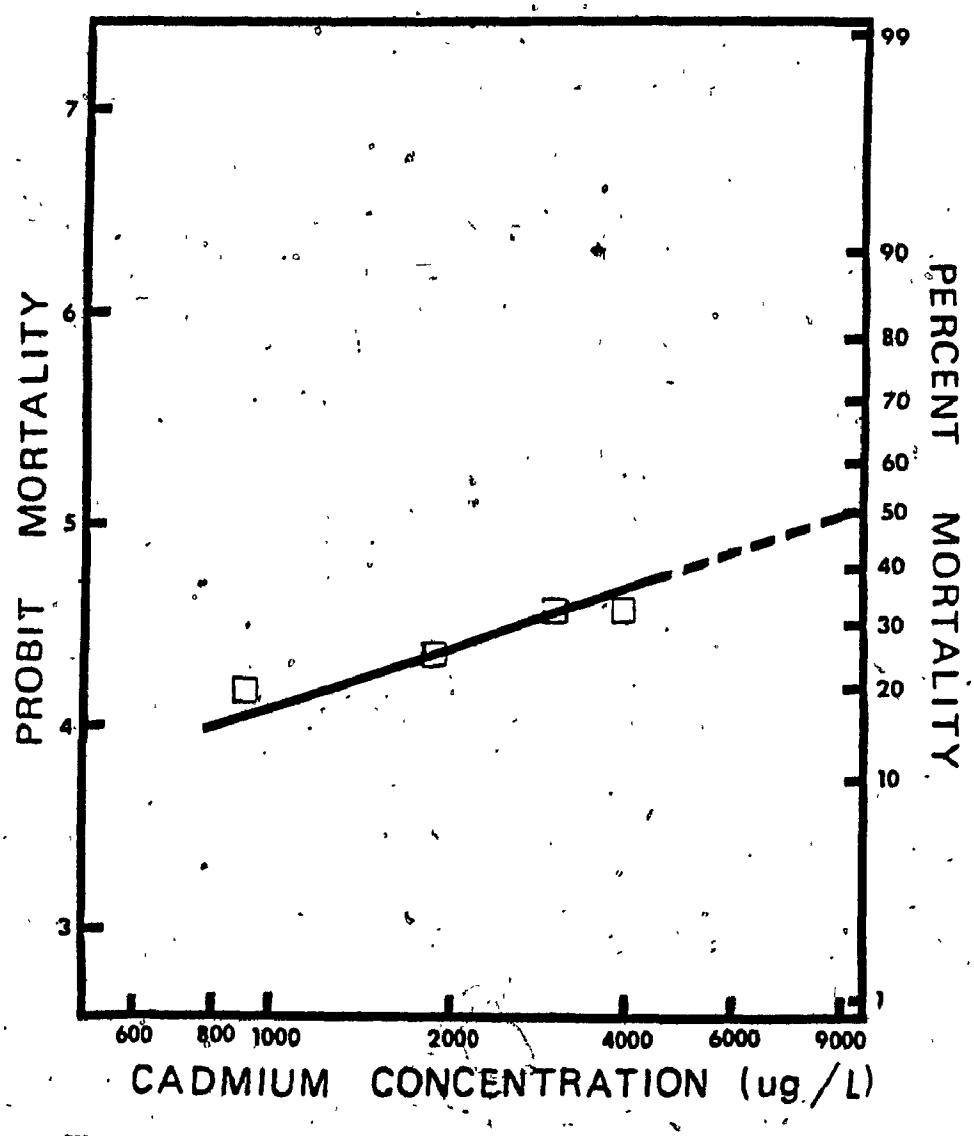
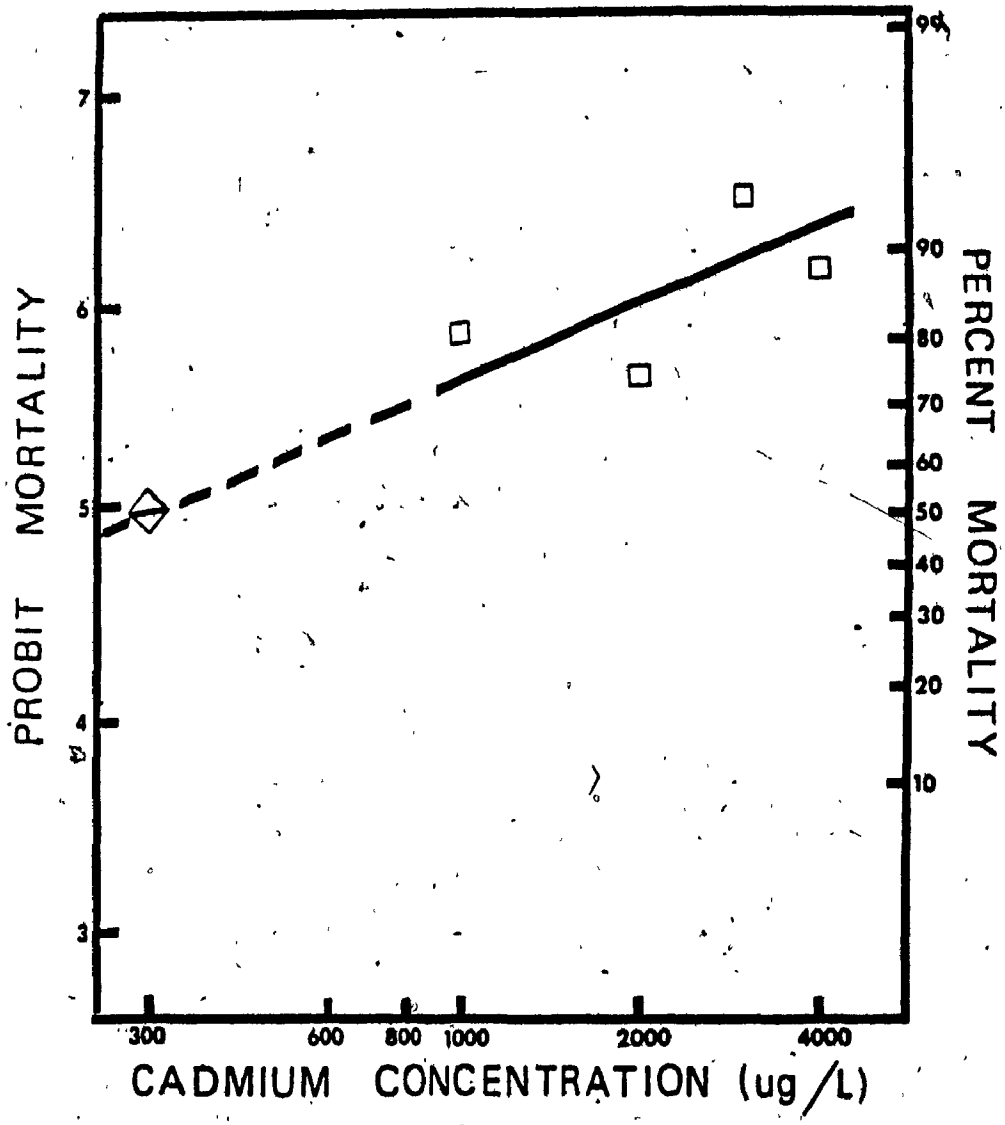

PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
1
600
800
1000
2000
4000
6000
9000
CADMIUM CONCENTRATION (ug/L)

Table 16

Dose response data for rainbow trout, mean wet weight 25 g, following exposure for 96 hours to lethal concentrations of cadmium, February 1985 experiment. Derivation of the LC_{50} value was by graphical interpolation.

Nominal cadmium concentration (ug/L)	Mean assayed concentration of cadmium (ug/L)	# of fish tested	Observed % mortality in 96 hours	96 hour LC_{50} (ug/L)
1,000	970	16	81	
2,000	1,980	16	75	300
3,000	2,970	16	94	
4,000	3,940	16	88	
control	0	16	0	

Figure 10. Dose response data for rainbow trout, mean wet weight 25 g, following exposure for 96 hours to lethal concentrations of cadmium, February 1985 experiment. Derivation of the LC_{50} value was by graphical interpolation. Roch's LC_{50} value is indicated by a diamond symbol (Roch, 1979).


PROBIT MORTALITY
7
6
5
4
3
PERCENT MORTALITY
99
90
80
70
60
50
40
30
20
10
300
600
800
1000
2000
4000
CADMIUM CONCENTRATION (ug/L)

experiments differed by about four fold. The mean wet weight of fish used in the February test was 25 g ± 4.4 g, while that of fish used in the May test was 7 g ± 1.6 g. Reported here are the dose response data at 64 and 96 hours for the test population with a mean weight of 25 g (Tables 15 and 16), since this weight approximated the weight of the test population used in the subsequent copper-cadmium mixture test.

It was apparent for the cumulative response values over a fourfold range in ambient cadmium concentrations that the dose response data for the cadmium test were insufficient for determining, by probit analysis, a linear response function at either 64 or 96 hours of exposure (Tables 15 and 16; Figures 9 and 10). Thus, LC_{50} values were estimated by graphical interpolation since it was impossible to compute by traditional means such as Probit Analysis and Litchfield and Wilcoxon (1959).

Mortality in cadmium solutions was first evident only after some 44 hours of exposure to the highest cadmium concentration (4000 Cd ug/L) (Table B, Appendix). During the initial stages of cadmium exposure, fish became hyperactive, swimming erratically about the tank. As time progressed, these fish became lethargic, swimming very slowly. Cearley and Coleman (1971) attributed behavior and death of bass and bluegills in cadmium solutions to the inhibition of acetylcholinesterase, causing death by paralysis of the muscles of respiration and/or depression of the respiratory center.

To depict progression of mortality in cadmium solutions a time

Table 17

Median mortality times of 7 g rainbow trout exposed to cadmium solutions, May 1983 experiment. Derivation of the LC_{50} values followed that of the 10% trim method of Spearman-Karber (Hamilton et al., 1977) and Litchfield and Wilcoxon (1959).

Mean assayed concentration of cadmium (ug/L)	# of fish tested	Observed % mortality	Time point (h)	LC_{50} (ugCu/L)
194	16	0		
390	16	0		
840	16	0	+48	7,330
1,225	16	7		
4,396	16	0		
6,200	16	33		
11,990	16	100		
196	16	0		
415	16	0		
865	16	0	+64	7,042
1,204	16	7		
4,315	16	7		
6,110	16	40		
11,845	16	100		
196	16	7		
415	16	1*		
865	16	3	72	6,720
1,204	16	7		
4,315	16	20		
6,110	16	47		
11,845	16	90*		
189	16	7		
435	16	7		
912	16	7	84	4,013
1,211	16	27		
4,570	16	27		
6,284	16	60		
11,990	16	92*		

Table 17 continued

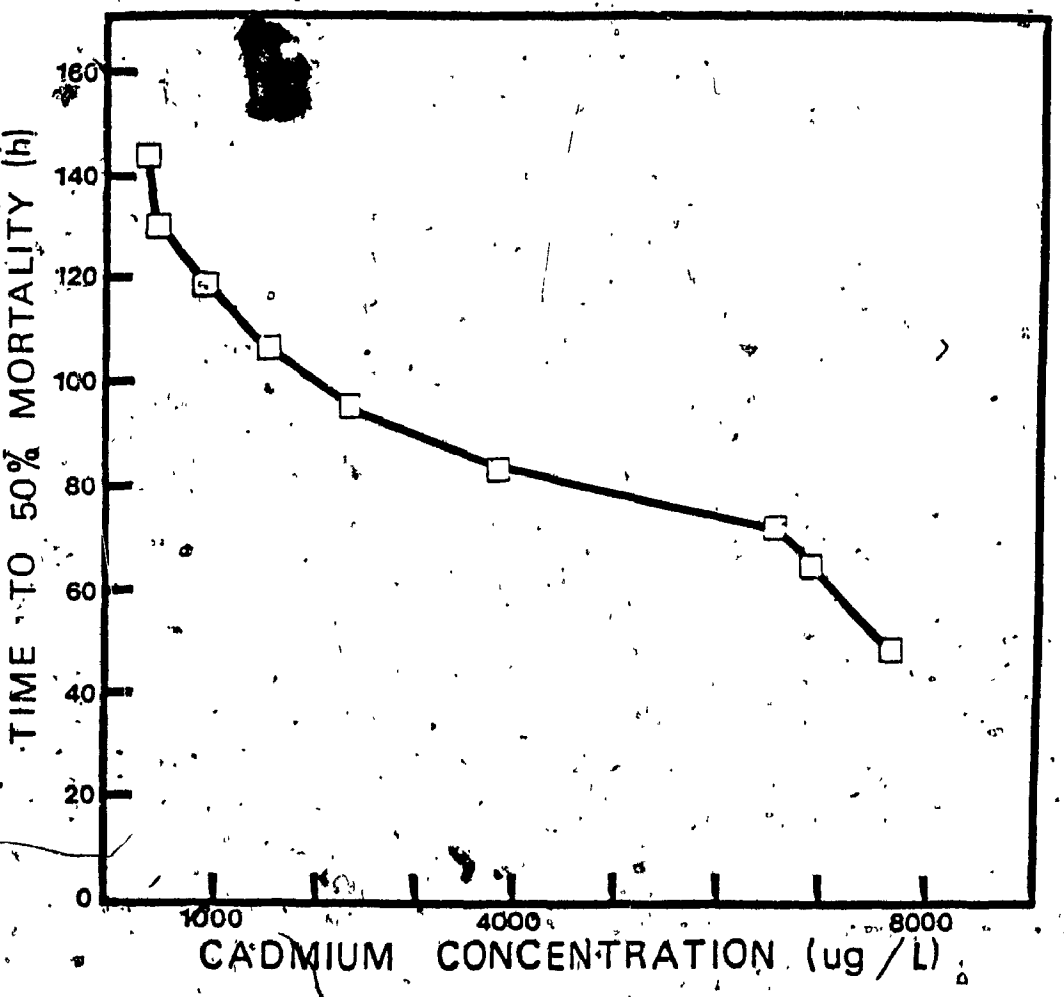
Median mortality times of 7 g rainbow trout exposed to cadmium solutions, May 1983 experiment.

Mean assayed concentration of cadmium (ug/L)	# of fish tested	Observed % mortality	Time point (h)	LC_{50} (ugCu/L)
189	16	7		
435	16	20		
912	16	13	96	2,440
1,211	16	40		
4,570	16	33		
6,284	16	80		
11,990	16	94		
198	16	27		
417	16	13		
914	16	33	108	1,667
1,204	16	60		
4,548	16	67		
6,240	16	80		
11,970	16	98*		
198	16	27		
417	16	20		
914	16	33	120	981
1,204	16	60		
4,548	16	80		
6,240	16	87		
11,970	16	97*		
210	16	33		
428	16	40		
890	16	40	132	571
1,215	16	80		
4,527	16	87		
6,308	16	93		
11,980	16	97*		
210	16	33		
428	16	47		
890	16	53	144	475
1,215	16	80		
4,527	16	87		
6,308	16	93		
11,980	16	97*		

+ LC50 estimated by Spearman-Karber.

* corrected values (Litchfield and Wilcoxon, 1959).

Figure 11 Time-mortality curve for 7 g rainbow trout exposed to cadmium solutions, May 1983 experiment. Derivation of the LC_{50} values followed that of the 10% trim method of Spearman-Karber (Hamilton et al., 1977) and Litchfield and Wilcoxon (1959).


TIME TO 50% MORTALITY (h)
160
140
120
100
80
60
40
20
0
1000
4000
8000
CADMIUM CONCENTRATION (ug / L)

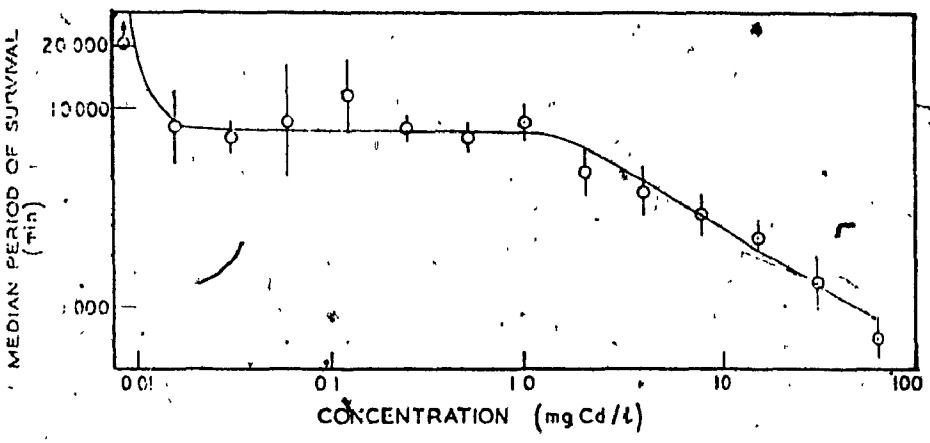


Figure 12 Toxicity of cadmium in hard water to rainbow trout (Ball, 1967).

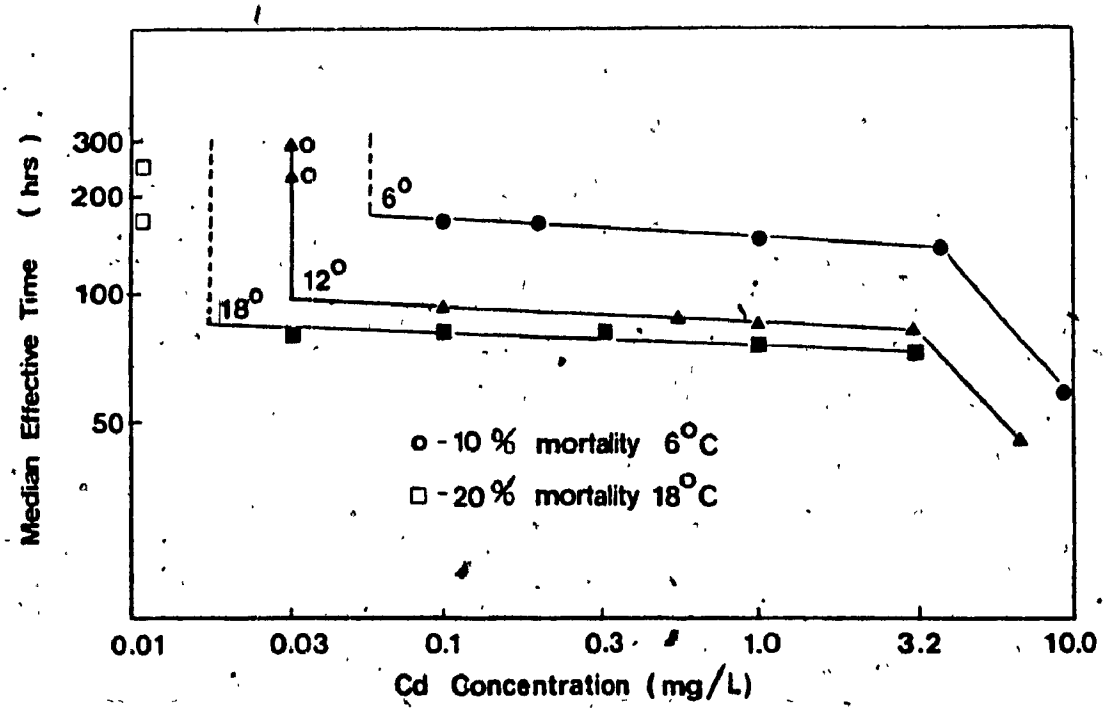


Figure 13 Median survival time of rainbow trout exposed to cadmium and acclimated to 6, 12 and 18°C (Roch, 1979).

mortality curve was computed from LC_{50} data at 48 hours up to 144 hours of exposure, using data collected on 7 g fish. This time mortality data is shown in Table 17 and Figure 11.

Discussion

Upon examination of time-mortality data (Table 17, Figure 11) it would appear that there may be major differences in the median tolerance parameter over a short portion of the exposure period concurring around 96 hours. This peculiar pattern has been observed for cadmium by other researchers (Ball, 1967; Roch, 1979) who reported cadmium toxicity curves for rainbow trout with a plateau in LC_{50} values ranging from 7,000 ug/L to less than 20 ug/L at approximately 4 days of exposure (Figure 12 and 13). Assuming that over this concentration range there is no significant change in the form and availability of cadmium to test fish, then this unique response pattern to cadmium may be interpreted to mean that rainbow trout can resist a wide range in cadmium concentrations in a manner that is not dose related.

I observed some disparity between my study and that of Ball's (1967). While the trends are similar, the area of disproportionality, manifested in the flat curve, is apparently shifted to the right. There are several possible contributing factors that can help explain this shift. Water quality is an important contributing factor in cadmium toxicity, and one readily capable of shifting the toxicity curve. In this case our water hardness was 125 mg/L as $CaCO_3$ while that of Ball's was 290 mg/L as $CaCO_3$. A relationship between toxicity

and water hardness was established in an EIFAC report which concluded that a decrease in water hardness (as well as dissolved oxygen and possibly pH) produced a lower cadmium LC_{50} (EIFAC, 1978). Other water quality factors that can modify toxicity of cadmium to aquatic organisms are alkalinity and mineral content of the water (Florence, 1982).

Another factor that can help explain the shift is weight. Our 96 hour LC_{50} was observed to be 2,440 ug Cd/L for 7 g fish while that of Ball's was observed to be 8,000 ug Cd/L. No specific comparisons can be made since Ball omitted to report the size and weight of the experimental fish (Ball, 1967).

Concentration response data recorded for the exposure period of 64 and 96 hours was incomplete in its distribution, being limited to that part of the response range below 30% and above 60% respectively (Tables 15 and 16). It was not possible to compute by traditional means (eg. Probit Analysis, Litchfield and Wilcoxon, 1959) a dose response function. However, if a line is fitted visually to the points, its gradual slope suggests a wide range in tolerance amongst members of the test populations in relation to a response which apparently is not markedly dose dependent (Figs 9 and 10).

A study by Roch (1979) in which the size of rainbow trout (21 g) were similar to those used in this experiment (25 g) and where conditions were virtually identical to those reported here, reported the 96 h LC_{50} for cadmium at 300 ug/L. If this value is combined with those in Table 16, it is possible to visually fit a line to the experimental response distribution that further validates the

data (Figure 10).

Thus, as shown, there is some suggestion that the slopes of dose response curves for juvenile rainbow trout exposed to cadmium are very gradual, at least within the exposure period of 64 to 96 hours. Such wide variance in lethal tolerance, as exemplified by the gentle slope, is normally associated with differences in a substance's toxicokinetics between test individuals (Anderson and D'Apollonia, 1978). The assumption is that individual differences between individuals in their uptake, distribution, excretion, storage and/or metabolism are reflected in dramatic differences in the level of effective dose reaching critical target tissue sites. It is considered far less likely that significant differences in the nature or number of critical target tissue sites may exist between individuals of the same population.

Where other cadmium LC_{50} values have been reported, there is considerable diversity. A cadmium 96 h LC_{50} of 95 ug Cd/L for juvenile rainbow trout was obtained by Chapman (1972) in water with hardness of 20-25 mg/L as $CaCO_3$. Ball (1967) was able to estimate a 7-day cadmium LC_{50} value of 8-10 ug Cd/L for rainbow trout, of unspecified size, age, or weight. Eisler (1971) examined the toxicity of cadmium on several species of Cyprinodonts. Ninety-six hour LC_{50} values of 21, 50, and 55 mg Cd/L were obtained for the striped killifish (Fundulus majalis), the sheepshead minnow (Cyprinodon variegatus) and the mummichog (Fundulus heteroclitus). All tests were conducted in 20°C water with a salinity of 20% (Eisler, 1971).

1.3 Copper-Cadmium Mixtures Lethal Response Study

One experiment was performed in February (1985) with the objective of determining the lethal potency of a series of copper-cadmium mixtures. Dose response data for the mixtures at 64 hours is presented in Table 18.

The levels of copper selected for the mixtures were approximately equivalent to 1/4x, 1/2x, and 1x toxic unit of the 96 hour copper LC_{50}. Due to the gentle slope of cadmium's discrete dose response lines, it was decided to choose a concentration range on the flat portion of the toxicity curve on the assumption that the unique dose response curve and toxicity for cadmium with a wide discrepancy between 64 hours and 96 hours would not lend itself to the empirical model for concentration addition.

The Cu-Cd combinations were arranged in order of increasing concentrations of each constituent. The lowest combination included cadmium at a level which was estimated to provide a 50% response upon 96 hours of exposure to cadmium, while the copper level in this combination was well below the lethal threshold for copper alone and would not have been expected to cause mortality. Upon testing, mortality amongst test organisms was nearly total (100%) at 96 hours of exposure to all of Cu-Cd combinations tested (Table C, Appendix).

At 64 hours of exposure to the Cu-Cd series, there were responses of 31%, 56%, 75% and 100% mortality respectively (Table 18). Lethal response data for Cd alone (Table 15) following 64 hours exposure indicates that pojected responses for cadmium alone were expected to be 19%, 25% and 31% respectively. Copper, as a constituent of

these mixtures may have contributed concurrently to the end-response, through some unknown physiological interaction.

The concentration of both metals was varied simultaneously relative to their individual potency to preserve equipotency of the metals in the combination. The alternative to keep one metal concentration constant while varying the other is in consideration for future studies.

To ascertain the toxicity of the mixtures at 64 hours the Concentration Addition Model was tested (Methods, 6.2.1). The isobologram in Figure 14 shows that higher levels of response may be predicted on the basis of the constituents of the Cu-Cd mixtures acting similarly as in the Concentration Addition model. However, this model may only be tested if the response lines for each constituent tested discretely are parallel. The dramatic differences in slope between copper and cadmium response lines negates testing this model as seen in Figures 6 and 9.

Another quantitative model which may be applied to data sets for copper and cadmium at 64 hours of exposure is the Response Addition Model (Methods, 6.2.2). This model is based on the assumption that the metals act on independent target sites. This model does not require parallelism between each toxicant's respective dose response curve. It simply attributes the probable contribution of a toxicant as a constituent of a mixture in proportion to its effect as observed alone.

On the assumption that the tests are not correlated in their tolerance to either copper or cadmium, the Response Addition Model was

Table 18

Dose-response data for rainbow trout, mean wet weight 25 g, following exposure for 64 hours to copper-cadmium mixtures, February 1985 experiment.

Mean assayed metal concentration (ug/L) Cu	Cd	# of fish tested	Observed % mortality in 64 hours
28	440	16	31
63	890	16	56
125	1,830	16	75
176	2,630	16	96*
6	0	16	0

* corrected value (Litchfield and Wilcoxon, 1959)

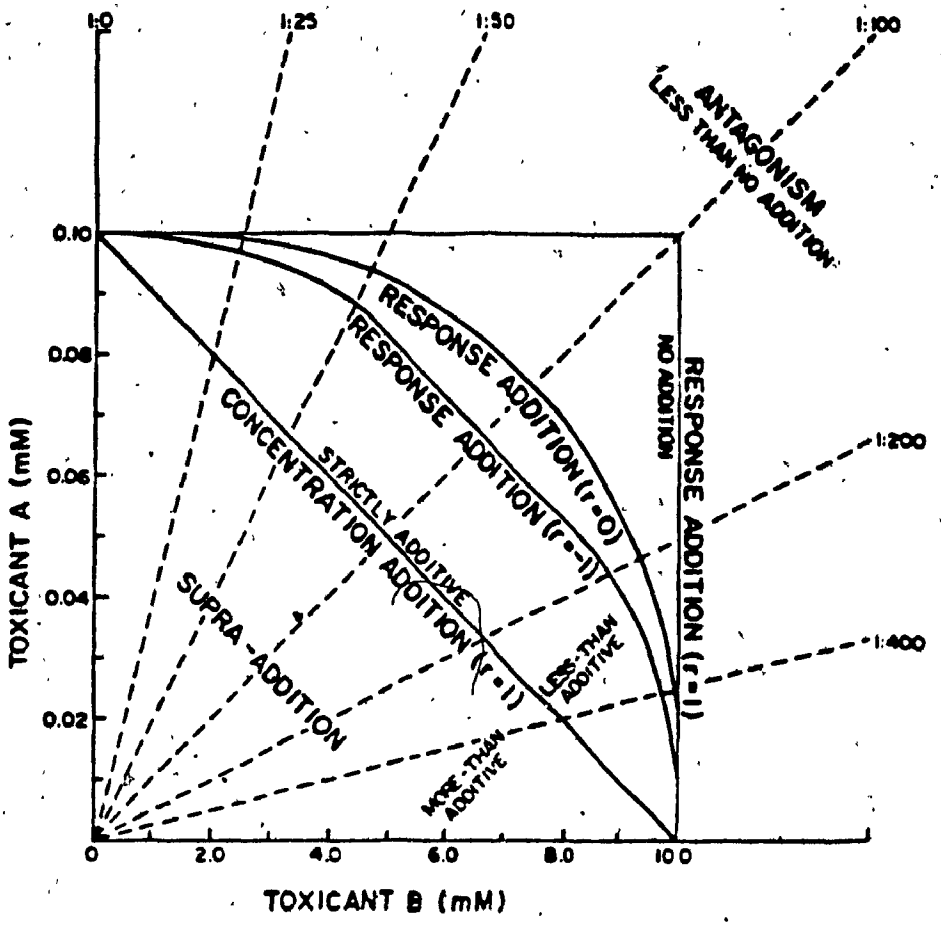


Figure 14 Isobole diagram depicting various types of lethal responses for the joint action of two toxicants displaying parallel concentration-response curves.

applied. Using 64 hour data sets the series of Cu-Cd mixtures would be expected to have results as shown in Table 19. Thus, in accordance with the Response Addition Model the highest concentration of Cu-Cd mixtures to which rainbow trout were exposed, should not have caused more than 50% mortality, however, a 96% mortality was observed. An additional aspect of the Response Addition Model analysis is worth noting. As seen in Table 19, copper in the mixtures, the lowest two combined concentrations were not expected to contribute to the joint toxicity. However, the Response Addition model could not be fitted to our experimental data. This model requires that the potencies of the toxicants be the same. As seen in Table 19, copper at the two lowest concentrations was not contributing to the toxicity of the mixture when compared to its potency as a single agent.

An alternative approach was thus necessary. The Toxic Unit method of Sprague (1970) was adopted to provide a quantitative estimate of concentration addition at the 50% level. This test may be applied to the highest combination of Cu and Cd used in this experiment since this combination yields a toxic unit value of 1.1 which approximates the required 1.0 unit for this test (Table 20). Rather than a slightly higher than 50% response level that the toxic unit principle predicts, a response of 100% was recorded (Table 20). More importantly almost all of the test organisms in the Cu-Cd mixtures tested succumbed by 96 hours (Table C, Appendix), indicating a reduction in resistance to that expected for copper or cadmium alone (Table A and B, Appendix).

The Mixture Toxicity Index was used to calculate the degrees of

Table 19

Response Addition Model for the copper-cadmium mixtures.

Mean daily concentration (ug/L) Cu	Cd	PCu	PCd	PC Expected	PC Observed
28	440	0	0.10	0.10	0.31
63	890	0	0.16	0.16	0.56
125	1,830	0.06	0.23	0.29	0.75
176	2,630	0.25	0.30	0.55	0.96

PC = proportion responding to the mixture
PCu = proportion responding to copper
PCd = proportion responding to cadmium

Table 20

Toxic unit values for a Cu-Cd mixture combination.

Mean daily concentration (ug/L)		Toxic Unit		Expected Toxic Unit at 64 h Cu-Cd mixtures	Expected % Response	Observed % Response
Cu	Cd	Cu	Cd			
176	2,630	0.84	0.27	1.11	50	100

enhancement of the mixture over that expected through toxic unit analyses (Methods, 6.2.4). The calculation of the MTI necessitated selection of the experimental mixture concentration which most closely approximated the LC_{50} value for the test series. This mixture concentration proved to be 63 ug Cu/L and 890 ug Cd/L which jointly resulted in a 56% mortality response (Table 18). An MTI value of 2.6 was calculated for this Cu-Cd mixture concentration. This value is greater than one, and is thus, according to the MTI scale, strongly synergistic with a supra-additive joint action.

The supra-additive response of Cu-Cd mixtures could not be shown graphically since the experimental design does not quantify the contribution of copper or cadmium to the lethal response.

Toxicity signs displayed by the test population exposed to Cu-Cd solutions were similar to those caused by cadmium in single metal exposures. These signs showed up earlier in the test, and as shown in Table C of the Appendix, mortality onset was earlier in the mixtures.

In contrast to my findings, Finlayson and Verrue (1982) working with coho salmon determined that Cu-Cd mixtures produced only an additive joint toxicity, falling short of the supra-additivity observed in my work. Unfortunately no concrete comparison can be made as they neglected to report the actual test concentrations. Thus, it is possible that their concentration range was such that a supra-additive joint toxic action may not have been feasible.

Supra-additivity of Cu-Cd toxicity mixtures in zebrafish (Brachydanio rerio) was demonstrated by Hewitt (1980). He determined that copper and cadmium when in combination were 2.1 times more potent

than predicted (Hewitt, 1980). Several other workers have noted supra-additivity resulting from metal mixture exposures. Sprague and Ramsay (1965) working with Atlantic salmon found that Cu-Zn mixtures decreased survival time by a factor of two to three when compared to the single metal toxicity. Similarly, Eisler and Gardner (1973) found that mixtures of Cu-Zn produced a supra-additive toxic effect, resulting in a greater-than-expected mortality rate in mummichogs, (Fundulus heteroclitus). Furthermore, the addition of 1 or 10 mg Cd/L to all Cu-Zn mixtures tested produced a still greater degree of mortality within the 96 hour toxicity test period indicating even more supra-additivity (Eisler and Gardner, 1973). Lewis (19[illegible]), determined the 96 hour acute toxicity of copper, zinc, and Cu-Zn mixtures in juvenile longfin dace (Agosia chrysogaster). Cu-Zn was the most lethal toxicant (210 ug Cu/L and 280 ug Zn/L) and exhibited a more than additive toxicity in contrast to 96 h LC_{50}'s single metal of 860 ug Cu/L and 790 ug Zn/L.

1.4 General Discussion

Individually, it was demonstrated that, on the basis of the 64 hour LC_{50}'s copper was more toxic to rainbow trout than was cadmium.

As shown through the experimental results the mode of action of copper and cadmium are apparently different. This inference is reinforced by the apparent differences in the slope distributions of the 64 hour dose response curves (Fig. 6 and 9) and the time-mortality curves (Fig. 8 and 11) of copper and cadmium. Furthermore, qualitative observations on the behavioral signs of toxicity for

copper and cadmium also suggest these metals respective modes of action may differ. The behavior of juvenile rainbow trout exposed to cadmium was sharply in contrast to that recorded for fish exposed to copper.

The results of the multiple toxicity studies indicated that mixtures of copper and cadmium have a supra-additive toxic action on rainbow trout. At this moment evaluation of environmental effects is usually based on toxicity data for single toxicants. Future attempts to describe or to predict the impact of pollutants on fish populations should consider the joint toxicity of Cu-Cd mixtures and should not be confined to the effects of copper and cadmium alone.

The observed supra-additive toxicity of copper-cadmium mixtures must result from some kinetic and/or dynamic interaction. Physiological interactions in the kinetic phase will alter mechanisms of toxicant uptake, distribution, degradation and excretion. A kinetic interaction may create the enhanced effect of increased availability of copper and/or cadmium at their respective target sites. In the dynamic phase physiological interactions of Cu-Cd mixtures may alter the sequence of events which lead to the binding of copper and cadmium to their target tissue.

2.0 Kinetic Studies

2.1 Copper Kinetic Studies

2.1.1 Copper Exposure and Clearance in the Gill

During the exposure phase, rainbow trout were exposed to three copper concentrations: 60, 115, and 228 ug/L. Maximum concentrations in the gill were seen at 4 hours and 16 hours respectively for the groups exposed to 60 ug Cu/L, 115 and 228 ug Cu/L (Table 21, Fig. 15). These levels of accumulation were proportionally related to the exposure concentration and represented a 200, 400 and 650% increase over the copper levels evidenced in controls. Past this initial rapid uptake, once a maximum concentration was attained in the gill, copper levels decreased to levels which were approximately common to all test concentrations and above levels seen in controls. Once they had decreased, metal levels showed little fluctuation for the remainder of the exposure phase.

During the clearance phase, copper levels remained approximately the same for the first four hours, increasing in levels at 8 hours of clearance (Table 22, Fig. 15).

2.1.2 Copper Exposure and Clearance in the Serum

Copper levels in the serum indicated that for the two lower exposure groups (60 and 115 ug Cu/L), uptake had been similar with maximum concentrations for both groups at 64 hours (Table 23, Fig. 16).

The pattern of uptake for the group exposed to the highest copper concentration (228 ug Cu/L) differed from that seen above. A maximum

Table 21

Mean copper concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase of the copper kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration of copper (ug/L)	Time of exposure (h)	Geometric mean-copper concentration in gills (ug/g of dry weight)	Standard deviation
60		4.3	1.53
115	2	6.1	1.26
228		8.7	1.22
7 (control)		2.6	1.91
60		10.7	1.32
115	4	6.2	1.62
228		8.1	1.49
7 (control)		3.5	1.43
60		7.0	1.42
115	8	7.9	1.21
228		8.9	1.19
7 (control)		2.9	1.55
60		4.6	1.21
115	16	14.0	1.32
228		23.1	1.47
7 (control)		3.0	1.07
60		6.3	1.70
115	32	5.0	1.32
228		5.0	1.40
7 (control)		2.9	1.05
60		4.5	1.38
115	64	6.8	1.39
228		6.4	1.61
7 (control)		3.2	1.24

Table 22

Mean copper concentration in rainbow trout gills, at various time intervals, during the 8 hour clearance phase of the copper kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of copper (ug/L)	Time of clearance (h)	Geometric mean-copper concentration in gills (ug/g of dry weight)	Standard deviation
60	2	5.5	1.10
115		6.7	1.39
228		6.0	1.11
7 (control)		2.4	1.21
60	4	5.9	1.32
115		6.0	1.11
228		5.0	1.43
7 (control)		2.7	1.37
60	8	5.4	1.50
115		10.2	1.21
228		20.5	1.40
7 (control)		3.0	1.14

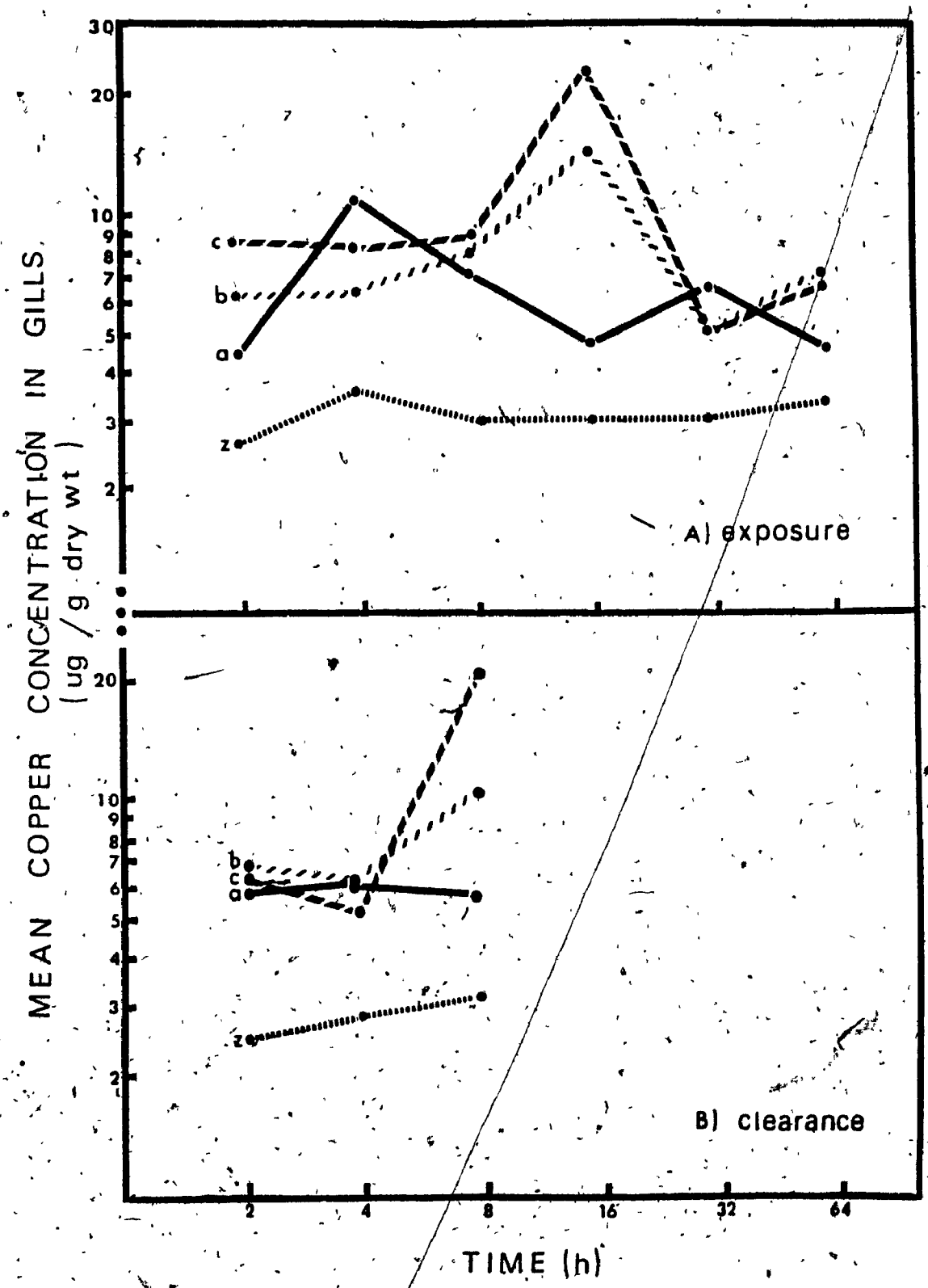
Figure 15 A and B Mean copper concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper kinetic studies. All values are the mean of 4 fish.

a = 60 ug Cu/L

b = 115 ug Cu/L

c = 228 ug Cu/L

z = 7 ug Cu/L (control)


MEAN COPPER CONCENTRATION IN GILLS
(ug / g dry wt)
A) exposure
B) clearance
TIME (h)
a
b
c
z
2
4
8
16
32
64

copper concentration was obtained at 2 hours of exposure. Following, a moderate decline in serum copper levels was observed with time to 32 hours of exposure. This was followed by an increase in serum copper levels from 32 to 64 hours. Levels at 64 hours were comparable to levels seen at 2 hours of exposure (Table 23, Fig. 16).

Accumulation of copper in the serum was apparently not related to the exposure concentration. Levels of copper for all of the test groups were approximately common throughout the entire exposure phase. The clearance phase revealed widely dispersed data (Table 24, Fig. 16).

Discussion

In both gills and serum, control levels of copper were found to be above 0 ug Cu/L. Copper is an essential metal in rainbow trout, normally found in the living animal (Spear, 1975). Furthermore, backgrounds levels of copper in the exposure water averaged 7 ug/L making it possible for control fish to absorb copper from the ambient water.

As shown in Figure 15, during the exposure phase, once maximum concentrations of copper were attained in the gills, levels of copper for all of the test groups decreased to concentrations slightly above those of controls groups. This strongly suggests that the gills are not a site of copper accumulation in rainbow trout during the 64 hour exposure period.

During the clearance phase in gills, maximum levels of copper were similar to maximum levels in the exposure phase for all three

Table 23

Mean copper concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase of the copper kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration of copper (ug/L)	Time of exposure (h)	Gedmetric mean-copper concentration in serum (ug/mL serum)	Standard deviation
60		3.8	1.16
115	2	3.2	1.33
228		5.4	1.07
7 (control)		2.4	1.31
60		4.6	1.32
115	4	3.3	1.34
228		5.1	1.40
7 (control)		2.5	1.03
60		5.4	1.36
115	8	5.2	1.41
228		4.4	1.33
7 (control)		3.1	1.15
60		4.8	1.51
115	16	5.4	1.12
228		4.4	1.34
7 (control)		2.2	1.22
60		4.1	1.23
115	32	5.4	1.25
228		3.2	1.60
7 (control)		2.8	1.16
60		12.3	1.12
115	64	10.1	1.79
228		5.6	1.60
7 (control)		3.4	1.21

Table 24

Mean copper concentration in rainbow trout serum, at various time intervals, during the 8 hour clearance phase of the copper kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of copper (ug/L)	Time of clearance (h)	Geometric mean-copper concentration in serum (ug/mL serum)	Standard deviation
60		12.1	1.14
115	2	4.1	1.32
228		7.0	1.65
7 (control)		3.7	1.23
60		4.9	1.33
115	4	4.4	1.58
228		7.0	1.46
7 (control)		4.6	1.33
60		10.2	1.52
115	8	7.6	1.43
228		9.8	1.18
7 (control)		3.3	1.05

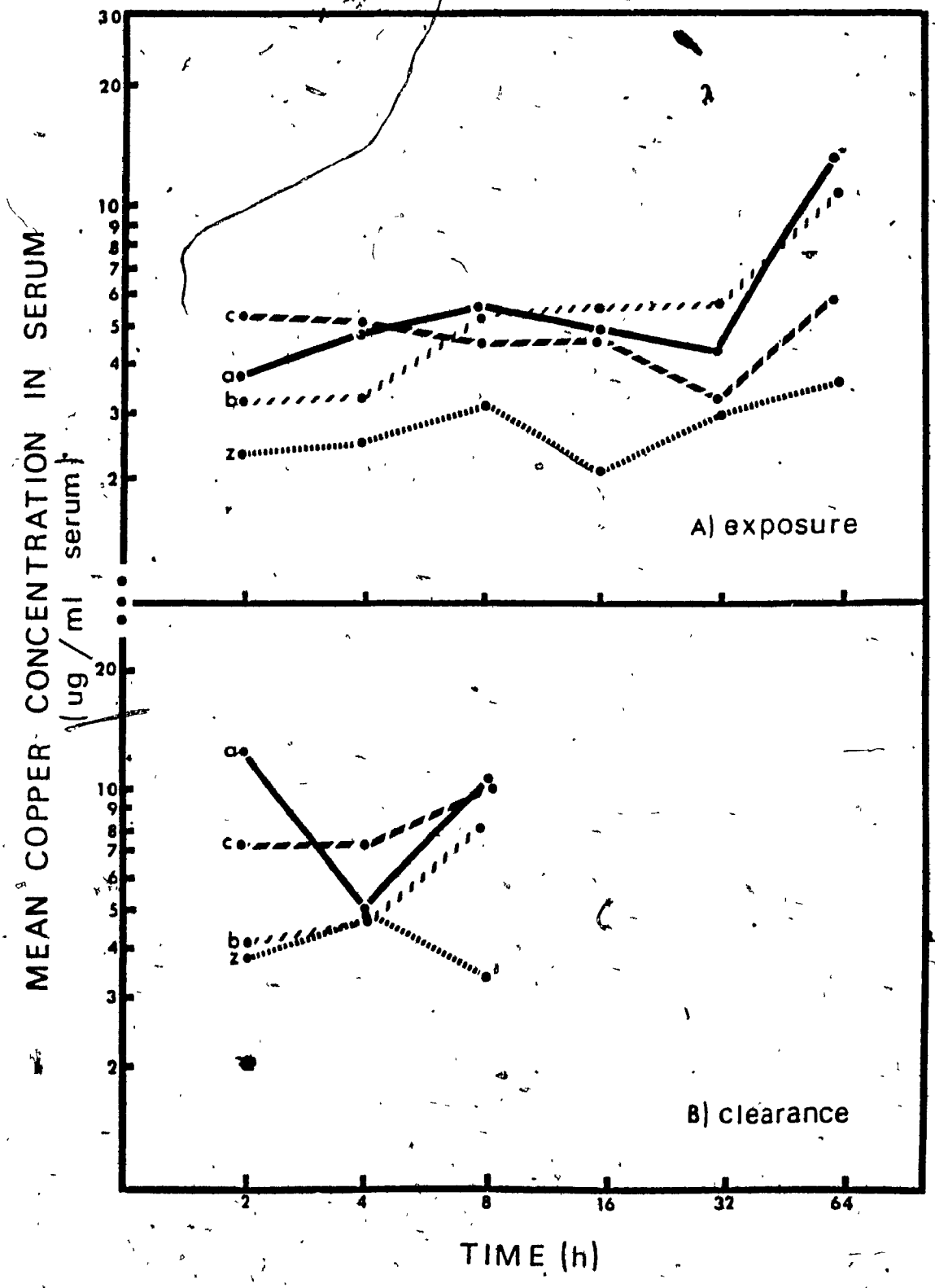
Figure 16 A and B Mean copper concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper kinetic studies. All values are the mean of 4 fish.

a = 60 ug Cu/L

b = 115 ug Cu/L

c = 228 ug Cu/L

z = 7 ug Cu/L (control)


MEAN COPPER CONCENTRATION IN SERUM
(ug/ml serum)
30
20
10
9
8
7
6
5
4
3
2
c
a
b
z
A) exposure
20
10
9
8
7
6
5
4
3
2
a
c
b
z
B) clearance
2
4
8
16
32
64
TIME (h)

exposure concentrations (Table 22, Fig. 15). This further indicates that copper absorbed during the uptake phase was translocated from the gills into other internal compartments.

No studies have been reported in fish dealing with kinetics of copper in internal compartments. Nevertheless, the initial rapid uptake of copper by gills has been observed by other authors. McConnell and Stokes (1985) reported that rainbow trout exposed to 1 and 5 mg Cu/L for a period of 96 hours showed significant increases in gill copper levels. Unfortunately, for comparison's sake, none of the actual levels were reported (McConnell and Stokes, 1985). Betzer and Pilson (1975), measuring levels of copper in the gills as a percentage of the total copper concentration in the body, reported a similar pattern of copper accumulation in the whelk (Busycon canaliculatum). Exposure levels of 6-9 ug Cu/L resulted in gill copper concentrations of approximately 90, 50 and 15 percent of the total at 1, 17, and 48 hours (Betzer and Pilson 1975).

The idea of copper translocation to internal organs can also be examined by looking at the serum data. At 32 hours, in the gills, copper levels would presumably reflect the point at which the rate of translocation to internal compartments surpassed the rate of copper absorption from the ambient water into the gills. This is reflected in a progressive increase of copper levels in the serum from 32 to 64 hours of exposure.

Studies of copper levels in fish serum are scarce, limiting interpretive efforts. Buckley (1982) performed a study of copper accumulation in the plasma of coho salmon as a result of exposure to

70 and 140 ug Cu/L. After 24 hours of exposure, plasma copper levels were 0.54 and 0.76 ug Cu/mL of plasma for the two exposure concentrations compared to the control level of 0.47 ug Cu/mL. This is in contrast to our data where serum Cu levels at 32 hours of exposure to 60, 115, and 228 ug Cu/L were 4.1, 5.4 and 3.2 ug/L of serum compared to the control level of 2.8 ug/L of serum.

2.2 Cadmium Kinetic Studies

2.2.1 Cadmium Exposure and Clearance in the Gills

Uptake of cadmium in rainbow trout gills was measured at exposure concentrations of 530, 1060 and 2070 ug Cd/L. Results clearly show that the amount of cadmium found in gills was dependent on the exposure concentration (Table 25, Fig. 17). A rapid initial uptake of cadmium by 4 hours of exposure was seen at all test concentrations. This was followed by moderate fluctuations in cadmium levels with a sharp increase in tissue levels beginning at 32 hours of exposure. Maximum concentrations for all three of the exposure groups were reached at 64 hours representing an increase of 300, 600 and 1000% over control levels.

Levels of cadmium for all of the test concentrations throughout the 8 hour clearance phase, remained at approximately the same levels achieved at the 64th hour of accumulation (Table 26, Fig. 17).

2.2.2 Cadmium Exposure and Clearance in the Serum

During the exposure phase, there was an initial rapid uptake of cadmium seen at 4 hours for all three of the exposure groups. This

Table 25

Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase of the cadmium kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration of cadmium (ug/L)	Time of exposure (h)	Geometric mean-cadmium concentration in gills (ug/g of dry weight)	Standard deviation
530	2	1.0	1.31
1,060		1.7	1.23
2,070		6.0	1.57
0 (control)		0.0	0.00
530	4	1.8	1.14
1,060		3.4	1.43
2,070		5.8	1.37
0 (control)		0.0	0.00
530	8	1.2	1.40
1,060		2.6	1.32
2,070		4.5	1.26
0 (control)		0.0	0.00
530	16	1.9	1.28
1,060		3.2	1.34
2,070		6.2	1.42
0 (control)		0.0	0.00
530	32	0.6	2.50
1,060		2.7	2.21
2,070		5.6	1.42
0 (control)		0.0	0.00
530	64	4.2	1.39
1,060		7.2	1.38
2,070		11.2	1.22
0 (control)		0.0	0.00

Table 26

Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 8 hour clearance phase of the cadmium kinetic studies. All values are the mean of 4 fish.

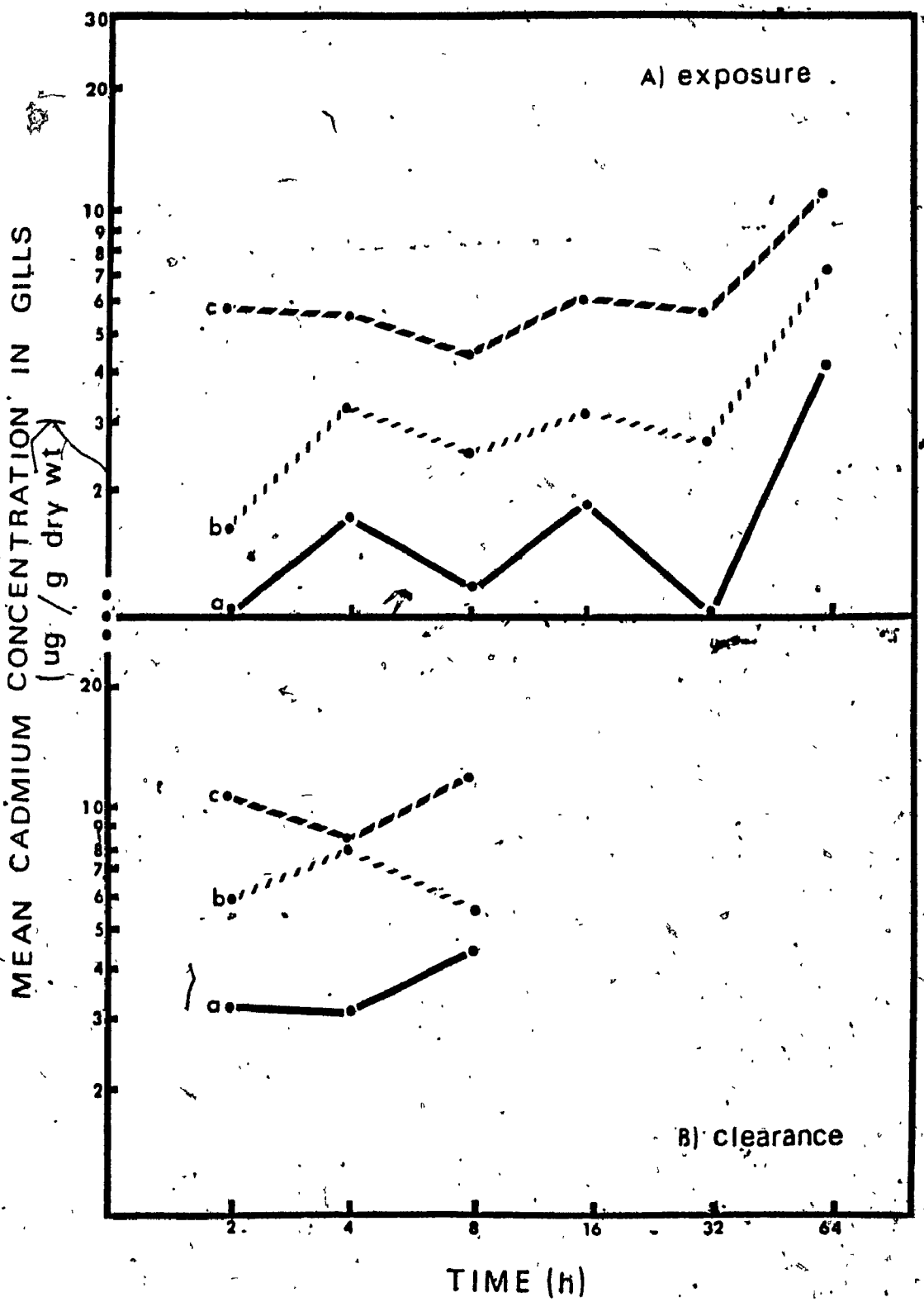
Previous exposure levels of cadmium (ug/L)	Time of clearance (h)	Geometric mean-cadmium concentration in gills (ug/g of dry weight)	Standard deviation
530		3.4	1.32
1,060	2	6.2	1.24
2,070		11.0	1.52
0 (control)		0.0	0.00
530		3.3	1.47
1,060	4	8.0	1.28
2,070		8.7	1.32
0 (control)		0.0	0.00
530		4.5	1.23
1,060	8	5.6	1.31
2,070		11.8	1.46
0 (control)		0.0	0.00

Figure 17 A and B Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the cadmium kinetic studies. All values are the mean of 4 fish.

a = 530 ug Cd/L

b = 1060 ug Cd/L

c = 2070 ug Cd/L


A) exposure
B) clearance
a
b
c
MEAN CADMIUM CONCENTRATION IN GILLS (ug / g dry wt)
TIME (h)
2
4
8
16
32
64

was followed at 8 hours by a decline in cadmium levels, for the two lower exposure groups a and b (Table 27, Fig. 18). During the remainder of the exposure phase, cadmium levels began to increase in an apparently concentration dependent fashion reaching maximum concentrations at 32 hours for the groups exposed to 500 and 2070 ug Cd/L (a and c) and at 64 hours for the group exposed to 1060 ug Cd/L (b).

Cadmium clearance from the serum showed different trends as illustrated in Figure 18 and Table 28.

Discussion

Cadmium is not present as an essential metal in rainbow trout (Cearley and Coleman, 1974). Furthermore, background levels of this metal in the control water are of 0 ug Cd/L. Consequently, the control values in tissue and serum were measured at 0 ug Cd/L.

Some translocation of cadmium from the gills to the serum most likely began at 16 hours of exposure for all of the test concentrations. Between 16 and 32 hours, gill cadmium levels showed minimal to moderate declines, while serum levels of cadmium were seen to increase (Fig. 17 and 18). However, levels of cadmium accumulation in the serum at any exposure time were lower than levels assayed in the gills. Furthermore, from 32 to 64 hours of exposure, cadmium levels in the serum remained relatively constant or decreased while those levels in the gills increased for all of the exposure concentrations. This would seem to indicate that the gill is a site of cadmium accumulation in the rainbow trout.

Table 27

Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase of the cadmium kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration of cadmium (ug/L)	Time of exposure (h)	Geometric mean-cadmium concentration in serum (ug/mL serum)	Standard deviation
530		0.14	1.18
1,060	2	0.19	1.22
2,070		0.35	1.13
0 (control)		0.00	0.00
530		0.32	1.62
1,060	4	0.38	1.56
2,070		0.39	1.54
0 (control)		0.00	0.00
530		0.15	1.12
1,060	8	0.23	1.13
2,070		0.40	1.17
0 (control)		0.00	0.00
530		0.26	1.11
1,060	16	0.47	1.32
2,070		0.44	1.35
0 (control)		0.00	0.00
530		0.37	1.22
1,060	32	0.45	1.27
2,070		1.21	1.24
0 (control)		0.00	0.00
530		0.24	1.07
1,060	64	0.53	1.31
2,070		1.16	1.22
0 (control)		0.00	0.00

Table 28

Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 8 hour clearance phase of the cadmium kinetic studies. All values are the mean of 4 fish.

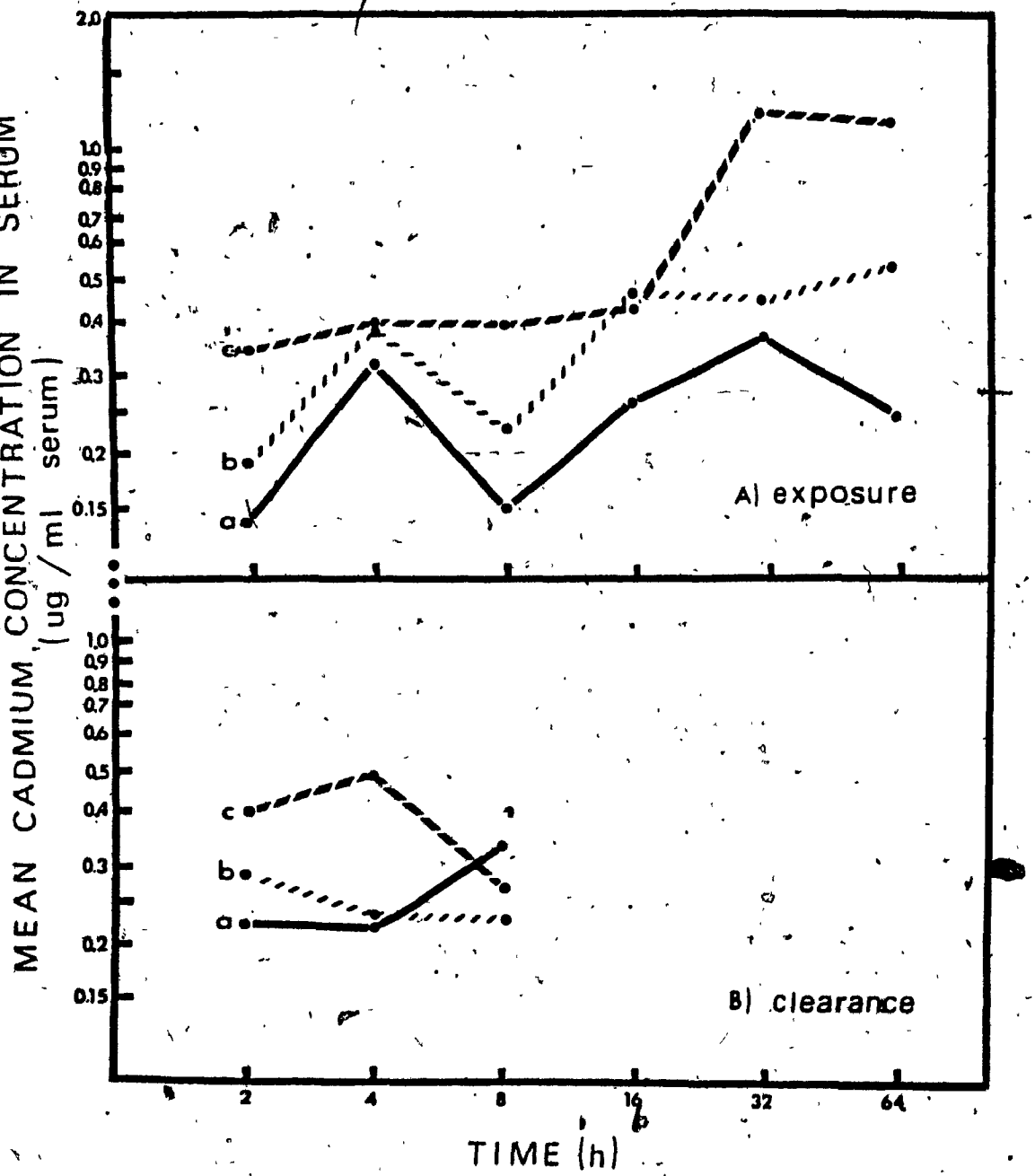
Previous exposure levels of cadmium (ug/L)	Time of clearance (h)	Geometric mean-cadmium concentration in serum (ug/g of dry weight)	Standard deviation
530		0.24	1.23
1,060	2	0.30	1.41
2,070		0.41	1.28
0 (control)		0.00	0.00
530		0.22	1.00
1,060	4	0.24	1.10
2,070		0.50	1.22
0 (control)		0.00	0.00
530		0.32	1.13
1,060	8	0.23	1.10
2,070		0.27	1.31
0 (control)		0.00	0.00

Figure 18 A and B - Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the cadmium kinetic studies. All values are the mean of 4 fish.

a = 530 ug Cd/L

b = 1060 ug Cd/L

c = 2070 ug Cd/L


MEAN CADMIUM CONCENTRATION IN SERUM
(ug / ml serum)
2.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.15
c
b
a
A) exposure
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.15
c
b
a
B) clearance
2
4
8
16
32
64
TIME (h)

There is little published data with which I can compare my findings; most work has concentrated on cadmium accumulation over longer exposure times. Roberts worked with rainbow trout, 170-340 g in weight, exposing them to 9 ug Cd/L for either 2 weeks or 3 months (Roberts et al., 1979). With 2 weeks of cadmium exposure, the gills were found to have the greatest cadmium concentration than any of the other body compartments, some four and a half times the cadmium levels found in either the liver or the kidneys (Roberts et al., 1979). This relationship apparently persisted, with 3 month cadmium level measurements of 17.7, 17.5 and 5.2 ug Cd/g dry weight in the gills, kidneys, and liver respectively (Roberts et al., 1979). Roberts also assayed cadmium levels at these same time points in the red blood cell fraction and plasma, finding no significant accumulation in either compartment at either time (Roberts et al., 1979). This finding is in agreement with our experimental data which shows levels of accumulation in the serum to be very low when compared to levels in the gills.

Clearance of cadmium from the gills seems to be a slow process as depicted in Figure 17 where tissue levels remained almost constant during the 8 hour clearance period. Cadmium clearance studies by Kumada withrainbow trout have yielded complementary information of cadmium kinetics in several internal compartments for longer exposure times. Following a 30 week exposure, a 10 week clearance period resulted in significant cadmium concentration decreases in the gill, but none whatsoever in either the kidneys or the liver (Kumada et al., 1973). Benoit et al. (1976) reported exposing rainbow trout to 3.4 ug

Cd/L for 105 weeks and then placing them in control water for 12 weeks. The loss of cadmium from the gill was 79% on a whole tissue basis. However, residues remained constant in the liver and kidney. Thus, longer term exposures ultimately implicate the kidney as the major target of cadmium kinetics, though both uptake and clearance rates are considerably less than those exhibited by the gills.

2.3 Copper-Cadmium Mixtures Kinetic Studies

2.3.1 Copper Exposure and Clearance in the Gill (Cu-Cd Mixtures)

Experimental results show that a maximum concentration of copper was observed for the lower exposure group at 4 hours (24 ug Cu/L, 198 ug Cd/L) (Table 29, Fig 19, a). The other two exposure groups showed maximum concentrations of copper at 16 hours (Fig. 19, b and c). In all cases, once a maximum concentration of copper was obtained in the gills it was followed by a decline in copper levels for the rest of the exposure period. This was true with the exception of the 52 ug Cu/L, 483 ug Cd/L mixture concentration (b) which displayed an apparent increase in accumulation at 64 hours (Table 29).

The single metal control level (50 ug Cu/L) (Fig. 19, d) was deemed to be similar to the level shown by a corresponding copper-cadmium mixture (52 ug Cu/L, 483 ug Cd/L) (b).

During the clearance phase, copper levels remained slightly above those of controls with some fluctuations in copper levels (Table 30, Fig. 19).

Table 29

Mean copper concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration (ug/L) Cu	Cd	Time of Exposure (h)	Geometric mean-copper concⁿ in gills (ug/g dry weight)	Standard deviation
24	198		2.8	1.23
52	483	2	3.6	1.03
108	1,023		4.0	1.15
7	0 (control)		2.3	1.12
24	198		4.4	1.26
52	483	4	3.6	1.23
108	1,023		2.1	1.52
7	0 (control)		3.3	1.28
24	198		3.2	1.38
52	483	8	4.2	1.32
108	1,023		7.2	1.20
7	0 (control)		2.4	1.20
24	198		3.8	1.31
52	483		6.4	1.22
108	1,023	16	9.7	1.32
50	0 (single metal control)		5.1	1.23
0	500 (single metal control)		3.0	1.10
24	198		3.5	1.14
52	483		5.8	1.17
108	1,023	32	6.9	1.32
50	0 (single metal control)		5.9	1.38
0	500 (single metal control)		4.0	1.35
24	198		4.1	1.39
52	483		8.6	1.11
108	1,023	64	6.2	1.28
50	0 (single metal control)		5.4	1.26
0	500 (single metal control)		3.2	1.21

Table 30

Mean copper concentration in rainbow trout gills, at various time intervals, during the 8 hour clearance phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of metal (ug/L) Cu	Cd	Time of Clearance (h)	Geometric mean-copper concⁿ in gills (ug/g dry weight)	Standard deviation
24	198		3.2	1.55
52	483	4	5.2	1.39
108	1,023		5.2	1.48
7	0 (control)		2.7	1.70
24	198		4.9	1.04
52	483	8	5.7	1.32
108	1,023		6.9	1.58
7	0 (control)		4.3	1.12
24	198		6.0	1.62
52	483	16	7.0	1.37
108	1,023		5.4	1.13
7	0 (control)		2.3	1.63

Figure 19 A and B — Mean copper concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper-cadmium kinetic studies. All values are the mean of 4 fish.

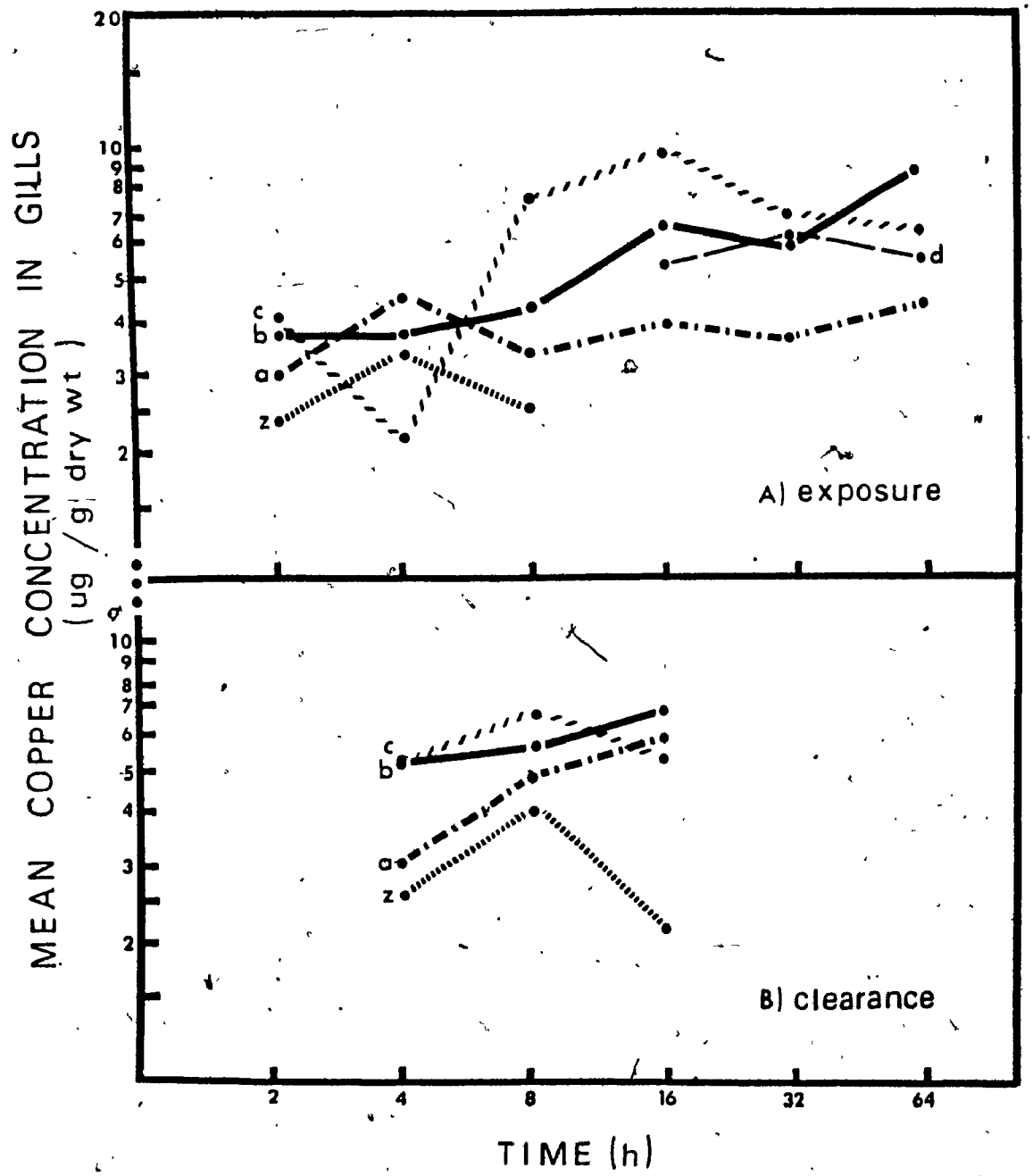
a = 24 ug Cu/L, 198 ug Cd/L

b = 52 ug Cu/L, 483 ug Cd/L

c = 108 ug Cu/L, 1023 ug Cd/L

d = 50 ug Cu/L (single metal control)

z = 7 ug Cu/L (control)


MEAN COPPER CONCENTRATION IN GILLS
(ug / g dry wt)
20
10
9
8
7
6
5
4
3
2
c
b
a
z
d
A) exposure
B) clearance
2
4
8
16
32
64
TIME (h)

2.3.2 Copper Exposure and Clearance in the Serum (Cu-Cd Mixtures)

Both groups exposed to the two lower copper-cadmium mixture concentrations (a and b) exhibited a similar pattern of uptake (Table 31, Fig. 20). Initially, from 2 to 4 hours of exposure, levels of copper in the serum increased, followed by a decline in levels at 8 hours. Throughout the rest of the exposure period, copper levels increased in a progressive manner to reach a maximum concentration for both groups at 64 hours.

The pattern of uptake for the group (c) exposed to the highest copper-cadmium mixture concentration differed from the pattern above (Table 31, Fig. 20). It resulted in the lowest copper level of the three test groups over most of the 64 hour exposure phase. A maximum concentration was reached at 64 hours and this concentration was similar to the level observed for the other two experimental groups at 64 hours.

The exposure level of 50 ug Cu/L (single metal control) (Fig. 20, d) at 64 hours of exposure resulted in a much lower serum concentration than did a similar level of the copper-cadmium mixture (52 ug Cu/L, 483 ug Cd/L)(b). A t-value of 4.1 was calculated ($p < 0.05$) and found to be significantly different from a t-critical value of 1.9. This result suggests that there is an enhanced uptake of copper in the serum at 64 hours when exposed to a Cu-Cd mixture concentration of 52 ug Cu/L, 483 ug Cd/L.

The clearance data showed different trends as illustrated in Table 32 and Figure 20.

Table 31

Mean copper concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration (ug/L) Cu	Cd	Time of Exposure (h)	Geometric mean-copper concⁿ in serum (ug/mL serum)	Standard deviation
24	198		5.5	1.23
52	483	2	4.5	1.19
108	1,023		4.1	1.12
7	0	(control)	2.6	1.32
24	198		12.4	1.59
52	483	4	11.4	1.32
108	1,023		2.8	1.71
7	0	(control)	3.0	1.07
24	198		2.8	1.29
52	483	8	3.4	1.19
108	1,023		5.9	1.20
7	0	(control)	3.1	1.10
24	198		11.0	1.30
52	483		16.2	1.20
108	1,023	16	6.3	1.19
50	0	(single metal control)	6.1	1.29
0	500	(single metal control)	9.6	1.19
24	198		9.3	1.30
52	483		20.0	1.26
108	1,023	32	4.3	1.18
50	0	(single metal control)	4.4	1.12
0	500	(single metal control)	8.2	1.05
24	198		19.3	1.15
52	483		20.1	1.06
108	1,023	64	18.9	1.15
50	0	(single metal control)	12.3	1.07
0	500	(single metal control)	3.4	0.57

Table 32

Mean copper concentration in rainbow trout serum, at various time intervals, during the 8 hour clearance phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of metal (ug/L) Cu	Cd	Time of Clearance (h)	Geometric mean-copper concⁿ in serum (ug/mL serum)	Standard deviation
24	198		7.3	1.41
52	483	4	17.8	1.14
108	1,023		17.2	1.38
7	0 (control)		3.4	1.20
24	198		20.5	1.20
52	483	8	18.4	1.12
108	1,023		13.7	1.23
7	0 (control)		4.9	1.24
24	198		13.1	1.16
52	483	16	14.7	1.28
108	1,023		3.6	1.10
7	0 (control)		4.0	1.40

Figure 20 A and B. Mean copper concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper-cadmium kinetic studies. All values are the mean of 4 fish.

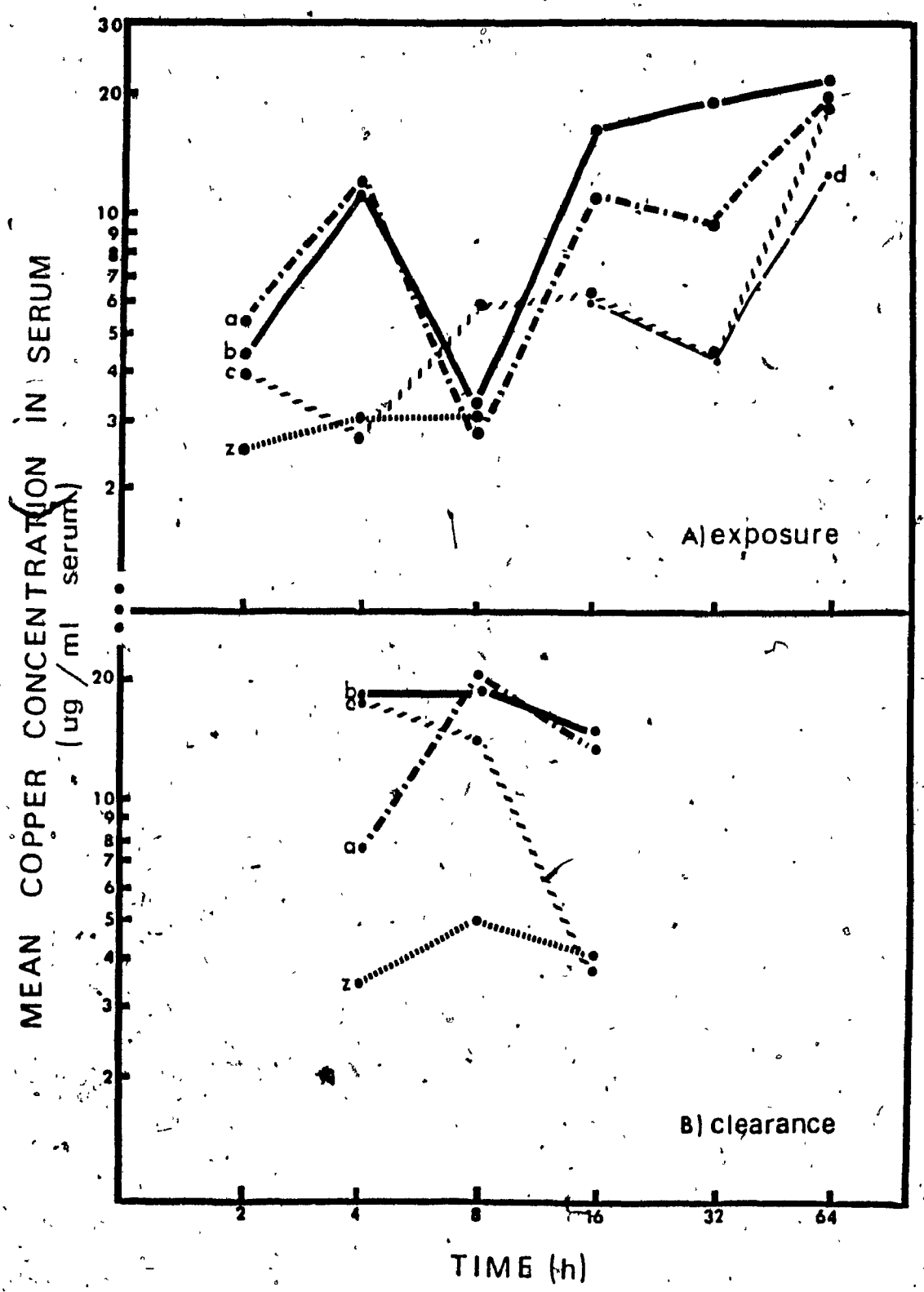
a = 24 ug Cu/L, 198 ug Cd/L

b = 52 ug Cu/L, 483 ug Cd/L

c = 108 ug Cu/L, 1023 ug Cd/L

d = 50 ug Cu/L (single metal control)

z = 7 ug Cu/L (control)


MEAN COPPER CONCENTRATION IN SERUM
(ug / ml serum)
30
20
10
9
8
7
6
5
4
3
2
a
b
c
d
z
A) exposure
20
10
9
8
7
6
5
4
3
2
a
b
z
B) clearance
2
4
8
16
32
64
TIME (h)

2.3.3 Cadmium Exposure and Clearance in the Gill (Cu-Cd Mixtures)

The level of cadmium observed in gills when exposed to mixtures of Cu-Cd was proportional to the exposure concentration and increasing in levels with time (Table 33, Fig. 21). Maximum concentrations were obtained at 64 hours for all of the test groups with the exception of group (c) (108 ug Cu/L, 1023 ug Cd/L) which peaked at 32 hours of exposure.

The single metal control (500 ug Cd/L) (Fig. 21, d) at 64 hours of exposure was determined to be different from a similar level in a Cu-Cd mixture (52 ug Cu/L, 483 ug Cd/L)(b). A t-value of 6.7 was calculated ($p = 0.05$) and found to be significantly different from a t-critical value of 1.9. This suggests a probable enhancement in the uptake of cadmium by gills at 64 hours of exposure when exposed to a Cu-Cd mixture similar to group b.

Clearance data from the gills showed little fluctuation in cadmium levels with the exception of group (a) exposed to the lower cadmium concentration (Table 34, Fig. 21).

2.3.4 Cadmium Exposure and Clearance in the Serum (Cu-Cd Mixtures)

In the serum, maximum cadmium concentrations were reached by 2 hours of exposure for groups b and c and at 8 hours of exposure for group a (Table 35, Fig. 22). After 8 hours, the pattern of cadmium uptake was different for each one of the test groups as shown in Figure 22. From 32 to 64 hours of exposure accumulation levels remained almost constant for all three of the exposure groups.

At 64 hours of exposure the single metal control (500 ug Cd/L)

Table 33

Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase of the copper-cadmium [redacted] kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration (ug/L) Cu	Cd	Time of Exposure (h)	Geometric mean-cadmium concⁿ in gills (ug/g dry weight)	Standard deviation
24	198		0.8	1.03
52	483	2	1.2	1.27
108	1,023		2.2	1.19
7	0	(control)	0.0	0.00
24	198		0.9	1.27
52	483	4	1.2	1.13
108	1,023		2.6	1.11
7	0	(control)	0.0	0.00
24	198		1.1	1.15
52	483	8	1.4	1.26
108	1,023		5.5	1.17
7	0	(control)	0.0	0.00
24	198		1.3	1.15
52	483		1.9	1.43
108	1,023	16	7.0	1.30
50	0	(single metal control)	0.0	0.00
0	500	(single metal control)	1.9	1.03
24	198		1.4	1.16
52	483		3.4	1.20
108	1,023	32	13.2	1.20
50	0	(single metal control)	0.0	0.00
0	500	(single metal control)	1.5	1.34
24	198		3.0	1.36
52	483		8.5	1.09
108	1,023	64	9.1	1.36
50	0	(single metal control)	0.0	0.00
0	500	(single metal control)	4.1	1.08

Table 34

Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 8 hour clearance phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of metal (ug/L) Cu	Cd	Time of Clearance (h)	Geometric mean-cadmium concn in gills (ug/g dry weight)	Standard deviation
24	198		2.9	1.08
52	483	4	3.5	1.12
108	1,023		5.3	1.20
7	0 (control)		0.0	0.00
24	198		1.6	1.19
52	483	8	4.3	1.09
108	1,023		5.2	1.03
7	0 (control)		0.0	0.00
24	198		2.0	1.33
52	483	16	3.5	1.26
108	1,023		4.8	1.13
7	0 (control)		0.0	0.00

Figure 21. A and B Mean cadmium concentration in rainbow trout gills, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper-cadmium kinetic studies. All values are the mean of 4 fish.

a = 24 ug Cu/L, 198 ug Cd/L

b = 52 ug Cu/L, 483 ug Cd/L

c = 108 ug Cu/L, 1023 ug Cd/L

d = 500 ug Cu/L (single metal control)

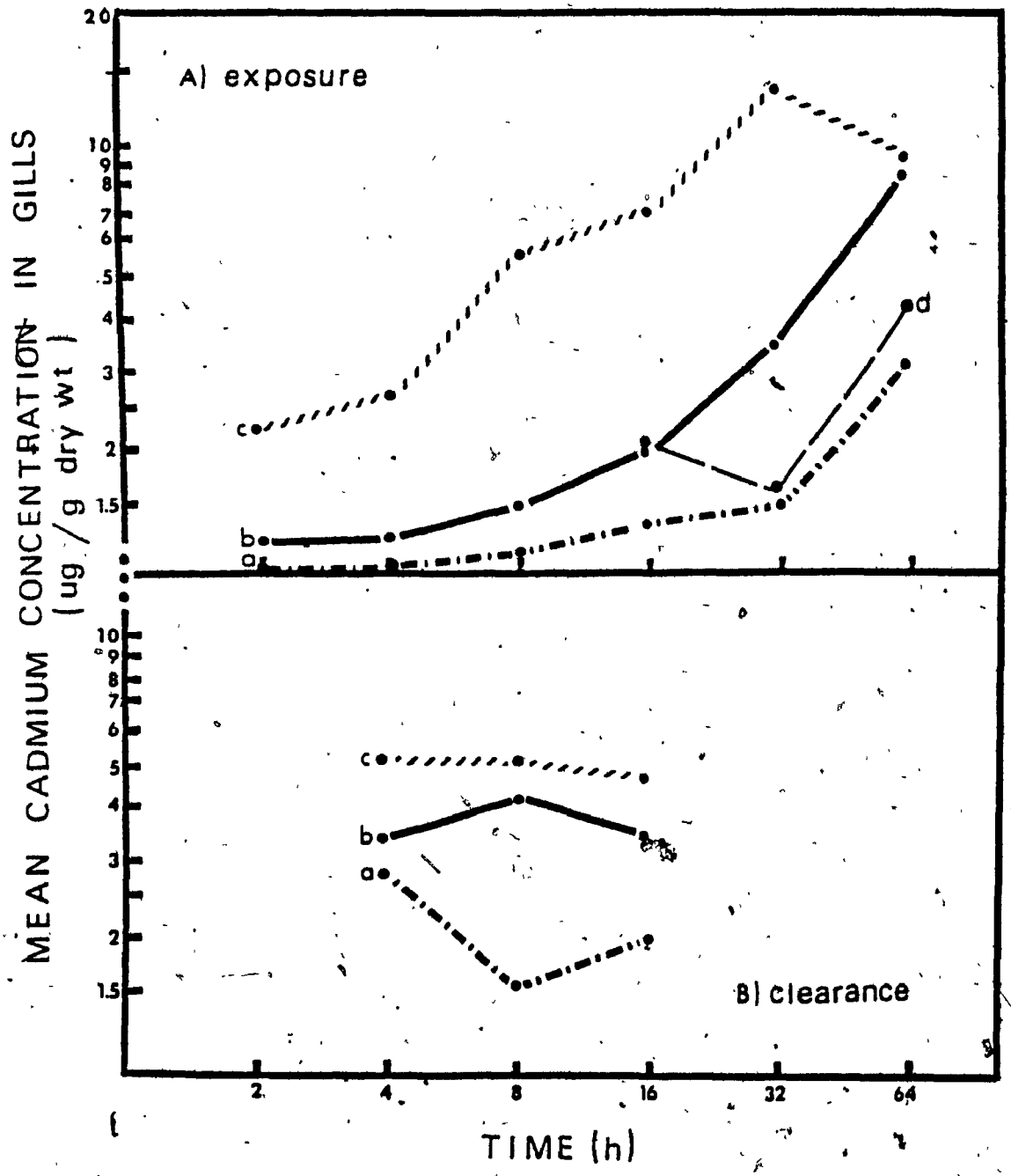

A) exposure
B) clearance
MEAN CADMIUM CONCENTRATION IN GILLS
(ug / g dry wt)
TIME (h)
a
b
c
d
20
10
9
8
7
6
5
4
3
2
1.5
2
4
8
16
32
64

Table 35

Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Mean assayed concentration (ug/L) Cu	Cd	Time of Exposure (h)	Geometric mean-cadmium concⁿ in serum (ug/mL serum)	Standard deviation
24	198		0.28	1.10
52	483	2	0.42	1.12
108	1,023		0.80	1.28
7	0 (control)		0.00	0.00
24	198		0.30	1.11
52	483	4	0.32	1.11
108	1,023		0.77	1.28
7	0 (control)		0.00	0.00
24	198		0.31	1.23
52	483	8	0.16	1.79
108	1,023		0.48	1.28
7	0 (control)		0.00	0.00
24	198		0.11	1.10
52	483		0.22	1.09
108	1,023	16	0.62	1.28
50	0 (single metal control)		0.00	0.00
0	500 (single metal control)		0.14	1.03
24	198		0.19	1.10
52	483		0.25	1.21
108	1,023	32	0.40	1.26
50	0 (single metal control)		0.00	0.00
0	500 (single metal control)		0.20	1.04
24	198		0.19	1.18
52	483		0.21	1.12
108	1,023	64	0.38	1.34
50	0 (single metal control)		0.00	0.00
0	500 (single metal control)		0.17	1.02

Table 36

Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 8 hour clearance phase of the copper-cadmium mixture kinetic studies. All values are the mean of 4 fish.

Previous exposure levels of metal (ug/L) Cu	Cd	Time of Clearance (h)	Geometric mean-cadmium concn in serum (ug/mL serum)	Standard deviation
24	198		0.16	1.32
52	483	4	0.29	1.20
108	1,023		0.21	1.05
7	0 (control)		0.00	0.00
24	198		0.20	1.30
52	483	8	0.33	1.05
108	1,023		0.35	1.03
7	0 (control)		0.00	0.00
24	198		0.21	1.77
52	483	16	0.19	1.09
108	1,023		0.23	1.34
7	0 (control)		0.00	0.00

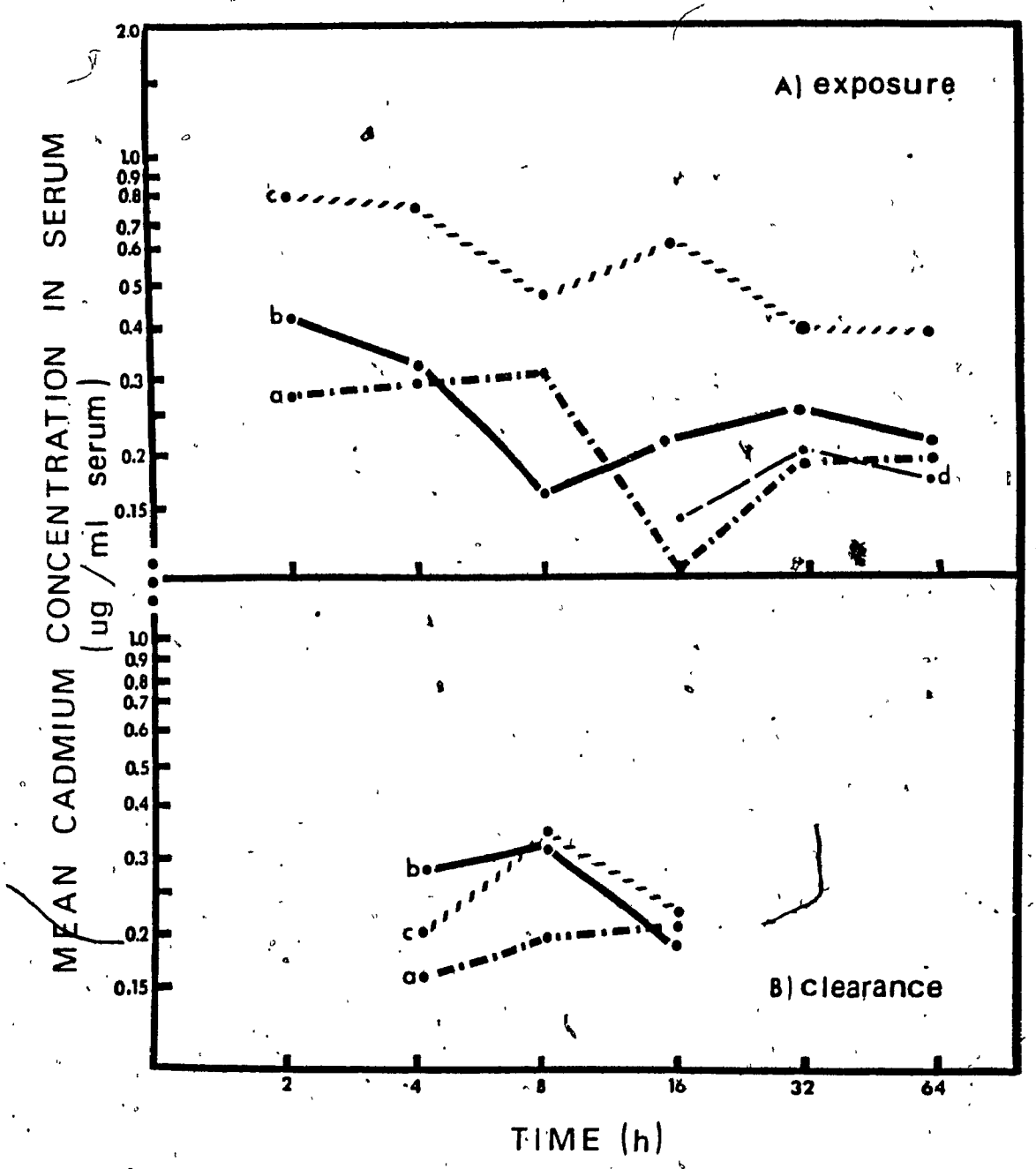
Figure 22 A and B Mean cadmium concentration in rainbow trout serum, at various time intervals, during the 64 hour exposure phase and the 8 hour clearance phase of the copper-cadmium kinetic studies. All values are the mean of 4 fish.

a = 24 ug Cu/L, 198 ug Cd/L

b = 52 ug Cu/L, 483 ug Cd/L

c = 108 ug Cu/L, 1023 ug Cd/L

d = 500 ug Cu/L (single metal control)


A) exposure
B) clearance
MEAN CADMIUM CONCENTRATION IN SERUM (ug / ml serum)
TIME (h)
2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.15
2
4
8
16
32
64
a
b
c
d

Cd/L) (Fig. 22, d) was estimated not to differ from the accumulation level of the Cu-Cd mixture concentration 52 ug Cu/L, 483 ug Cd/L (b).

Throughout the 16 hours of clearance, cadmium levels in the serum for all of the test groups displayed moderate fluctuations (Table 36, Fig. 22).

Discussion (Cu-Cd Mixtures)

It appears that the pattern of copper accumulation in the gill was not affected by the presence of cadmium. This is supported by comparing the pattern of the copper single metal study with that of copper in the Cu-Cd mixtures. In both studies, an initial accumulation of copper was followed by a decline in gill levels which stabilized for the remainder of the 64 hour exposure period (Figs. 15 and 19). Furthermore, the actual level of copper accumulation in the gill was not affected by the presence of cadmium. No apparent difference was observed between the single metal control in the gill (52 ug Cu/L) (Fig. 15, d) and a similar level in the Cu-Cd mixture (52 ug Cu/L, 483 ug Cd/L) at 64 hours of exposure.

The pattern of cadmium uptake by the gills in the presence of copper appeared to be different from the pattern seen in the single metal cadmium study (Fig. 17 and 21). Although in both cases, cadmium uptake was dose dependent and increasing in levels with time, in the Cu-Cd mixture study the uptake did not show the fluctuations seen in the single metal study. This may indicate that the presence of copper alters the pattern of cadmium uptake.

Furthermore, the single metal control (500 ug Cd/L) (Fig 21, d),

kinetic interaction between the two metals which may result in increased levels of cadmium in gill tissue. The apparent increase in cadmium levels in the gill may help explain the observed displacement of the dose response curve of Cu-Cd mixtures (expressed as cadmium) compared to the cadmium single metal dose response curve. However, no conclusive evidence for synergism of Cu-Cd mixtures in the gill can be offered as the single metal control corresponds only to one of three Cu-Cd mixture concentrations tested. Thus, the use of only one single metal control in mixture tests limits interpretations of the experimental data. Further studies should follow where single metal levels are monitored with a corresponding Cu-Cd mixture concentration.

Few studies have addressed the subject of tissue accumulation of ambient heavy metals present in mixtures. McConnell and Stokes found that rainbow trout exposed to 1 mg/L of copper in conjunction with 8 mg/L of nickel for a period of 30 days had similar copper levels in gill and liver tissue to those observed for copper single metal exposure tests. Thus nickel did not appear to modify the pattern of copper accumulation. On the other hand, nickel accumulated at a greater than single metal control level in the gill, but not the liver, in the presence of copper.

The results of this study complies with what was observed in the kinetics experiments where the single metal uptake for copper in the gills was the same as the uptake in the mixtures.

In contrast to these findings, an ambient test concentration of 1 mg Cu/L in the presence of 100 mg Ni/L caused the gill copper levels

to double from the 29 ug Cu/g dry weight observed in copper single metal control studies to approximately 58 ug Cu/g dry weight in the roach, Rutilus rutilus (Van Hoof and Nauwelaers, 1984). Liver and kidney copper concentrations dropped from 39 and 31.7 to 16 and 13.6 ug Cu/L respectively when the nickel was introduced into the copper test solution.

It is very likely that some of the differences between the single metal studies and the Cu-Cd mixture studies are due to the differences in fish weight between the three experiments, 50 g mean wet weight in the single metal studies vs 60 g mean wet weight in the mixture studies. The seasons during which the tests were conducted are another source of variation. The kinetic tests were conducted over the months of February, April and May (Methods, Table 3).

2.4 General Discussion

During the 64 hour exposure period, the gill was shown to be a site of cadmium accumulation in rainbow trout. This was supported by the serum data where concentrations were 1/10 of the levels seen in gills.

The gills did not appear to be a site of copper accumulation at 64 hours of exposure. In this case, to get an index of copper loading other organs such as liver or kidney should be assayed in the future. This can serve as a caution in water management projects to discriminate the organs being used as indicators of pollutant action since in this case the gill was not a useful indicator of copper loading levels.

It is presumed from the results shown above that the mode of toxicity of copper and cadmium may differ since the critical target sites appear to be different.

In the Cu-Cd mixture studies, copper accumulation in gills apparently was not affected by the presence of cadmium and did not change in pattern from the single metal study. On the other hand, experimental results for cadmium uptake in the gills suggest there may be a physiological interaction between copper and cadmium which results in a potentiation of the cadmium uptake in the gill. It stems from this result that the action and toxicity of mixtures of potentially harmful substances to aquatic ecosystems must be evaluated if water quality criteria are to be meaningful.

3.0 Enzyme Studies

3.1 Na^+-K^+ ATPase Activity in Gills following Exposure to Copper

Exposure to 60, 115, and 228 ug Cu/L for a duration of 64 hours resulted in unchanged activity of the Na^+-K^+ ATPase system when sampled at 4, 8, 16, 32, and 64 hours, as noted in Table 37.

3.2 Na^+-K^+ ATPase Activity in Gills following Exposure to Cadmium

Concentrations of 530, 1060 and 2070 ug Cd/L were used in this study. Results listed in Table 38 show that at the three cadmium concentrations tested, no inhibition or stimulation of the enzyme activity was detected when compared with activity in controls.

3.3 Discussion

Cellular water balance and osmoregulation of the whole animal is fundamentally dependent on the Na^+-K^+ ATPase enzyme (Haya et al., 1980; Witherspoon and Wells, 1975). This particular enzyme is located in the chloride cells at the inner border of the gill epithelia (Kerstteter et al., 1970; Houston and McCarty, 1978). The chloride cells are considered as sites of electrolyte transport.

This membrane bound protein hydrolizes ATP to ADP and inorganic PO_4. The energy released from the high energy based PO_4 bond is used to drive the active transport system for cations which is primarily dependent on ATP for energy (Witherspoon and Wells, 1975; Haya et al., 1980). This active transport system serves to concentrate nutrients within the cell, to maintain the proper level of inorganic electrolytes, and to maintain the correct osmotic pressure and volume

Table 37

Na^+-K^+ ATPase activity in rainbow trout gills following exposure to copper. Activity was assayed at 37° with a 20 min 37°C preincubation.

Time of Exposure (h)	Mean copper exposure concentration (ug/L)	Mean Na^+-K^+ ATPase activity (u mole Pi/mg/h)	Standard Deviation
8	60	9.2	2.9
	115	11.25	2.9
	228	8.8	2.2
	(control) 7	7.2	2.4
16	60	9.5	1.1
	115	10.2	2.9
	228	10.5	2.0
	(control) 7	11.2	1.9
32	60	12.8	2.3
	115	11.0	2.4
	228	9.8	2.6
	(control) 7	10.0	2.1
64	60	8.8	1.9
	115	8.5	2.4
	228	11.5	1.1
	(control) 7	11.5	2.1

Table 38

Na^+-K^+ ATPase activity in rainbow trout gills following exposure to cadmium. Activity was assayed at 37° with a 20 min 37°C preincubation.

Time of Exposure (h)	Mean cadmium exposure concentration (ug/L)	Mean Na^+-K^+ ATPase activity (u mole Pi/mg/h)	Standard Deviation
	530	7.8	2.3
8	1,060	6.0	0.7
	2,070	5.3	1.3
	(control) 0	7.0	1.4
	530	7.0	1.6
16	1,060	6.5	2.1
	2,070	9.5	2.3
	(control) 0	8.6	2.5
	530	10.0	2.2
32	1,060	9.5	2.5
	2,070	13.2	2.6
	(control) 0	9.3	1.8
	530	8.3	2.0
64	1,060	7.8	2.8
	2,070	7.8	0.8
	(control) 0	8.6	1.2

of intracellular fluid (Witherspoon and Wells, 1975).

Electron microscopy has revealed that the purified enzyme is a polypeptide with a mass of 90-100 thousand daltons found in the form of vesicles, rods and rings (Jorgensen, 1974). These vesicles formed are impermeable, of different sizes and contain the enzyme within them. The fact that vesicles formed are of different sizes explains why the enzyme is found in all sediments after conventional centrifugation (Sips et al., 1982; Jorgensen, 1975). As a result, the specific activity of the Na^+-K^+ ATPase in a single subcellular fraction such as the microsomal pellet is not deemed to be a safe measure of the amount of enzyme in the tissue (Jorgensen, 1974). Accordingly, Jorgensen (1974) recommends analysis of the activity in whole homogenates. In our studies, however, assaying the activity of whole homogenates was not possible. Preliminary studies showed rainbow trout whole homogenates to be without any enzyme activity under varying assay conditions. Only with the microsomal fraction isolated could successful Na^+-K^+ ATPase assays be performed. The above restriction has already been reported in the literature by Pfeiler and Kirschner (1972) and Kamiya and Utida (1969) when dealing with whole homogenates of rainbow trout. The inability to work with crude homogenates of the enzyme introduced the use of the procedure of subcellular fractionation and it limited the number of samples per assay.

The Na^+-K^+ ATPase in salmonids has been reported to be partially ouabain insensitive (Schwartz et al., 1962; Davis, 1970; Pfeiler and Kirschner, 1972). This was confirmed by assays performed in our

laboratory. Preliminary studies revealed that Na^+-K^+ ATPase activity in rainbow trout gill tissue was 62% ouabain insensitive (see Appendix, section D). Because of its apparent ineffectiveness as an inhibitor of this enzyme in rainbow trout, ouabain was not used in our assays.

Determination of teleostean Na^+-K^+ ATPase activities are frequently conducted at temperatures ranging from 30 to 40°C corresponding to the thermal optimum for the system. Such temperatures, however are far higher than those tolerated by salmonids. While the activities obtained in this way are presumably close to the maximum to be expected for the crude preparations used in this and other studies, their relationship to activity under in vivo temperature conditions is uncertain (McCarty and Houston, 1977). In our studies however, an incubation temperature of 37°C was chosen to eliminate Mg-ATPase activity, which is a thermo-labile enzyme. In this way, isolation of the Na^+-K^+ ATPase activity was presumably magnified (Pfeiler and Kirschner, 1972; Kamiya and Utida, 1969).

A large Na^+-K^+ ATPase enzyme activity variability has been reported inherent between individual gill arches and between individual fish (Pfeiler and Kirschner, 1972; Kamiya and Utida, 1969; Johnson et al., 1977). In our studies, considerable individual variability in control activity occurred during the course of time over which these experiments were carried out. Perhaps this reflects not only differences between individual animals but diurnal and seasonal variability as well. Johnson et al. (1977) reported this variation as a reflection of the natural biological variation inherent

within the population, which does not represent problems with the precision of the assay itself.

There is not much information in the literature reporting on the effects of metals on the Na^+-K^+ ATPase enzyme. Lorz et al, (1978), conducted studies exposing coho salmon (Oncorhynchus kisutch) to levels of 4, 8, or 12 ug Cd/L over a period of 170 hours. Cadmium exposure did not affect gill Na^+-K^+ ATPase activity when compared to controls (Lorz et al., 1978). Lobsters (Homarus americanus) exposed to 6 ug/L of cadmium for a duration of 30 days had cadmium levels in the gills of 70 ug Cd/L. However, Na^+-K^+ ATPase activity and that of residual ATPases were the same as those in controls (Haya et al., 1980). Another very similar study with lobsters (Homarus americanus) exposed to 6 ug Cd/L for 30 days showed no differences in Na^+-K^+ ATPase activity. However, the activity of residual ATPases was increased by 25% (Tucker, 1979). These results are comparable with my experimental data where exposure to copper or cadmium did not affect the activity of the Na^+-K^+ ATPase enzyme.

Another investigation has reported the Na^+-K^+ ATPase activity to be stimulated under zinc exposure. An increase in the Mg^{++}, Ca^{++} and Na^+-K^+ ATPase activity of coho salmon was noted under in vivo exposure to 0.29 and 1.98 mg /L Zn after 20 days (Watson and Beamish, 1981). Branchial Na^+-K^+ ATPase activity, measured at 37°C, was 14.5 u moles PO_4/h/mg protein compared to control values of 9 u moles PO_4/h/mg protein (Watson and Beamish, 1981). These control values comply favorably with the values obtained for controls in my study.

As discussed above, there is considerable variability in the

effect of xenobiotics on the Na^+-K^+ ATPase enzyme. These effects vary with species, habitat and concentration of the toxicant. Many chemicals may affect the activity of many enzymes only to a moderate degree and it may be presumed that the ultimate debilitating effect on the whole organism develops from a variety of non-specific biochemical malfunctions (McCarty and Houston, 1977; Haya et al. 1980).

CONCLUSION

Toxicity of copper-cadmium mixtures could not be predicted from a simple additive rationale. This thesis has presented evidence which shows that mixtures of copper and cadmium are synergistic in their toxic action. Furthermore, the results of the kinetic studies suggest that copper and cadmium may be interacting at the level of the gill to potentiate cadmium uptake.

Further study is needed to determine the type and degree of interaction of toxicants on both an acute and chronic basis. Results from these tests would help identify general characteristics of certain pollutant mixtures and may provide data for new methods and possibly the rationale for deriving water quality criteria for combined pollutants.

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APPENDIX

A) Lethal Response Data: July 1982 experiment, copper exposure

Table A

Lethal response data for rainbow trout, 14 g, exposed to solutions of copper, July 1982 experiment. The number of fish per experimental concentration was 17

Time (h)	Cu exposure concentration (ug/l) 79	100	196	247	290
*15				1	
16					
17					
18					
19			1		
20					1
21					
22				1	
23					
24					
25					
26					
27					1
28					1
29					1
30					
31					
32					
33					2
34					2
35				1	
36		1			
37				1	2
38					
39					
40				1	
41					
42					
43					2
44					
45					
46					
47					
48	1				
49					
50					
51					
52				1	
53					
54					
55					

Time (h)	Cu exposure concentration (ug/l) 79	100	196	247	290
56				1	
57					2
58					
59					
60					
61					
62					
63					
64					
65					
66					
67					
68					
69					
70					
71					
72			2		
73					
74					
75					
76					
77					
78					
79					
80					
81		1	1		
82					
83					
84					
85					
86					
87					
88					
89					
90					
91					
92					
93					
94					
95					
96	1				

* no mortalities were observed before the 15th hour of exposure.

B) Lethal Response Data: February 1985 experiment, cadmium exposure.

Table B

Lethal response data for rainbow trout, 25 g, exposed to solutions of cadmium, February 1985 experiment. The number of fish per experimental concentration was 16.

Time (h)	Cd exposure concentration (ug/l) 970	1,890	2,970	3,940
*43			1	
44				1
45				
46				
47				
48			1	
49				
50				
51	1			
52				
53			1	
54			1	
55		1		1
56				
57				
58				
59				
60			1	
61		1		
62				
63		1		2
64	1	1		1
65			1	
66				
67	2		1	
68		3	1	
69		2		
70				
71				
72				
73				
74				
75	3			3
76				
77	1	2		1
78				
79	1		1	2
80		1	1	
81				
82				
83			1	2
84			1	1
85				
86				
87				
88				
89				
90				
91				
92	1	1	3	
93				
94				
95				
96	2			
97				
98				
99				
100				
101	1	2		
102				
103				
104				
105		1	1	
106				
107				
108				
109				
110				
111				
112				
113				
114				
115				
116	2	1	1	

* no mortalities were observed before the 40th hour of exposure.

C) Lethal Response Data: February 1985 experiment, copper-cadmium exposure.

Table C

Lethal response data for rainbow trout, 25 g, exposed to solutions of copper-cadmium mixtures, February 1985 experiment. The number of fish per experimental concentration was 16.

Time (h)	Cu-Cd exposure concentration (ug/l)							
	Cu	Cd	Cu	Cd	Cu	Cd	Cu	Cd
	28,440		63,890		125,1830		176,2630	
3							1	
4								
5							2	
6								
7								
8								
9							1	
10								
11					1		2	
12							3	
13								
14					1			
15							3	
16							1	
17							1	
18							2	
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								
29								
30								
31								
32								
33								
34								
35								
36								
37								
38								
39								
40								
42								

Time (h)	Cu-Cd exposure concentration (ug/l)							
	Cu	Cd	Cu	Cd	Cu	Cd	Cu	Cd
	28,440		63,890		125,1830		176,2630	
43								
44						4		
45								
46			1			4		
47						1		
48			1					
49	1							
50								
51			1			1		
52								
53			1					
54								
55								
56								
57								
58								
59			2					
60	1							
61								
62			1					
63	1		1					
64	1		1					
65	1		1					
66								
67	1							
68			1			1		
69								
70								
71								
72								
73								
74								
75	2		1					
76								
77	1		1					
78								
79						1		
80								
81			1					

Table C cont'd

Lethal response data for rainbow trout, 25 g exposed to solutions of copper-cadmium mixtures, February 1985 experiment. The number of fish per experimental concentration was 16.

Time (h)	Cu-Cd exposure concentration (ug/l)							
	Cu	Cd	Cu	Cd	Cu	Cd	Cu	Cd
	28,440		63,890		125,1830		176,2630	
82								
83					1			
84								
85								
86								
87								
88								
90								
91	2		2		1			
92								
93								
94								
95								
96	1							
97								
98								
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D) The Ouabain Insensitive Fraction of the Na^+-K^+ ATPase Enzyme

The purpose of this study was to examine the effect of ouabain on the Na^+-K^+ ATPase enzyme activity. It was noticed in preliminary assays that the inhibitory action of ouabain was minimal. The sample size was of twelve fish with a wet weight of 25 g. There were three sets of duplicate tubes: The first set, "a" tubes contained the Na^+-K^+ ATPase enzyme with 200 mM Na and 50 mM K; the "b" tubes had the same components as the "a" tubes plus ouabain; the "c" tubes had the enzyme Na^+-K^+ ATPase plus the homogenate medium with no stimulatory salts and no ouabain present.

Results shown in Table D show enzyme absorbance in the "a" tubes, ranging from 0.768 to 0.926. Enzyme absorbance in tubes "b", having ouabain present, was slightly reduced from that seen in tubes "a" with values between 0.544-0.742. However, a much larger reduction of enzyme absorbance was noted in tubes "c" where neither Na nor K were present with values between 0.263-0.384. It was calculated that an average 62% of the Na^+-K^+ ATPase activity was insensitive to the action of ouabain. Accordingly, it was decided to omit ouabain from the Na^+-K^+ ATPase assays of rainbow trout.

Table D

Effect of ouabain on Na^+-K^+ stimulated ATPase activity in gills of rainbow trout, assayed at 37°C with a 20 min 37°C preincubation.

Sample no	Gill wet weight (g)	Total ATPase absorbance a	ATPase absorbance with ouabain present b	ATPase absorbance with no Na^+ or K^+ present c	Ouabain sensitive fraction a-b	Ouabain insensitive fraction (a-c)-(a-b)	Total Na^+-K^+ ATPase absorbance a-c	% ouabain sensitive fraction a-b	% ouabain insensitive fraction (a-c)-(a-b)
1	0.76	0.863	0.654	0.263	0.209	0.391	0.600	35	65
2	0.60	0.923	0.742	0.384	0.181	0.358	0.539	34	66
3	0.63	0.768	0.596	0.321	0.172	0.275	0.447	38	62
4	0.81	0.926	0.544	0.315	0.382	0.229	0.611	38	62